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Steroid–Protein Interactions. XVIII. Isolation and Observations on the Polymeric Nature of the Corticosteroid-Binding Globulin of the Rat*

Gerald J. Chader† and Ulrich Westphal

ABSTRACT: Chromatographic techniques in combination with gel filtration resulted in the isolation of a corticosteroid-binding globulin from pooled rat serum. The isolated corticosteroid-binding globulin–corticosterone complex was homogeneous by sedimentation velocity ($s^0_{20,w}$ = 3.56 S), paper electrophoresis, and immunoelectrophoresis (α_1-globulin). A molecular weight of 61,000 ± 1100 was obtained by the approach to sedimentation equilibrium method whereas the corticosterone content indicated a molecular weight of approximately 53,000 for the active steroid-binding species. A carbohydrate content of 27.8% was found. Distinct differences in certain amino acid residues such as half-cystine may account for differences in steroid-binding and polymeric properties between rat corticosteroid-binding globulin and corticosteroid-binding globulin from the human and rabbit. The number of high-affinity binding sites for corticosterone in the pure rat corticosteroid-binding globulin was determined to be $n = 1$. The association constants of the corticosterone complex at 4 and 37° were $k = 5.1 \times 10^8$ and 2.8×10^7 M^{-1}, respectively. Thermodynamic calculations gave a negative enthalpy change and a negative entropy change for the interaction. Rat corticosteroid-binding globulin was found to be polymeric in nature. Removal of steroid from the isolated steroid–protein complex ("stripping") resulted in at least four polymeric peaks discernible during ultracentrifugation; a concomitant loss in steroid-binding affinity was observed. Recombination with 1 mole of corticosterone/mole of corticosteroid-binding globulin restored most of the corticosteroid-binding globulin activity and also reversed the polymerization resulting in one homogeneous sedimentation peak of the $s_{20,w}$ value of the original monomeric complex. Polyacrylamide disc electrophoresis of "stripped" rat corticosteroid-binding globulin showed a polymeric pattern similar to that observed in the ultracentrifuge. Considerably less polymeric banding of corticosteroid-binding globulin occurred before stripping substantiating the conclusion that dissociation of the complex and removal of corticosteroid from the protein led to polymerism. The reactions are an example of the regulatory control by a steroid hormone of the quaternary structure of a steroid-binding protein.

Soon after the discovery of the corticosteroid-binding globulin (Daughaday, 1956; Bush, 1957) or transcortin (Slaunwhite and Sandberg, 1959) in human plasma, it was observed that the blood of the rat also contains macromolecules which bind glucocorticoid hormones with high affinity (Daughaday, 1958; Slaunwhite and Sandberg, 1959). This finding was of particular significance to the biochemist since it opened the way for experimental study of the factors involved in this highly specific association between steroid and protein (Westphal, 1961).

Corticosteroid-binding activity in rat serum was found to be dependent upon the corticosterone level (Westphal *et al.*, 1963); the binding ability is associated with an α_1-globulin (Westphal and DeVenuto, 1966). The corticosteroid-binding globulin level is influenced

* From the Biochemistry Department, University of Louisville, School of Medicine, Reynolds Building, Louisville, Kentucky 40208. *Received May 27, 1968.* The present work was supported by Grants AM-04040 and AM-06369 from the National Institute of Arthritis and Metabolic Diseases, by Research Career Award GM-K6-14,138 from the Division of General Medical Sciences of the U. S. Public Health Service, and by Contract DA-49-193-MD-2263 from the U. S. Army Medical Research and Development Command. Some of the present results have been reported (Chader and Westphal, 1968). For the preceding paper of this series, see Ganguly and Westphal (1968).

† Present address: Department of Biological Chemistry, Harvard Medical School, Boston, Mass. 02115.

by sex steroid hormones (Gala and Westphal, 1965); it is also mediated by hormones other than the steroid hormones (Gala and Westphal, 1966a). Of particular importance is the thyroid-stimulating hormone (Gala and Westphal, 1966b,c) acting through the thyroid hormone (Labrie *et al.*, 1967; Labrie, 1967).

Isolation and biochemical characterization of rat corticosteroid-binding globulin would permit comparison with human corticosteroid-binding globulin which has been characterized chemically (Seal and Doe, 1962a,b; Slaunwhite *et al.*, 1966; Muldoon and Westphal, 1967) as well as biologically (Sandberg *et al.*, 1966; Seal and Doe, 1966). Rabbit corticosteroid-binding globulin was characterized as a homogeneous glycoprotein (Chader and Westphal, 1967, 1968a; Ganguly and Westphal, 1968). The study of the corticosteroid-binding globulin from a third species is of interest because the corticosteroid-binding globulin molecules from different species differ distinctly in their relative binding affinity to steroids of different "polarity;" *e.g.*, the human glycoprotein follows the polarity rule at 37° in that the steroid with the smallest number of polar groups is bound most firmly, whereas the opposite is true for rabbit corticosteroid-binding globulin (Westphal, 1967). The significant differences between the affinities for cortisol, corticosterone, and progesterone among the glycoproteins from several species, including the rat (Westphal, 1967), suggest a high specificity of binding for individual steroids which have identical structure except for the number of hydroxy groups present.

The objective of the present study was to determine some of the physical and chemical parameters that might be involved in the interaction of rat corticosteroid-binding globulin with steroids. The results are expected to contribute to an elucidation of the molecular basis for steroid–protein interaction and for the differences in binding affinities for individual steroids. By analogy such investigation may also be significant for an understanding of interactions at receptor sites in steroid-sensitive tissues.

Materials and Methods

Experimental procedures and materials, including radiolabeled steroids, were applied as previously described (Muldoon and Westphal, 1967; Chader and Westphal, 1968; Ganguly and Westphal, 1968), except for additions and changes outlined below. All experiments were conducted at 4° unless otherwise noted. Column flow rates were maintained at approximately 1 ml/min except for the hydroxylapatite chromatography where the maximum rate was about 0.5 ml/min.

Protein Source. The serum pool was prepared from blood of male and female Sprague–Dawley rats obtained from commercial suppliers. Part of the serum was from rats which had been subjected to experimental procedures such as adrenalectomy or hypophysectomy while the major amount was from normal males and females. Animals which had received steroid hormone injections were generally excluded. On the basis of all available evidence, one may assume that the treatment which some of the donor rats had received affects only the level of corticosteroid-binding globulin but not its chemical nature; adrenalectomy (Westphal *et al.*, 1962) and estrogen treatment (Seal and Doe, 1962b) result in changes in corticosteroid-binding globulin concentration with no apparent qualitative alterations (Westphal and DeVenuto, 1966). The serum pool was stored at −85°.

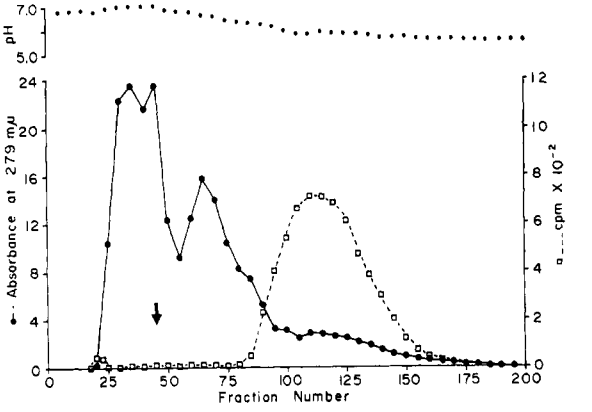

FIGURE 1: DEAE-cellulose chromatography of the partially purified rat corticosteroid-binding globulin–corticosterone-4-^{14}C complex after DEAE-Sephadex separation. Except for maxima and minima, absorbance and radioactivity were measured for every fifth fraction. Arrow indicates start of gradient.

Isolation Procedure. Techniques and specific conditions including buffer strengths, column sizes, etc., were applied as described (Chader and Westphal, 1968; Ganguly and Westphal, 1968). Rat serum (1100 ml) was dialyzed against distilled, deionized water overnight, equilibrated with 8.0 μg of corticosterone-4-^{14}C, and subjected to DEAE-Sephadex chromatography. The resulting partially purified protein was applied to a DEAE-cellulose column and rechromatographed under the same conditions. It was then subjected to gel filtration over Sephadex G-200 and to hydroxylapatite chromatography. Stepwise elution of protein from the hydroxylapatite column (4.5 × 12 cm), equilibrated with 5 mM phosphate buffer (pH 6.8), was performed with 0.05 and 0.2 M phosphate buffer of pH 6.8. The highly purified preparation was then subjected to three further gel filtrations over Sephadex G-200 until a homogeneous product was obtained.

Steroid-Binding Studies. Equilibrium dialysis studies on the isolated steroid–protein complex for the determination of association constants were performed as reported (Chader and Westphal, 1968; Ganguly and Westphal, 1968) for 72 hr at 4° and for 48 hr at 37° with 0.20 mg of corticosteroid-binding globulin/ml of inside solution (3 ml). In other experiments, corticosterone-4-^{14}C was added in successive increments from 0.1 to 0.4 μg in the outside solutions (6 ml).

In conjunction with the ultracentrifugation studies on polymerism (see next section), equilibrium dialysis was carried out at 37° with a corticosteroid-binding globulin concentration of 0.20 mg/ml; the corticosteroid-binding globulin preparation used was cleared ("stripped") of 87% of its corticosterone (as measured by loss of bound radiolabel from the protein) by passage of 5-mg amounts

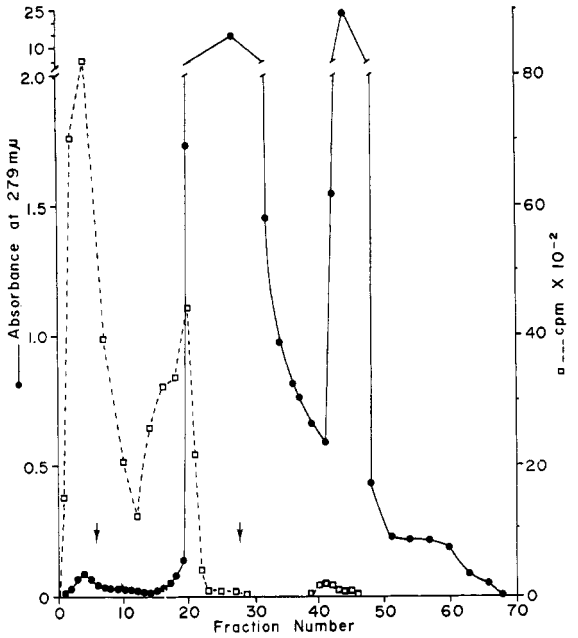

FIGURE 2: Hydroxylapatite chromatography of partially purified rat corticosteroid-binding globulin after Sephadex G-200 filtration. Arrows indicate changes from 0.005 to 0.05 M and to 0.2 M phosphate buffer (pH 6.8).

of the isolated corticosteroid-binding globulin–steroid complex over a 2.5 × 27 cm column of Sephadex G-75 at 23°. Similar equilibrium dialyses were performed on the "stripped" corticosteroid-binding globulin preparation after recombination with 1 mole of corticosterone/53,000 g of protein and equilibration for approximately 12 hr at 4°. The specific radioactivity of the added corticosterone was the same as that of the originally isolated corticosteroid-binding globulin–steroid complex. This procedure essentially reconstituted the original steroid–protein complex.

Methods of Characterization. The general characterization techniques have been described (Muldoon and Westphal, 1967; Chader and Westphal, 1968; Ganguly and Westphal, 1968). A water content of 5.1% was determined by drying the lyophilized protein to constant weight at 110°. The extinction coefficient at 279 mμ, corrected for water content, was found to be $E^{1\%}_{1\,cm}$ 6.2. This value was used to adjust concentrations in all experiments involving quantitative measurements.

The approach to sedimentation equilibrium was performed in a Spinco Model E ultracentrifuge as outlined by Schachman (1957), using a protein concentration of 0.9% in 0.1 M NaCl and a speed setting of 8766 rpm. The temperature was controlled at 20°. Pictures were taken periodically up to 3 hr and 20 min at angles of 80, 70, 60, and 50° for the sedimentation equilibrium, and up to 1 hr 45 min for the synthetic boundary run. At the end of the latter run, the speed was raised to 59,780 rpm and the protein was allowed to sediment for verification of homogeneity throughout the run. Sedimentation velocity measurements for the $s^0_{20,w}$ determination and the studies on polymerism were carried out in 0.1 M NaCl at 20° with a speed setting of 59,780 rpm using the double-sector cell.

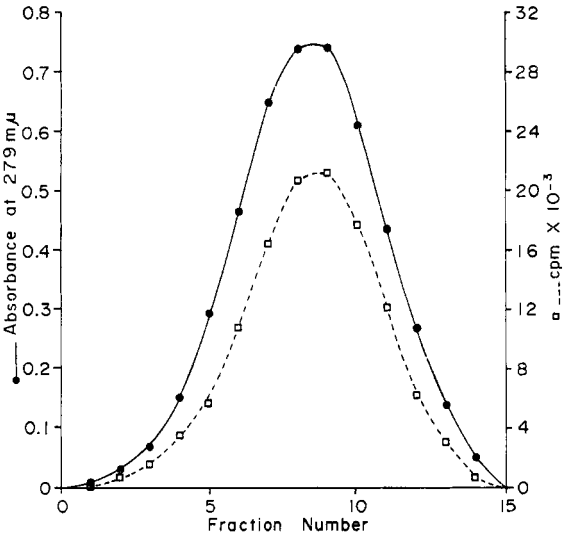

FIGURE 3: Final Sephadex G-200 filtration of rat corticosteroid-binding globulin–corticosterone complex. Phosphate buffer (0.05 M, pH 7.4). For further details, see text.

In the polymerism studies, the corticosteroid-binding globulin used was that which had been stripped of 87% of its endogenous corticosterone as reported in a previous section. After the run, the corticosteroid-binding globulin–steroid complex was reconstituted with 1 mole of corticosterone/53,000 g of protein as described above. It was then allowed to equilibrate overnight at 4° and subjected to a second ultracentrifugation after removal of an aliquot for the equilibrium dialysis study.

Amino acid analysis was performed on a Technicon Model NC-1 analyzer after HCl digestion *in vacuo* for 22 hr at 110°. The amino acid and the carbohydrate analyses were controlled by test runs with a well-characterized α_1-acid glycoprotein preparation as well as with appropriate standards. Disc gel electrophoresis in polyacrylamide was performed at room temperature according to Davis (1964) using the Model 12 apparatus of Canalco, Inc., Rockville, Md. Samples were run in 0.025 M Tris–glycine buffer at 5 mA/sample tube. A total of 100 μg of corticosteroid-binding globulin in 5 μl was applied in each case. Samples of rat serum and of Versatol were included for comparison.

Results

Isolation of Rat Corticosteroid-Binding Globulin. The DEAE-Sephadex chromatography gave the same general fractionation pattern as observed in the isolation of the rabbit protein (Chader and Westphal, 1968; Ganguly and Westphal, 1968).

A small amount of free, nonprotein-bound corticosterone-4-^{14}C in the breakthrough volume indicated oversaturation of the binding sites; the major portion of the steroid was eluted in association with a protein peak at the end of the separation. Since the procedure did not approach the 15–20-fold purification usually obtained with rabbit serum, the partially purified rat corticosteroid-binding globulin was applied to DEAE-cellulose column as shown in Figure 1. A small amount of

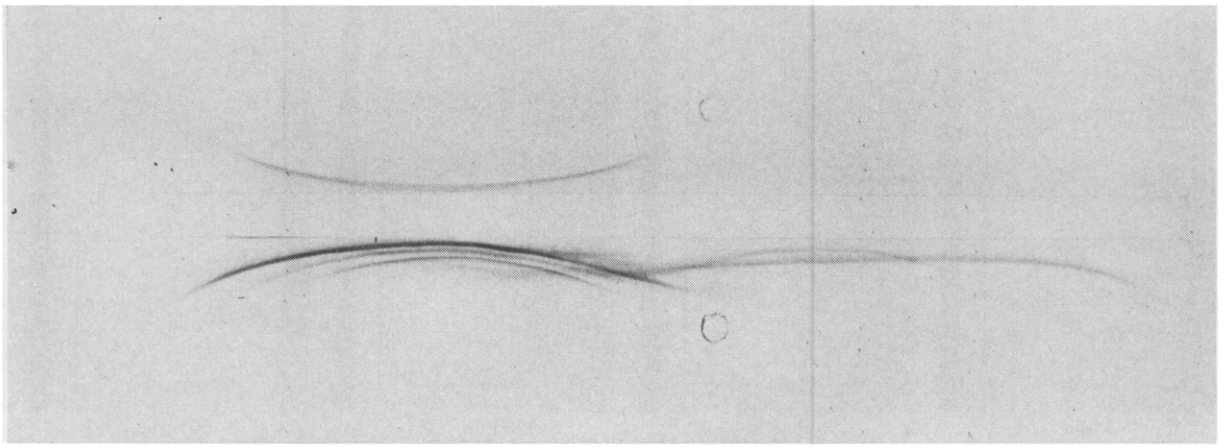

FIGURE 4: Immunoelectrophoresis of pure rat corticosteroid-binding globulin–corticosterone complex. Top well contains 8% corticosteroid-binding globulin, bottom well normal rat serum. Rabbit antiserum to whole rat serum was added in central trough. High-resolution Tris buffer, pH 8.9, 23°.

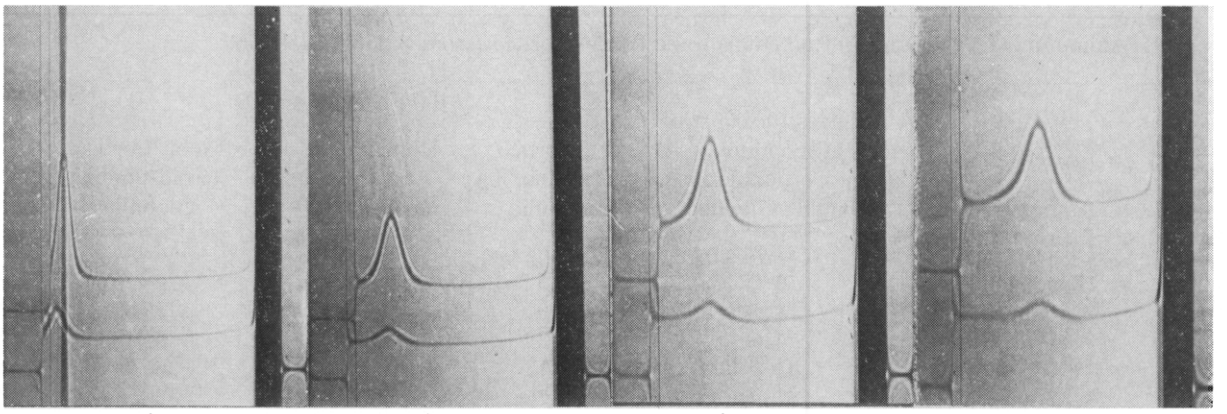

FIGURE 5: Ultracentrifugation patterns of 0.9 and 0.2% solutions of rat corticosteroid-binding globulin–corticosterone complex in 0.1 M NaCl; 20°; 59,780 rpm. Bar angles, 70, 70, 60, and 55°.

unbound corticosterone-4-^{14}C was again observed in the breakthrough volume while over 96% of the radioactivity was eluted with the protein toward the end of the gradient. A total of 5.3 g of protein of high specific radioactivity was obtained.

The material was subjected to gel filtration on Sephadex G-200. The fractions of high specific activity were combined, dialyzed overnight, lyophilized (yield 1.4 g), and directly applied to the hydroxylapatite column. Stepwise elution resulted in the pattern seen in Figure 2. The fraction of high specific activity (tubes 1–21) which contained 110 mg of protein was subjected to three final Sephadex G-200 filtrations which resulted in a single, homogeneous steroid–protein peak as shown in Figure 3. A total of 31.5 mg of protein was obtained from the 1100 ml of pooled serum used as starting material. Immunoelectrophoresis of the purified corticosteroid-binding globulin preparation from the final Sephadex G-200 filtration indicated the presence of a single homogeneous α_1-globulin in high-resolution Tris buffer (pH 8.9) (Figure 4) as did paper strip electrophoresis.

The boundary pattern of the isolated steroid–protein complex in each of five sedimentation velocity runs in the ultracentrifuge was found to be symmetrical for concentrations ranging from 1.4 to 0.2%. Figure 5 shows representative exposures taken during the development of the patterns of a 0.9 and 0.2% solution of the corticosteroid-binding globulin in the double-sector cell. Figure 6 shows the linear relationship between the reciprocal $s_{20,w}$ values and the protein concentrations. Ex-

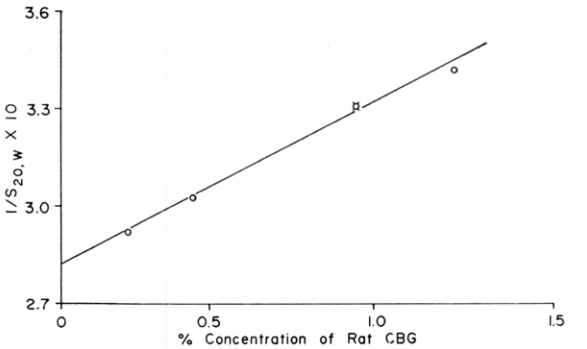

FIGURE 6: Reciprocal $s_{20,w}$ values *vs.* concentration for rat corticosteroid-binding globulin–corticosterone complex in 0.1 M NaCl; 20°. Two runs at 0.9% concentration gave identical $s_{20,w}$ values.

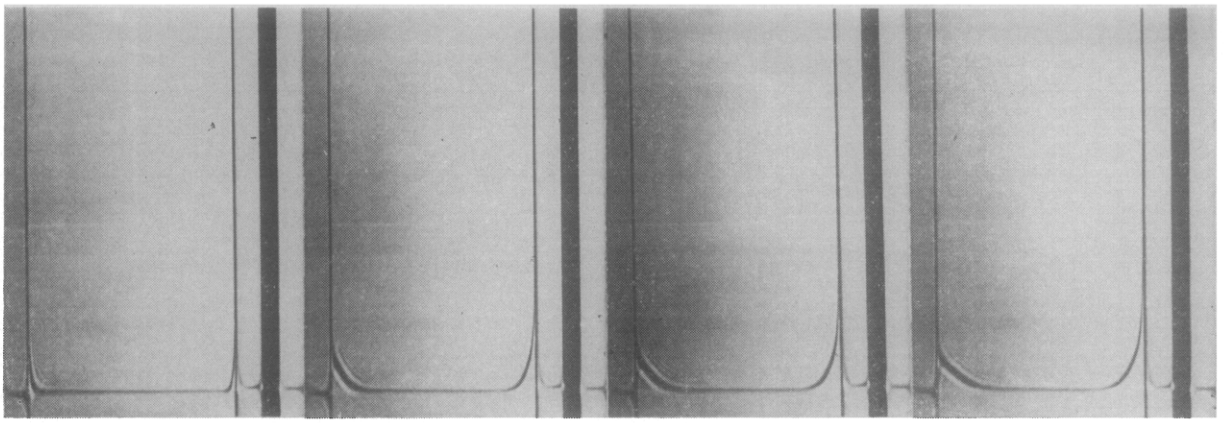

FIGURE 7: Representative ultracentrifugation patterns for rat corticosteroid-binding globulin–corticosterone complex during the approach to equilibrium in 0.1 M NaCl; 8766 rpm; bar angle, 60°.

TABLE I: Amino Acid Composition of Rat, Human, and Rabbit Corticosteroid-Binding Globulin.[a]

Amino Acid	Residues/100,000 g of Glycoprotein Rat Corticosteroid-Binding Globulin	g/100 g of Polypeptide		
		Rat Corticosteroid-Binding Globulin	Human Corticosteroid-Binding Globulin	Rabbit Corticosteroid-Binding Globulin
Lysine	37.4	6.64	5.03	4.60
Histidine	13.5	2.57	3.29	2.89
Arginine	18.5	4.01	3.99	5.22
Aspartic acid	74.6	11.89	9.72	9.84
Threonine	43.8	6.13	5.73	6.70
Serine	51.9	6.26	6.15	6.21
Glutamic acid	80.6	14.42	12.77	13.33
Proline	31.9	4.29	5.48	5.31
Glycine	40.0	3.16	2.86	3.67
Alanine	43.3	4.26	4.34	5.12
Half-cystine	16.4	2.32	0.54	2.10
Valine	37.5	5.15	6.66	6.10
Methionine	14.9	2.70	3.52	1.51
Isoleucine	24.9	3.90	4.76	3.74
Leucine	63.9	10.02	11.98	10.05
Tyrosine	19.0	4.30	4.22	4.41
Phenylalanine	30.2	6.16	7.29	5.80
Tryptophan	7.1	1.82	1.49	3.39

[a] Values for human and rabbit corticosteroid-binding globulin from Muldoon and Westphal (1967) and Chader and Westphal (1968; Ganguly and Westphal, 1968), respectively.

trapolation to zero protein concentration gave $s_{20,w} = 3.56$ S.

For determination of molecular weight, the approach to sedimentation equilibrium method was used. Figure 7 gives four representative exposures for the equilibrium run at various times after reaching a speed of 8766 rpm. Two exposures for the synthetic boundary run are given in Figure 8, (left-hand side). The other two exposures in this figure show the homogeneous sedimentation pattern which developed when the speed was increased to 59,780 rpm after completion of the run in the synthetic boundary cell. From measurements at the meniscus and bottom of the equilibrium run for 80, 70, and 60° bar angles, a molecular weight of 61,000 ± 1100 was calculated.

Table I gives the amino acid composition of rat corticosteroid-binding globulin and for comparison that of human corticosteroid-binding globulin (Muldoon and Westphal, 1967) and of rabbit corticosteroid-binding globulin (Chader and Westphal, 1968; Ganguly and

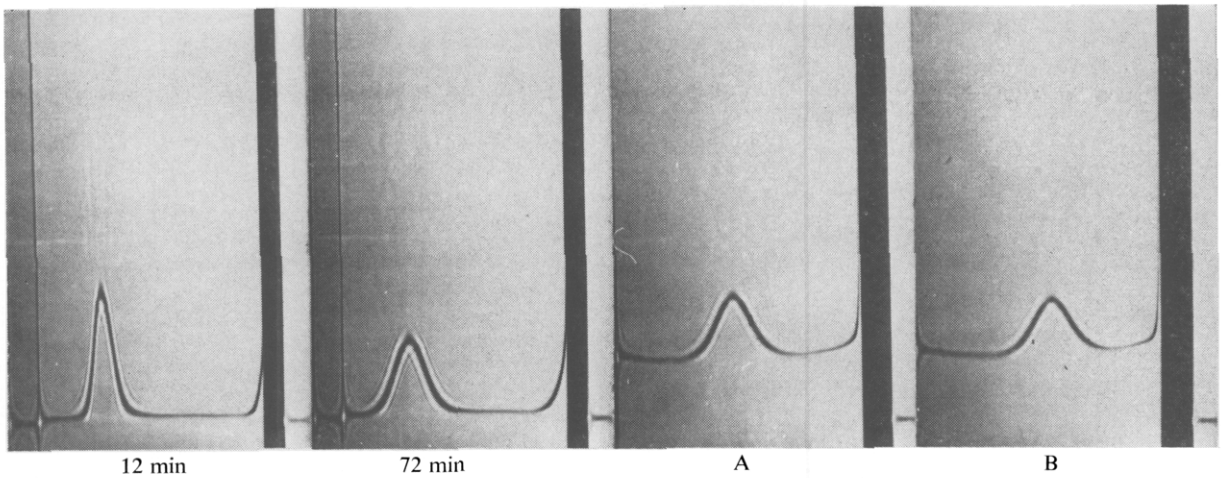

FIGURE 8: Representative ultracentrifugation patterns for rat corticosteroid-binding globulin–corticosterone complex during synthetic boundary run after 12 and 72 min. Parts A and B, sedimentation patterns 18 and 38 min after speed was increased to 59,780 rpm.

Westphal, 1968). The analysis shows the patterns to be similar (exceptions discussed below). The carbohydrate content of the rat corticosteroid-binding globulin was found to be 9.8% hexose, 9.5% hexosamine, 6.4% sialic acid, and 2.1% fucose for a total of 27.8% carbohydrate as compared with 26.1 and 29.2% for the human and rabbit protein, respectively. From these data, the partial specific volume was calculated to be 0.711 ml/g. The A_{280}/A_{260} ratio for the isolated steroid–protein complex was found to be 1.58; after removal of approximately 90% of the steroid (as described above) the ratio was 1.71.

Steroid-Binding Studies. The serum pool used in the isolation procedure contained 32 μg of corticosterone/100 ml and no significant amount of cortisol. The isolated corticosteroid-binding globulin–steroid complex had 6.6 μg of corticosterone/mg of protein and also essentially no cortisol. When this complex was subjected to equilibrium dialysis at 4 and 37° without further addition of steroid, apparent association constants of 4.56×10^8 and 2.86×10^7 M^{-1} were obtained, respectively. When 0.1 μg of corticosterone-4-^{14}C was added to the outside solutions in a second series of dialysis experiments in which 0.6 mg of corticosteroid-binding globulin complex was applied, the association constants were approximately the same, *i.e.*, 5.59×10^8 M^{-1} at 4° and 2.68×10^7 M^{-1} at 37°. Further addition of 0.1-μg portions of radiolabeled steroid resulted in the appearance of correspondingly higher amounts of unbound corticosterone due to oversaturation of binding sites as will be discussed below. The association constants and the values for change of free energy, enthalpy, and entropy for the corticosterone–corticosteroid-binding globulin complex are compiled in Table II, together with other physicochemical properties.

Attempts at removing ("stripping") the steroid from the isolated corticosteroid-binding globulin–corticosterone complex for a more complete determination of the association constant were unsuccessful. Dissociation of the complex by Sephadex G-50 gel filtration at 45° resulted in complete loss of steroid-binding activity even though such a procedure applied to whole serum removed the steroid without loss of corticosteroid-binding globulin activity (Westphal, 1967). Filtration of the corticosteroid-binding globulin complex over Sephadex G-75 at 23° resulted in removal of 87% of the steroid and an apparent loss of about 60% of the binding affinity. An association constant of 1.20×10^7 M^{-1} was obtained at 37° with this protein. However, after reconstitution of the steroid–protein complex by readdition of corticosterone as described above, an association constant of 2.03×10^7 M^{-1} was calculated from dialysis data obtained at 37°. This result shows the reversible nature

TABLE II: Physicochemical Properties of Rat Corticosteroid-Binding Globulin.

$s^0_{20,w}$ (S)	3.56
$\bar{v}$ (ml/g)	0.711
Molecular weight (g) (approach to sedimentation equilibrium)	61,000 ± 1100
Molecular weight (g) (from corticosterone content)	52,600 ± 3000
$E^{1\%}_{1\text{ cm}}$, 279 mμ	6.2
$A_{280}:A_{260}$ (corticosterone complex)	1.58
$A_{280}:A_{260}$ (complex stripped)	1.71
k_{assoc}, corticosterone, 4° (M^{-1})	5.1×10^8
k_{assoc}, corticosterone, 37° (M^{-1})	2.8×10^7
$\Delta F°$, corticosterone complex, 4° (kcal/mole)	−11.0
$\Delta F°$, corticosterone complex, 37° (kcal/mole)	−10.6
$\Delta H°$, corticosterone complex (kcal/mole)	−14.8
$\Delta S°$, corticosterone complex (cal mole^{-1} deg^{-1})	−14

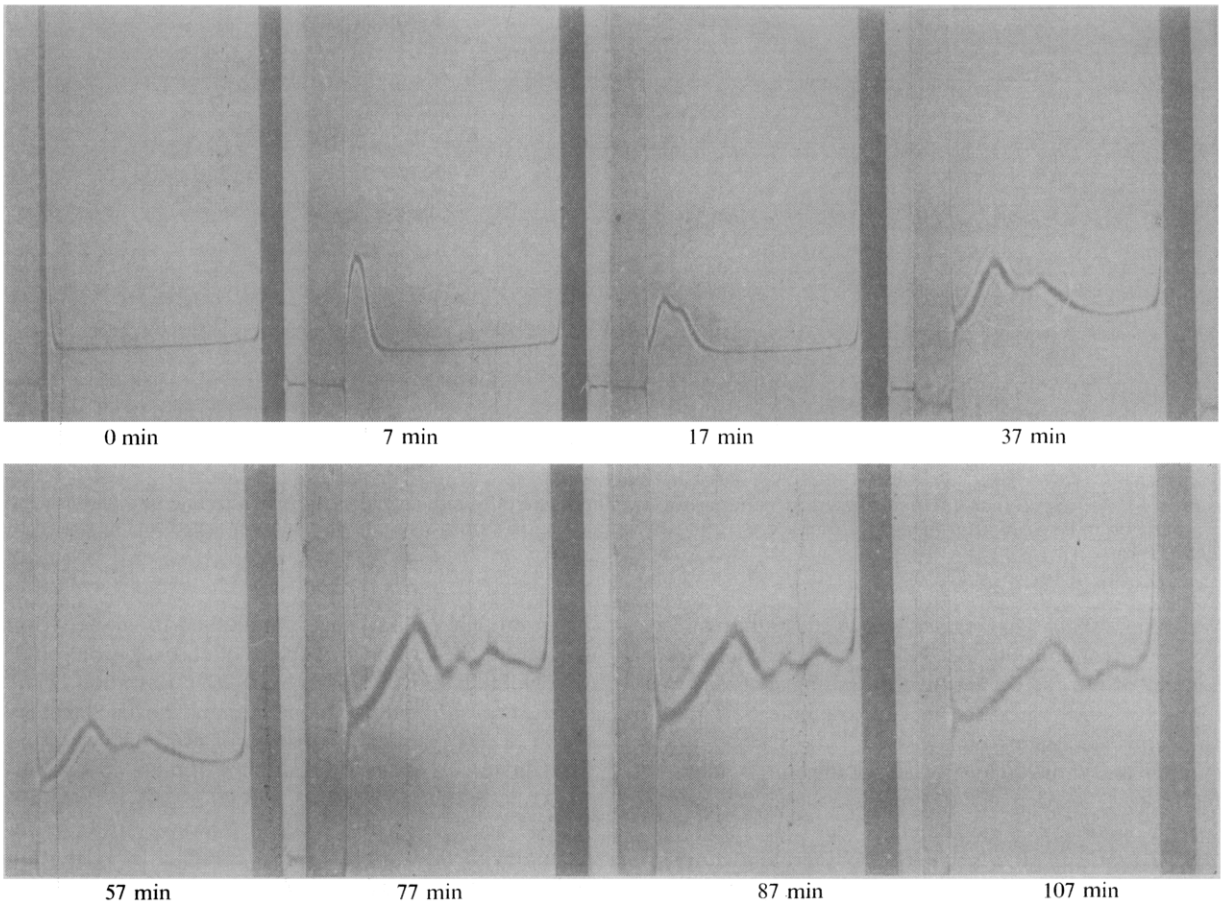

FIGURE 9: Ultracentrifugation pattern of rat corticosteroid-binding globulin after removal of 87% of the associated corticosterone at various times after speed of 59,780 rpm was reached. 0.1 M NaCl; 20°. Bar angles up to 17 min, 70°; at 37 and 57 min, 50°; remaining exposures, 30°.

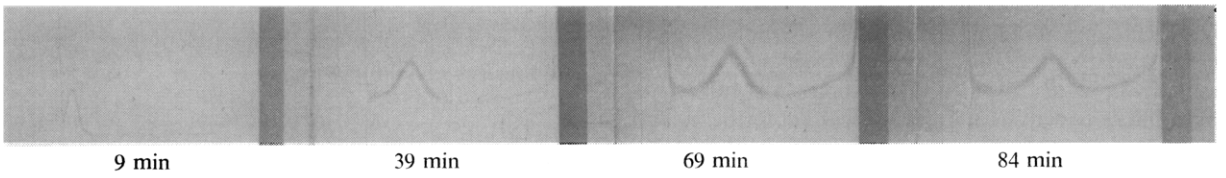

FIGURE 10: Ultracentrifugation pattern of rat corticosteroid-binding globulin used for study shown in Figure 9 after recombination with corticosterone, at various times after speed of 59,780 rpm was reached. 0.1 M NaCl; 20°. Bar angles were 70, 50, 40, and 40°.

of the inactivation. In these calculations a molecular weight of 53,000 was used as will be discussed below.

Ultracentrifugation Studies of Corticosteroid-Binding Globulin after Removal and Readdition of Corticosterone. Figure 9 shows the sedimentation pattern of a rat corticosteroid-binding globulin preparation described above from which approximately 90% of bound corticosterone was removed by gel filtration at 23°. After 77 min, four peaks, one major and three minor ones, can be distinguished. The sedimentation coefficients were calculated to be 3.4, 5.4, 6.8, and 8.1, respectively. After prolonged centrifugation, a fifth species appears as a shoulder on the trailing edge of the 3.4S peak. When this corticosteroid-binding globulin preparation, after recovery from the ultracentrifuge cell, was recombined with approximately 1 mole of corticosterone/53,000 g of protein, the sedimentation pattern changed to that of the original corticosteroid-binding globulin–corticosterone complex (Figure 10). One homogeneous peak was observed with a sedimentation coefficient of 3.4 S, a value in agreement with that found for the original corticosteroid-binding globulin–corticosterone complex at a similar concentration. The same polymeric and monomeric sedimentation patterns, respectively, were obtained in a second experiment in which rat corticosteroid-binding globulin was stripped and later recombined with corticosterone.

Disc Gel Electrophoresis Studies. Figure 11 shows the polymeric pattern developed when samples of corticosteroid-binding globulin were subjected to electrophoresis in polyacrylamide gel at room temperature. Tube 4 was a sample of the isolated corticosteroid-binding glob-

ulin–corticosterone complex while tube 9 was a sample of corticosteroid-binding globulin from which steroid had been removed as described above. Migration is toward the bottom of the tube. Densitometry tracings of the two runs are given beside the pictures. The ratios of the migration rates of the electrophoretic bands to those of their clearly separated faster migrating neighboring bands were for tube 9 as follows: 1.19, 1.21, 1.28, and 1.32; the ratios for tube 4 were 1.26 and 1.32. This is a maximal deviation of ±5% from the theoretical value of 1.26 for the ratio of monomer to dimer velocity as will be discussed below.

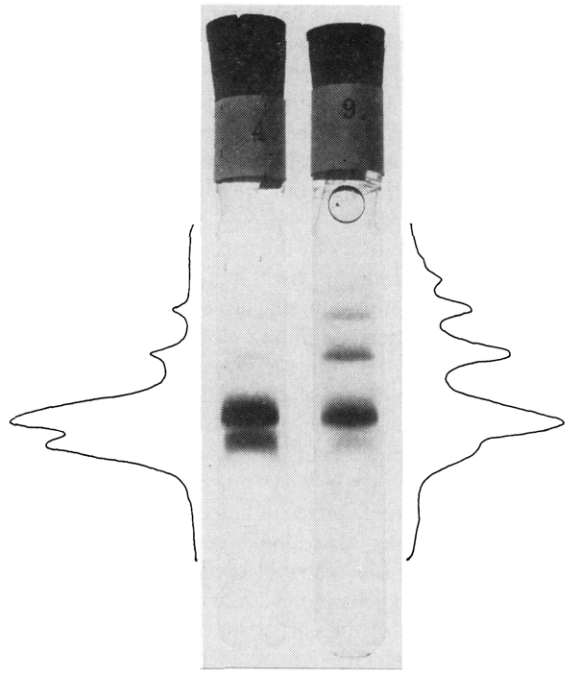

FIGURE 11: Disc electrophoresis patterns with microdensitometer curves of rat corticosteroid-binding globulin samples. Sample 4, isolated corticosteroid-binding globulin–corticosterone complex; sample 9, the same complex after removal of 87% of the steroid. The peaks of the absorption curves, drawn from projections of the microdensitometer tracings, coincide accurately with the protein discoids in the original tracings.

Discussion

Isolation and Physicochemical Properties. In the isolation of rat corticosteroid-binding globulin, chromatography on DEAE-Sephadex and DEAE-cellulose was first used to achieve a preliminary concentration of corticosteroid-binding globulin activity from a relatively large amount of serum. Sephadex G-200 gel filtration was then applied to eliminate the bulk of the remaining contaminating proteins. The use of several hydroxylapatite steps (Seal and Doe, 1962a) in the final phase of purification was found to be successful with rabbit (Chader and Westphal, 1968; Ganguly and Westphal 1968) and human (Muldoon and Westphal, 1967) corticosteroid-binding globulin. Here only one hydroxylapatite chromatography was carried out; it yielded a product of α_1 mobility which appeared homogeneous by paper strip electrophoresis but which was found to contain three components in the ultracentrifuge. This active material was subjected to further Sephadex G-200 filtrations until a single protein peak was obtained with a symmetrical corticosterone-4-^{14}C pattern (Figure 3). Sephadex G-200 filtration affords a test procedure for corticosteroid-binding globulin activity, demonstrates relative size homogeneity, and provides chromatographic purification as well (Chader and Westphal, 1968; Ganguly and Westphal, 1968). Virtually all of the radiolabeled steroid remained protein bound throughout the isolation as verified in the various filtration steps; this indicates that the integrity of the original steroid–protein complex was maintained.

The physicochemical properties of the isolated rat corticosteroid-binding globulin–steroid complex (Tables I and II) showed certain similarities with those obtained for the human (Seal and Doe, 1962a,b; Slaunwhite *et al.*, 1966; Muldoon and Westphal, 1967) and rabbit (Chader and Westphal, 1968) glycoproteins. All three preparations behaved as α_1-globulins when tested by paper strip, gel, or immunoelectrophoresis; the ultracentrifuge patterns were similar and gave comparable $s_{20,w}$ values.

Table I shows that, in spite of a general similarity, the amino acid composition of rat corticosteroid-binding globulin differs significantly in certain amino acid residues from that of the rabbit and human proteins. Differences are seen in the two sulfur-containing amino acids, half-cystine and methionine, the half-cystine value of the rat protein being more than four times that in human corticosteroid-binding globulin. This difference may be of importance for the binding properties since sulfhydryls have been shown to be involved (directly or indirectly) in the interaction of steroid with the rabbit protein (Chader and Westphal, 1968; Ganguly and Westphal 1968). The lysine content of rat corticosteroid-binding globulin was also found to be higher than that of human or rabbit corticosteroid-binding globulin; likewise, glutamic and aspartic acid are present in somewhat greater amounts.

Molecular Weight and Binding Parameters. In the present study, the approach to sedimentation equilibrium technique yielded a value of 61,000 ± 1100 for the molecular weight. Calculations at the meniscus and bottom of the cell were in good agreement indicating apparent homogeneity of the isolated protein complex. Increasing the speed at the end of the synthetic boundary run resulted in the appearance of a single symmetrical descending boundary pattern and thus demonstrated the maintenance of homogeneity throughout the determination.

The molecular weights of human corticosteroid-binding globulin determined by different methods have been reported to be 45,000 (Seal and Doe, 1962a), 52,000 ± 1500 (Seal and Doe, 1966), 58,500 (Slaunwhite *et al.*, 1966), and 51,700 (Muldoon and Westphal, 1967). In view of these differences obtained for presumably the same human corticosteroid-binding globulin entity, it was desirable to assess the molecular weight of rat corticosteroid-binding globulin by a second, independent method involving steroid content and association con-

stant calculations. The isolated rat corticosteroid-binding globulin complex contained 6.6 μg of corticosterone/mg of protein. If one assumes a single binding site, n, and saturation, a molecular weight of approximately 53,000 would correspond to the observed corticosterone content in rat corticosteroid-binding globulin. The approximate error arising from the steroid analysis would presumably be ±3000.

Evidence for validity of the assumption of one binding site and saturation is given by the following results. In each of the equilibrium dialysis experiments with the pure rat corticosteroid-binding globulin–corticosterone complex, a total of 0.6 mg of protein was employed in association with 4.0 μg of corticosterone bound in the complex. Without further addition of steroid, or after addition of not more than 0.10 μg of radiolabeled corticosterone to the dialysis system, high and consistent association constants were obtained at 4 and 37°. Evidently, the number of available corticosteroid-binding globulin binding sites was not exceeded. When greater quantities of corticosterone (0.20 and 0.40 μg) were added to 0.6 mg of the corticosteroid-binding globulin complex in other dialysis experiments, the binding sites were exceeded and the fraction of unbound steroid rose markedly in a manner similar to that previously described for rabbit corticosteroid-binding globulin (Chader and Westphal, 1968; Ganguly and Westphal, 1968). This rapid increase of unbound steroid when the total corticosterone content of the corticosteroid-binding globulin complex was raised above 4.1 μg/0.6 mg indicated that the high-affinity binding sites were saturated at a value of $n = 1$ per mole of corticosteroid-binding globulin of an approximate molecular weight of 53,000.

The association constants (Table II) calculated using a molecular weight of 53,000 were close to those reported from studies with unfractionated serum (Westphal, 1967). They were also similar to the association constants of the cortisol complexes of human corticosteroid-binding globulin and rabbit corticosteroid-binding globulin (see Table VIII in Chader and Westphal, 1968; Ganguly and Westphal, 1968). Correspondingly, the changes in free energy, enthalpy, and entropy of the rat corticosteroid-binding globulin–corticosterone complex (Table II) were found to be in close agreement with those previously obtained for the corticosteroid–corticosteroid-binding globulin complexes of the other two species (see Table VIII in Chader and Westphal, 1968; Gangley and Westphal, 1968) where an interpretation of the thermodynamic data has been given).

For a molecular weight of 61,000, the calculated amount of corticosterone bound at equimolar ratio would be 5.7 μg/mg of protein, whereas 6.6 μg was actually found. The following explanations are to be considered. (1) The result of the corticosterone determination is too high. This is not likely on the basis of our experience with the analytical method over the years. (2) The number of high-affinity binding sites is greater than one. This assumption has been disproved in the preceding section. (3) Rat corticosteroid-binding globulin possesses, in addition to the one high-affinity binding site, a set of secondary binding sites of lower affinity. This property has recently been found for α_1-acid glycoprotein, another steroid-binding serum protein, in its interaction with several C_{21} steroid hormones (Kerkay and Westphal, 1968). This explanation is a possibility for rat corticosteroid-binding globulin, although difficult to assess since the lability of the protein does not permit standard binding analysis by reciprocal or Scatchard plots. (4) The molecular weight of 61,000 determined by approach to sedimentation equilibrium is somewhat different from the size of the molecular species responsible for steroid binding; this latter entity corresponds to a molecular weight of approximately 53,000, which is compatible with the observed sedimentation coefficient of 3.56 S. Possible reasons for this fourth explanation will be discussed below in connection with the polymeric nature at rat corticosteroid-binding globulin.

Observations on Polymeric Nature. The ultracentrifugation pattern of steroid-depleted rat corticosteroid-binding globulin (Figure 9) indicates the polymeric nature of the protein, as described under Results. The single homogeneous peak obtained after readdition of corticosterone (Figure 10) clearly demonstrates the reversibility of the polymerization and its control by the steroid hormone, as shown in repeated experiments. This readiness of rat corticosteroid-binding globulin to form polymeric structures after removal of corticosterone may well be able to explain the apparent discrepancy between the molecular weights found by the approach to sedimentation equilibrium, and that calculated from the corticosterone content. The ultracentrifugation was performed at 20°. Since the dissociation of the rat corticosteroid-binding globulin–corticosterone complex increases rapidly with temperature rising above 4° (Westphal, 1966), it is possible that dissociation of corticosterone and subsequent partial polymerization occurred during the ultracentrifugation. This could result in the calculation of a somewhat enhanced molecular weight if one assumes a rapid dynamic equilibrium between monomer and polymer in the single peak observed (Figure 8).

Some of the differences in the reported molecular weights of human corticosteroid-binding globulin, which were detailed above, may have a similar basis as the discrepancy between the two values found in the present study for rat corticosteroid-binding globulin. It should be noted that the lowest molecular weight value obtained for human corticosteroid-binding globulin, 45,000 (Seal and Doe, 1962a), was calculated from the cortisol content in a similar way as the lower rat corticosteroid-binding globulin value of 53,000. The highest molecular weight observed for human corticosteroid-binding globulin, *i.e.*, 58,500 (Slaunwhite *et al.*, 1966), was determined by a sedimentation equilibrium technique similar to that which gave the higher value of 61,000 for rat corticosteroid-binding globulin in the present work.

The results obtained by disc gel electrophoresis for the original isolated corticosteroid-binding globulin–steroid complex and for the stripped protein (Figure 11) further substantiate the conclusions drawn from the ultracentrifugation studies. The stripped glycoprotein

(tube 9) demonstrated at least four distinct bands in a regular stepwise pattern with a somewhat diffuse band (shoulder on microdensitometer tracing) constituting the fastest moving component. By analogy, these bands may correspond to the 8.1S, 6.8S, 5.4S, and large 3.4S peaks seen in the ultracentrifuge with the slowly sedimenting shoulder corresponding to the small, fast-moving electrophoretic species. The relative velocities of the migrating bands are in agreement with the values postulated by Marinis and Ott (1965) on the assumption that each electrophoretic species is followed by a dimer of its own molecular size. According to this concept, the ratio of dimer to monomer velocity in polyacrylamide gel is $1:\sqrt[3]{2}$ so that each band should migrate with a rate approximately 1.26 times greater than that of the following protein species (Schultze and Heremans, 1966). The individual ratios of migration rates for each two neighboring, clearly separated bands in tube 9 (Figure 11) have been given under Results. Their average value is 1.25, in close agreement with the ratio of 1.26 calculated for dimerization.

The isolated complex (tube 4) before removal of steroid does not reveal the slowest moving polymer seen with the stripped protein. The two discernible slow-moving bands appear less concentrated than their counterparts in tube 9; concomitantly, the main band and the fastest moving component contain practically all of the protein. The ratios of migration rates of the three clearly separated bands are 1.26 and 1.32 again in close agreement with the theoretical value of 1.26 for dimer formation. The presence of steroid thus appeared to inhibit the formation of the slower moving polymeric forms and enhance the concentration of the smaller, faster moving components. Since the electrophoresis was conducted at room temperature under heat-producing conditions resulting in temperatures of about 35–40° (Davis, 1964), it is assumed that the polymeric pattern observed with the original isolated complex can essentially be ascribed to loss of steroid from the protein due to dissociation during electrophoresis; the particularly high-temperature dependency of the rat corticosteroid-binding globulin–corticosterone complex has been mentioned above. Polyacrylamide electrophoresis also affords separation of interacting species by gel filtration, thus removing steroid from protein; this would further enhance the dissociation of the steroid–protein complex.

The general similarity between rat, human, and rabbit corticosteroid-binding globulin is evident. However, the specific affinity of rat corticosteroid-binding globulin for individual steroids differs from that demonstrated by the human or the rabbit protein. The glycoproteins also vary significantly in amino acid composition. The higher half-cystine content in the rat and rabbit proteins in comparison with human corticosteroid-binding globulin may be a significant factor in the greater polymeric tendency of the two animal proteins. The human corticosteroid-binding globulin apparently does not readily polymerize (Seal and Doe, 1962a; Muldoon and Westphal, 1967); the polymeric nature of rabbit corticosteroid-binding globulin as well as factors influencing the determination of its molecular weight will be discussed elsewhere. To what extent the observed differences in the sulfur-containing and certain other amino acids are related to the different steroid-binding affinities of the three species (Westphal, 1967) is not clear. This question as well as the nature of corticosteroid-binding globulin polymerization are being further investigated.

The similarity in chemical and general physical properties between the three corticosteroid-binding globulin proteins and the thyroxine-binding globulin of human blood as described by Seal and Doe (1964) deserves mention. The amino acid compositions are close, and the carbohydrate content of about 32% in thyroxine-binding globulin approximates that observed for rat, rabbit (Chader and Westphal, 1968; Ganguly and Westphal, 1968), and human (Muldoon and Westphal, 1967) corticosteroid-binding globulin. A sedimentation coefficient of 3.6 S for thyroxine-binding globulin (Seal and Doe, 1964) is indistinguishable from values found for rat and rabbit corticosteroid-binding globulin, and the molecular weight of 59,000 is close to those determined for the isolated corticosteroid-binding globulin preparations. Both types of glycoproteins have one high-affinity binding site for their respective hormones. It thus appears that one class of proteins with closely related characteristics is responsible for the binding in serum of several steroid hormones as well as that of thyroxine. It is worthwhile to consider the role this type of ligand-binding glycoprotein might play in the movement of vitally important hormones at the cellular level.

The observed control of the polymerism of the corticosteroid-binding globulin molecule by the steroid hormone as demonstrated by ultracentrifugation and polyacrylamide gel electrophoresis is considered of particular significance since the interaction between steroid and corticosteroid-binding globulin is of a highly specific nature and fulfills a distinct physiological function.

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