Transcortin. A Comparison of the Cortisol-Binding Globulin from Human and Cavian Plasma*

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ABSTRACT: The isolation and purification of corticosteroid-binding globulin (transcortin) from the blood plasma of man and guinea pig was accomplished. The human and guinea pig proteins were homogeneous when examined in disc electrophoresis and in the analytical ultracentrifuge. The amino acid and carbohydrate compositions of the two proteins were determined. Human transcortin contained 18% carbohydrate by weight and guinea pig (cavian) 25% carbohydrate. The molecular weight of human transcortin was determined by sedimentation equilibrium to be 55,700 with an $s_{20,w}$ of 3.16 S.

An $s_{20,w}$ value of 3.25 S was determined for cavian transcortin.

A spontaneous self-association of cavian transcortin in solution was observed which was promoted by the presence of *p*-chloromercuribenzoate and by the absence of cortisol. The association of cavian transcortin with cortisol was inhibited by *p*-chloromercuribenzoate. Association constants at 6° were determined for both human transcortin (1.7×10^8 M^{-1}) and cavian transcortin (0.74×10^7 M^{-1}) with cortisol by equilibrium dialysis.

The examination of structure–function relationships involved in the interaction of steroid hormones with proteins will be facilitated by investigation of the corticosteroid-binding globulin present in low concentration in the plasma of man and other species (Sandberg *et al.*, 1966). The preparation of a highly purified CBG, which has been named transcortin (Slaunwhite and Sandberg, 1959), from the plasma of normal and estrogen-treated human subjects was first reported by Seal and Doe (Seal and Doe, 1962a). Preliminary efforts by Slaunwhite (Slaunwhite *et al.*, 1966) provided information regarding the molecular weight and chemical composition of transcortin isolated from plasma of male subjects treated with diethylstilbestrol.

Limited evidence exists for the presence of a corticosteroid binding capacity in the plasma of over 130 different species (Slaunwhite and Sandberg, 1959; Seal and Doe, 1963; Westphal and DeVenuto, 1966). Recently, Westphal and coworkers have reported the isolation and characterization of corticosteroid-binding globulin from the rabbit and the rat (Chader and Westphal, 1968a,b). The reported presence of a binding globulin with a high association constant for cortisol in guinea pig plasma prompted the isolation of transcortin from the plasma of pregnant guinea pigs in which the amount of this protein is markedly elevated (Rosenthal *et al.*, 1969).

In order to obtain sufficient amounts of material for study, it was necessary to modify methods previously reported. The use of hydroxylapatite is critical in the isolation of transcortin. Remarkably specific elution analysis of both human and guinea pig proteins can be achieved when attention is given to the quality of the adsorbent. The method used in the present study controls methodological variations more effectively than any of the reported methods. Application of a modified purification procedure to human and cavian plasma resulted in the preparation of homogeneous human and cavian transcortin. A comparison of the properties of pure human and cavian transcortins was considered to be of great interest since it can provide a preliminary basis for an understanding of the characteristics of a protein which are relevant to interaction of a specific steroid, cortisol.

Materials and Methods

All water used throughout the entire procedure was passed through a mixed-bed ion-exchange column (Barnstead 0808) which was attached to a distilled water outlet. Crystalline cortisol was purchased from Merck, Sharpe, and Dohme and [1,2-^{3}H]cortisol (123 μCi/mg) and [4-^{14}C]cortisol (0.15 μCi/mg), dissolved in benzene, from the New England Nuclear Corp. *N,N*-Diethylaminoethylcellulose was purchased from the Eastman Chemical Co. of Rochester, N. Y. Sephadex G-25 in the bead form and G-100 in the block form were obtained from Pharmacia Fine Chemicals, Inc., New Market, N. J. Hydroxylapatite as the dry powder was purchased from Bio-Rad Laboratories, Richmond, Calif. Variations in the adsorptive quality of the commercial product required use of an adsorption assay procedure which was adopted as a routine step in the preparation of the adsorbent. The hydroxylapatite used for the isolation of the cavian transcortin was prepared in accord with the detailed instructions of Tiselius (Tiselius *et al.*, 1956) and Levin (Levin, 1962). It was found necessary to test each preparation of hydroxylapatite utilized in the procedure to give assurance that material of the same adsorptive capacity was used in all experiments. Human serum albumin (American Red Cross, salt poor as a 25% aqueous solution) at a concentration of 5 mg/ml was dialyzed against 0.001 M sodium phosphate buffer (pH 6.8) for 12–16 hr at 6°. One milliliter was added to a tracer amount of [4-^{14}C]-cortisol (0.001 μCi, 8.4 ng) and placed on a 0.9×10 cm column of hydroxylapatite. Since cortisol is not adsorbed by hydroxylapatite, the elution of radiolabeled cortisol can be used as a convenient indication of the solvent volume of the

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column. Elution with 0.001 M sodium phosphate buffer was continued until approximately twice the total bed volume of the column had been collected. If the adsorbent was acceptable, albumin was eluted exclusively with 0.1 M sodium phosphate buffer (pH 6.8).

Human blood plasma was obtained from heparinized blood drawn from female subjects who had been maintained on a regimen of 15 mg of diethylstilbesterol/day for 5 days. The plasma was stored in the frozen state until used. Guinea pig plasma was obtained from two different sources. Blood was withdrawn from pregnant animals obtained from Riverside Aviaries, Holland, N. Y., on approximately the 60th day of a 65-day gestation period. Plasma was stored frozen for approximately 5 months before use. Additional plasma, drawn after 50–55-days pregnancy, was obtained from Pel-Freez Biological, Inc., Rogers, Ark. The plasma was shipped frozen and used within 2 weeks of its arrival.

Isolation of Transcortin. All chromatographic steps were performed at 6°. DEAE-cellulose chromatography and subsequent desalting on Sephadex G-25 were performed as previously described (Slaunwhite *et al.*, 1966). The resin was equilibrated with starting buffer which was 0.025 M in NaCl and 0.015 M in NaH_2PO_4 at pH 5.0. The pH of the starting buffer was modified to accommodate the cavian plasma. The DEAE-cellulose used for guinea pig plasma was adjusted to pH 5.5 prior to the packing of the column and this pH was maintained throughout the entire elution process. The column was equilibrated with initial buffer which contained 1.3 μCi of [4-^{14}C]-cortisol per ml (0.43 μCi/nmole) in order to minimize the dissociation of cortisol from the cavian transcortin. The presence of radioactive cortisol was detected in only the most active fractions in the DEAE step.

Approximately 400 ml of human plasma was applied to a 4 × 40 cm column and 1 μCi of [4-^{14}C]cortisol (7–8 μg) in 10 ml of starting buffer was added directly to the top of the column after the plasma had completely filtered into the adsorbent bed. Because of the lowered affinity of cortisol for cavian transcortin, the cavian plasma (200 ml) was equilibrated prior to charging with 1 mg of carrier cortisol and 1 μCi (8 μg) of [^{3}H]cortisol for 5 hr at 6°. Elution with the starting buffer at a flow rate of 0.8–1 ml/min was continued until the absorbance of the eluate at 280 nm was between 0.2 and 0.3. A linear gradient was started which consisted of 1:9 (v/v) 0.2 M NaH_2PO_4–0.09 M NaCl at pH 5.0 for human plasma and pH 5.5 for cavian plasma. All fractions eluted after the start of the gradient were analyzed for radioactivity and absorbance at 280 nm. The cortisol-binding activity of each lyophilized pool was determined.

Sephadex G-100 columns were packed at 6° in 0.001 M sodium phosphate buffer (pH 6.8) under downward-flow conditions utilizing a combination of a syphon outlet and a facsimile of a constant-pressure Mariotte flask for the buffer feed. Lyophilized, desalted Sephadex G-25 eluates were dissolved in an appropriate volume of water which contained an additional 0.22 μCi (20 μg) of [4-^{14}C]cortisol/ml. Sample volume was never in excess of 1% of the total bed volume. The concentration of the sample was maintained at 8–25 *A* units/ml for a 3.7 × 120 cm column. The cortisol–protein solution was equilibrated for a period of 3–5 hr at 6° and applied to the column. Elution with 0.001 M phosphate (pH 6.8) was accomplished at a flow rate of 10–15 ml/hr. After approximately two-thirds of one void volume had been eluted, 1-ml fractions were collected. Since the lower affinity of cavian transcortin for cortisol resulted in the complete dissociation of the protein–steroid complex, the fractions were pooled in sequence according to their absorbance at 280 nm. All pools were lyophilized the same day they were eluted and stored at −5°. Equilibrium dialysis against cortisol was performed on all lyophilized pools to determine which pools had significant binding activity.

Measurement of radioactivity was performed in a Packard Tri-Carb liquid scintillation spectrometer Model 3375. The following scintillation medium was used: 833 ml of dioxane, 176 ml of 2-ethoxyethanol, 50 g of naphthalene, and 5 g of 5-diphenyloxazole. This medium permitted assay of [^{14}C]-cortisol at a maximum efficiency of 86% and of [^{3}H]cortisol at 27%. An aliquot of each lyophilized fraction pool was examined in polyacrylamide gel electrophoresis in the pH 9.5 system (Ornstein, 1964; Davis, 1964).

Hydroxylapatite chromatography of human protein was performed on columns which were packed at 6° under conditions which permitted a final gravity flow rate of 0.3–0.5 ml/min. If the removal of fines was complete, this flow rate was readily established. Hydroxylapatite was equilibrated in 0.001 M phosphate (pH 6.8) and columns were packed 1 day prior to their use. The dimensions of the column were chosen to provide a height to diameter ratio of not less than 20:1 nor greater than 30:1 and to provide 1 ml of bed volume for each 0.5–1 *A* unit of protein applied. Columns of 1.5 × 40 cm dimensions were loaded with 2–6 ml of protein solution containing 20–60 *A* units. The lyophilized eluate pools from Sephadex G-100 columns which contained the [4-^{14}C]cortisol peak and coincident binding activity as assayed by equilibrium dialysis at 6° were applied to hydroxylapatite columns. The material for use was dissolved at room temperature in an aqueous solution which was 0.0005 M in sodium phosphate and 0.001 M in DL-ascorbic acid adjusted to a pH of 6.8. The protein solution was used to dissolve 0.01 μmole of cortisol and [^{14}C]cortisol for each 5 *A* units of protein. The protein–steroid solution was dialyzed against 200 times its volume of ascorbate–phosphate buffer for a period of 3–5 hr at 6°. Elution was performed initially with 0.001 M sodium phosphate buffer at a flow rate of not more than 30 ml/hr. Fractions of 3–4 ml were collected. When a [4-^{14}C]cortisol peak had been eluted from the column, a stepwise increase to 0.02 M sodium phosphate buffer (pH 6.8) was performed. The fractions which contained both the material absorbing at 280 nm and radiolabeled cortisol were pooled, lyophilized, and stored at −5°.

For the isolation of cavian transcortin hydroxylapatite was thoroughly equilibrated in the cold with 0.005 M phosphate buffer prior to use. The preparation of a cavian sample differed from that of the human protein. The concentration of protein was maintained at 25 *A* units/ml and the volume of the packed bed was chosen so that 0.35 *A* unit was applied per ml of total bed volume. This resulted in the application of 1 ml containing 25 *A* units to a 1.5 × 40 cm column. A large excess of cortisol (280 μg, 0.08 μCi) was added to the protein solution to stabilize the transcortin. The cortisol–protein solution was dialyzed for 5–9 hr at 6° against 100 volumes of 0.001 M phosphate buffer. Elution was accomplished with 0.005 M phosphate buffer in 3-ml fractions at a flow rate of 15 ml/hr.

Amino acid and carbohydrate composition analyses were performed on transcortin desalted on columns of Sephadex G-25. Lyophilized protein was equilibrated with air, and the moisture content was estimated by heating duplicate samples to constant weight *in vacuo* at 105°. Hydrolysis of both transcortins for amino acid composition was performed at a concentration of 0.05% in 6 N HCl for 22 hr at 110°. The analysis was performed at 50° on a Spinco Model 120C automatic re-

cording amino acid analyzer.[1] Tryptophan was determined spectrophotometrically (Beaven and Holiday, 1952).

The neutral monosaccharides were determined by the method of gas–liquid chromatography of their *O*-acetylglycitols (Lehnhardt and Winzler, 1968).[2] The amino sugars were determined using the amino acid analyzer. The sialic acid content of human transcortin was determined using Ehrlich's reagent while that of cavian transcortin was determined by the thiobarbituric acid method of Warren (1959).

Sedimentation velocity measurements of human and cavian transcortin were performed in a Spinco Model E ultracentrifuge.[3] Human transcortin was examined at a concentration of 0.78% in 0.1 M sodium phosphate buffer (pH 6.8). Measurements of cavian transcortin were performed at a concentration of 0.85% in 0.1 M sodium phosphate buffer (pH 6.8). The apparent $\bar{V}$ of human and cavian transcortin was calculated from the compositional analysis (Cohn and Edsall, 1943; Gibbons, 1966).

The molecular weight of human transcortin prepared in the present study was determined as previously described (Slaunwhite *et al.*, 1966).

Measurements of the association constants of cortisol with human and cavian transcortin were performed by equilibrium dialysis at 6° (Sandberg *et al.*, 1966). Human transcortin eluted from hydroxylapatite columns contained less than 1 mole of cortisol per mole of protein. The removal of endogenous cortisol prior to equilibration with measured increments of cortisol was not attempted since preliminary experiments indicated that complete removal inactivated the protein. Five quantities (0.31–1.57 nmoles) of cortisol and [^{3}H]cortisol contained in 3 ml were equilibrated with 0.16 mg of transcortin–cortisol complex which contained approximately 1.1 nmoles of cortisol and [4-^{14}C]cortisol. The use of a three-channel liquid scintillation counter permitted the calculation of disintegrations per minute of both [^{3}H]cortisol and [4-^{14}C]cortisol from one sample. The *total* amount of cortisol in each duplicate set of samples was 1.42, 1.64, 1.87, 2.04, and 2.30 nmoles equilibrated with 2.9 nmoles of human transcortin.

Binding parameters for the interaction of cortisol with cavian transcortin were determined in a slightly different manner. Cortisol-free cavian transcortin (220 μg) was equilibrated with five different amounts of cortisol for 30 min at room temperature and 30 min in a 0° ice bath. The steroid was preequilibrated with the protein solution to prevent inactivation throughout the duration of the 48-hr dialysis. Each of the protein solutions contained either 3.45, 4.14, 5.48, 8.22, or 10.96 nmoles of cortisol. The protein–cortisol complex solutions were dialyzed at 6° for 48 hr against 3 ml of 0.1 M sodium chloride–0.01 M sodium phosphate (pH 7.4). At the end of this time 0.5-ml aliquots of the contents of the dialysis casing and of the dialysate were assayed for radioactivity as previously described.

Cavian transcortin which had been preequilibrated with 5 μg of cortisol/mg of protein was also dialyzed to equilibrium in the presence of cortisol and *p*-chloromercuribenzoate (PCMB).[4] The association of 1–2.5 μg of added and [^{3}H]-cortisol with transcortin (250 μg) was measured in the presence of 0.45 μmole of PCMB under the routine conditions of equilibrium dialysis.

Polyacrylamide gel electrophoresis was used to examine the behavior of cavian transcortin in the presence of PCMB. The 7.5% acrylamide gel (pH 9.5) was prepared from the Canal Corp. premixed solutions. The gels were stained in 1% aniline blue-black and excess dye was removed by bleaching in 7% acetic acid. Approximately 250 μg of cavian transcortin was used for each gel column. The examination of the effect of PCMB upon the electrophoretic pattern of cavian transcortin was done in the presence and absence of cortisol. The order of addition of PCMB and cortisol to solutions of the steroid-free protein was varied in order to evaluate any protective effect of the steroid. Approximately equimolar quantities of transcortin[5] and PCMB were equilibrated at room temperature for 1 hr prior to electrophoresis. Aliquots of the same transcortin solution were equilibrated with cortisol both prior to the addition of PCMB and after the addition of the reagent to the protein. In a typical experiment 250 μg of transcortin was equilibrated with 0.03 μmole of PCMB and 0.06 μmole of cortisol. All samples with the exception of the control were examined after standing at room temperature for 1 hr. Samples of cavian transcortin (200 μg) which had stood in solution for 5 days at 6° were also examined.

Results

Chromatography of human and guinea pig plasma on DEAE-cellulose produced elution patterns which have been described previously (Slaunwhite *et al.*, 1966; Sandberg *et al.*, 1966). The Sephadex G-100 step increased the purity of the transcortin to an extent which permitted the use of a single hydroxylapatite column step to obtain homogeneous human and cavian transcortins. The elution patterns of radioactive cortisol and protein from typical Sephadex columns are presented in Figure 1. Human protein fractions which contained radiolabeled cortisol were pooled. All binding activity was found in the protein eluted with the [4-^{14}C]cortisol. Cavian plasma protein fractions eluted from G-100 in the second absorbance peak seen in Figure 1 were active.

The specific desorption of both human and cavian transcortin from hydroxylapatite at a low molarity of phosphate buffer is unique among the plasma proteins. This behavior has been observed to be characteristic of the corticosteroid-binding globulins from different species (Seal and Doe, 1963) and has been successfully applied in the recently reported purification of rabbit and of rat corticosteroid-binding globulin (Chader and Westphal, 1968a,b).

The desorption of transcortin from hydroxylapatite requires careful coordination of the adsorptive capacity of the hydroxylapatite, of the effect of the mutual displacement of proteins, and of the molarity of the eluting buffer. Figure 2 presents the results of a typical hydroxylapatite column fractionation of human and cavian protein eluted from Sephadex G-100. Desorption of human transcortin occurred at a concentration of 0.02 M. The cortisol present in the protein solution applied to the column was eluted exclusively with the 0.02 M peak. The protein recovery in this eluate from any individual hydroxylapatite column was always between 8 and 12% of the protein applied. Elution of cavian transcortin–steroid complex from a column of hydroxylapatite occurred at a concentration

[1] The amino acid compositions were determined in a laboratory of Dr. Richard Winzler under the direct supervision of Dr. Peter Weber.

[2] The carbohydrate compositions were determined in the laboratory of Dr. Richard Winzler.

[3] Sedimentation velocity experiments were performed in the laboratory of Dr. Michael Laskowski, Sr.

[4] Abbreviation used is: PCMB, *p*-chloromercuribenzoate.

[5] A molecular weight of 60,000 was assumed on the basis of a preliminary sedimentation equilibrium analysis of cavian transcortin.

TABLE I: Recoveries of Protein and Cortisol in Each Step in Purification of 750 ml of Human Plasma and 200 ml of Cavian Plasma.

	Human						Cavian		
	Protein			Radioactivity			Protein		
		Recovd in Act. Fractions		Applied (dpm × 10^6)	Recovd in Act. Fractions			Recovd in Act. Fractions	
Step	Applied (g)	g	%		dpm × 10^6	%	Applied (g)	g	%
DEAE-cellulose	52.50	1.37	2.6	4.22	3.65	86	14	0.279	2
Sephadex G-100	1.180	0.27	23	3.35	2.18	65	0.142	0.074	52
Hydroxylapatite	0.106	0.011	10	1.31	0.92	70	0.035	0.016	49

of 0.005 M phosphate (Figure 2). Protein–steroid association was maintained since neither the steroid nor the cavian transcortin is adsorbed at the buffer concentration of 0.005 M.

Recovery of protein and cortisol at each step in the purification procedure are pesented in Table I. Calculation of grams of protein was done using a value of $E^{1\%}_{1\text{ cm}}$ at 280 nm of 7.4. Losses due to analytical procedures performed at each step are not included in the calculations. The yield of human transcortin was equal to approximately 0.06% of the total protein in the original plasma. A higher yield of cavian transcortin of 0.5% of the total protein was obtained from the plasma of pregnant animals.

Examination of preparations of human and cavian transcortins in polyacrylamide gel electrophoresis revealed the presence of one band in both cases.

Examination of final preparations of human and cavian transcortins in the ultracentrifuge demonstrated the presence of one sedimenting species. An $s_{20,w}$ value of 3.16 S for human transcortin and a 3.25 S for cavian transcortin was calculated. Sedimentation equilibrium analysis of a 0.05% solution of human transcortin permitted the calculation of a molecular weight of 55,700.

The amino acid and carbohydrate compositions of transcortins isolated from the plasma of humans, guinea pig, and other species are presented in Table II–IV. Calculation of values for tyrosine from spectrophotometric measurements resulted in a value of 2.46 g/100 g of human transcortin and of 1.89 g/100 g of cavian transcortin.

The association constants for cortisol and human and cavian transcortin were determined from a modified Scatchard plot analysis of data obtained from equilibrium dialysis at 6°. The results of the equilibration of both transcortins with increasing amounts of cortisol and [^{3}H]cortisol are presented in Figure 3. From the slopes of the straight lines calculated by the method of least-squares k_{assoc} values of 1.7 × 10^8 M^{-1} for human transcortin and of 0.97 × 10^7 M^{-1} for cavian transcortin were obtained. The cavian value is recognized as an estimate. Due to the shortage of pure cavian transcortin, these experiments could not be extended.

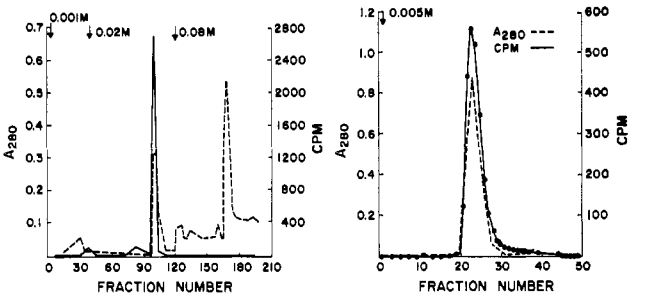

FIGURE 2: Hydroxylapatite chromatography of human (left) and cavian (right) transcortin on 1.5 × 40 cm columns. Human transcortin was eluted with 0.02 M phosphate and cavian transcortin by 0.005 M phosphate.

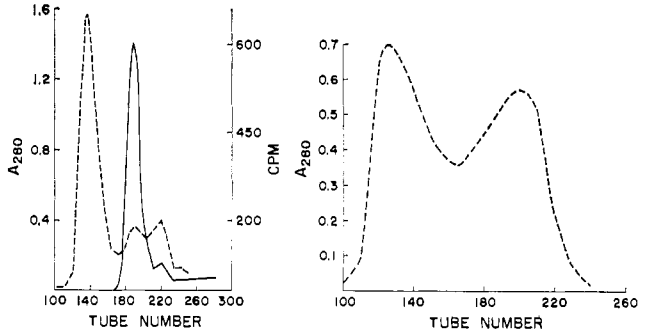

FIGURE 1: Sephadex G-100 column chromatography of purified human (left) and cavian (right) transcortins. Column dimensions were 3.7 × 120 cm, flow rate was 10 ml/hr, and fraction volume was 1 ml. (---) A_{280}, (—) counts per minute. Fractions 180–200 were pooled from each column for further purification. See text for details.

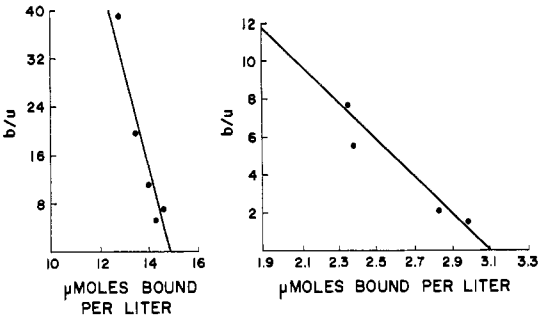

FIGURE 3: Scatchard plot analysis of the results of equilibrium dialysis of 2.7 nmoles of human transcortin (left) and 250 μg of cavian transcortin (right) against cortisol at 6°. The straight lines were calculated by the method of least squares. From the slopes of each line, k_{assoc} was computed and $n[P_t]$ was calculated from the intercept on the abscissa.

TABLE II: Amino Acid and Carbohydrate Composition of Human and Cavian Transcortin.

Residue	Human: g of Residue/100 g of Glycoprotein[a]	Human: No. of Residues/Mole of Glycoprotein[b]	Cavian: g of Residue/100 g of Glycoprotein[a]
Lysine	3.79	16.5	3.29
Histidine	2.03	8.5	2.65
Arginine	3.47	12.3	4.27
Aspartic acid	9.37	45.3	9.06
Threonine	4.97[c]	27.4	4.40[c]
Serine	5.14[d]	32.9	5.76[d]
Glutamic acid	8.56	36.9	7.94
Proline	3.33	19.0	1.77
Glycine	2.80	27.3	1.86
Alanine	4.01	31.3	3.74
Half-cystine	0.38[e]	2.1	0.33[e]
Valine	4.96[f]	27.9	2.66[f]
Methionine	1.71[e]	7.3	0.57[e]
Isoleucine	3.53	17.4	3.88[f]
Leucine	9.65	47.5	10.52
Tyrosine	2.70	9.2	1.71
Phenylalanine	5.94	22.5	5.91
Tryptophan	1.26[g]	3.7	0.65[g]
Galactose	2.90	9.9	3.89
Mannose	2.88	9.9	4.44
Glucose	1.07	3.7	
Fucose	0.34	1.2	0.13
Galactosamine			0.25[h]
Glucosamine	5.06[h]	17.5	5.26[h]
N-Acetylneuraminic acid	3.29	6.3	10.73

[a] Hydrolyzed in 6 N HCl at 110° for 22 hr. [b] Calculated on the basis of a molecular weight of 55,700. [c] Corrected for destruction during hydrolysis (5.3%). [d] Corrected for destruction during hydrolysis (10.5)%. [e] Uncorrected for destruction during hydrolysis. [f] Uncorrected for incomplete hydrolysis. [g] Determined by spectrophotometric method. [h] Calculated as the free base.

Self-Association of Cavian Transcortin. Polyacrylamide gel electrophoresis of 200 μg of cortisol-free cavian transcortin, which had been stored at −5° in the lyophilized state for 2 months, revealed the presence of a minor second component. Subsequent attempts at removal of the second component by rechromatography on G-100 and on hydroxylapatite produced material with an increased concentration of the second slower component. The results of the examination of the protein in polyacrylamide gel electrophoresis (Figure 4) demonstrated the spontaneous formation of aggregate forms of the protein upon aging in solution. Transcortin (cortisol free) was left in dilute solution at pH 6.8 (2 mg/ml) at room temperature for 1 and 2 hr and at 5° for 5 days. The appearance of an increased amount of a second band after 2 hr at room temperature can be seen by comparison of gel A with gel B in Figure 4. After 5 days at 5° the same material displayed two to three additional bands as presented in gel C.

TABLE III: Amino Acid Composition of Transcortins Isolated from Different Species.

Residue	No. of Residues/10^5 g of Polypeptide: Human	Cavian	Rat[a]	Rabbit[b]
Lysine	35.80	35.02	51.79	35.88
Histidine	18.54	27.18	19.30	21.70
Arginine	27.01	37.32	25.67	33.41
Aspartic acid	98.78	107.64	103.30	85.49
Threonine	59.63	59.53	60.62	66.25
Serine	71.65	90.36	71.87	71.37
Glutamic acid	80.46	84.02	111.67	103.22
Proline	41.49	42.52	44.16	54.66
Glycine	59.40	44.68	55.37	64.30
Alanine	68.36	72.02	59.92	72.02
Half-cystine	4.45	2.95	22.48	20.35
Valine	60.71	36.71	51.94	61.52
Methionine	15.85	5.94	20.51	11.50
Isoleucine	37.81	46.91	34.45	33.04
Leucine	103.46	127.05	88.53	88.79
Tyrosine	20.03	14.33	23.73	24.33
Phenylalanine	48.98	54.89	37.30	35.11
Tryptophan	8.10	4.77	9.77	18.20

[a] Chader and Westphal (1968b). [b] Chader and Westphal (1968a).

TABLE IV: Carbohydrate Composition of Transcortin from Different Species.

Residue	No. of Residues/10^5 g of Glycoprotein: Human	Cavian	Rat[c]	Rabbit[d]
Galactose	17.8	23.9		
Mannose	17.7	27.7		
Glucose	6.6			
Fucose	2.3	1.06	14.3	5.5
Galactosamine		1.55		
Glucosamine	31.4	32.6		
N-Acetylneuraminic acid	11.3	36.8	21.9	29.2
Total hexose	42.1[a]	51.6[a]	60.4[b]	64.1[b]
Total hexosamine	31.4[a]	34.2[a]	58.9[b]	58.9[b]

[a] Arithmetic sum of individual values. [b] Determined as total. [c] Chader and Westphal (1968b). [d] Chader and Westphal (1968a).

It has been previously noted that it was not possible to maintain saturation of the cavian protein with cortisol during Sephadex chromatography. The material examined in gel D of Figure 4 was depleted of cortisol by passage through a Sephadex G-200 column. When excess cortisol was added to an aliquot of the same protein prior to chromatography, a reduction in the number and a change in the intensity of the bands were observed as presented in gel E of Figure 4. The

TABLE V: Physicochemical Properties of Transcortin from Different Species.

Parameter	Human				Cavian	Rabbit	Rat
	Seal and Doe (1966)	Slaunwhite *et al.* (1966)	Muldoon and Westphal (1967)	Present Study	Present Study	Chader and Westphal (1968a)	Chader and Westphal (1968b)
M_{app}	52,000	58,500	51,700	55,700		40,700	61,000
$s_{20,w}$ (S)	3.0	4.1		3.16	3.25		
$s^0_{20,w}$ (S)			3.8			3.55	3.56
V			0.708	0.718	0.703	0.695	0.711
k_{assoc}, 4° (M^{-1})	5×10^8		5.2×10^8	1.7×10^8	0.97×10^7	9×10^8	5.1×10^8
$E^{1\%}_{1\ cm}$ 280 nm	7.4		7.4	7.1	7.1	8.4	6.2
$A_{280/260}$	1.74[a]		1.13[b]	1.31[b]	1.46[a]	1.38[a]	1.71[a]

[a] Steroid free. [b] Steroid complex.

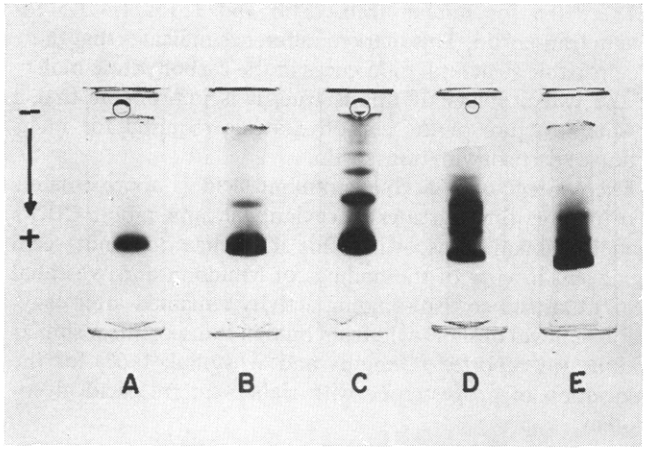

FIGURE 4: Disc electrophoresis of cavian transcortin showing the effect of aging in solution. Electrophoresis was in 7.5% acrylamide gel pH 9.5 from cathode to anode. Gel A is 200 μg of cavian transcortin after 1 hr and gel B is the same preparation after standing 2 hr at room temperature prior to electrophoresis. Gel C is the same preparation after standing in solution for 6 days at 5°. Gel D is 325 μg of a preparation of cavian transcortin which was depleted of cortisol by passage through a Sephadex G-200 column and gel E is the same material which was equilibrated with excess cortisol prior to the same treatment.

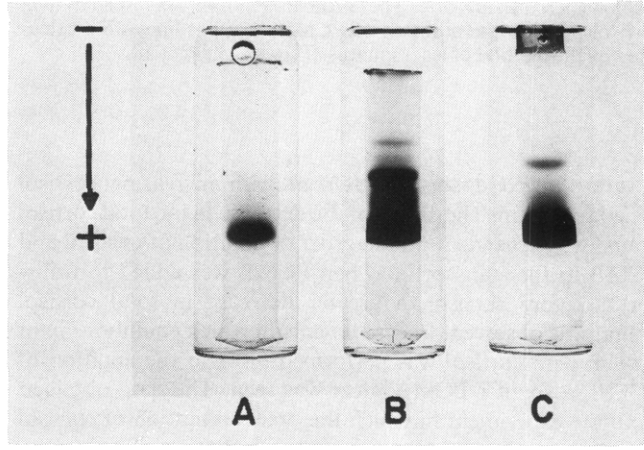

FIGURE 5: Analytical disc electrophoresis of cavian transcortin in the presence of *p*-chloromercuribenzoate and cortisol. Gel A is 200 μg of a final preparation of transcortin; gel B is 250 μg of transcortin mixed with a 10-fold molar excess of PCMB; and gel C is 250 μg of transcortin equilibrated with excess cortisol prior to addition of 10-fold molar excess of PCMB.

amount of protein analyzed on both gels D and E of Figure 4 is the same. The increase in intensity of the fastest band of gel E of Figure 4 appears to correlate with the disappearance of the slower migrating components. The apparent increase in the band second from the front is evident when gel C is compared to gel A (Figure 4). These additional, "slower" bands seen in disc electrophoresis gels indicate that a spontaneous aggregation of the protein occurs in solution in the absence of cortisol. The formation of aggregate forms has been observed by Chader with corticosteroid-binding globulin from the rat (Chader and Westphal, 1968b).

Cavian transcortin was examined in the presence of a 10-fold molar excess of PCMB.[6] A comparison of the control with a gel A sample containing a 10-fold molar excess of PCMB (Figure 5, gel B) demonstrates the increase in intensity of a second band as well as the appearance of a third. However, when a 10-fold molar excess of cortisol was added to the protein prior to the addition of PCMB, this effect diminished (Figure 5, gel C). The addition of PCMB increases the number of bands detected as did the aging of the protein in solution. Cortisol did not prevent aggregation unless equilibrated with the protein prior to the addition of PCMB. The aggregation of cavian transcortin was not observed to be reversed by cortisol. The presence of the steroid reduced the number of bands seen in polyacrylamide gel only when it was added to the transcortin before self-association occurred.

Inactivation of Cavian Transcortin by Aggregation. The aggregate forms of cavian transcortin produced by aging or by the action of PCMB were found to reduce the cortisol binding activity of the protein. Examination of the dilute solutions stored overnight at 4° in the absence of cortisol revealed complete loss of high binding affinity. Equilibrium dialysis of 3 nmoles of cavian transcortin in the presence of a 30-fold molar excess of PCMB produced a substantial decrease in the amount of cortisol bound by the transcortin. Since inhibition of binding was never complete and cortisol was present in slightly less than saturation amounts, the total cortisol bound

[6] A molecular weight of 60,000 was assumed on the basis of a preliminary sedimentation equilibrium analysis of cavian transcortin.

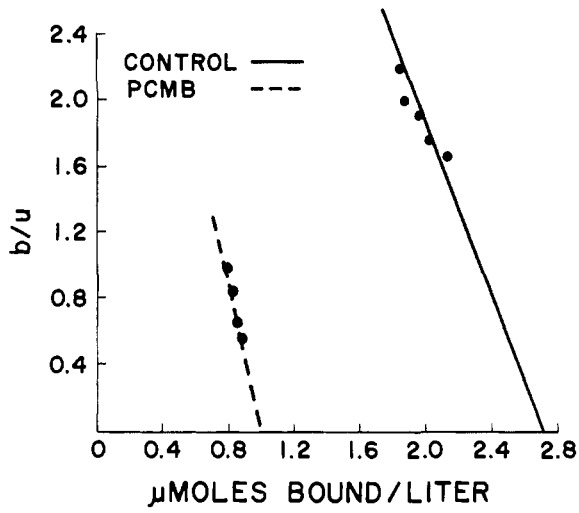

FIGURE 6: Scatchard plot analysis of the results of equilibrium dialysis of cavian transcortin at 6° in the presence and absence of *p*-chloromercuribenzoate. The protein solution was mixed with cortisol prior to the addition of PCMB. Straight lines were calculated by the method of least squares. [P_t] was 250 μg/3 ml.

always showed a small increment with increasing cortisol added to system. The extent of the decrease in the total cortisol bound was dependent on the order of addition of cortisol and PCMB to the transcortin; when PCMB was added to transcortin before cortisol, a greater decrease in total cortisol bound was observed. In experiments in which equilibration of protein with cortisol was performed prior to the addition of PCMB, a smaller PCMB effect was seen. The data obtained from the experiment in which the preequilibration of cortisol with transcortin was done were submitted to graphical analysis using the modified Scatchard plot (Figure 6). From the control points a value for $n[P_t]$ (total binding sites) of 2.71 μmoles was calculated. The data obtained from samples containing PCMB produced an estimate for $n[P_t]$ of 0.99 μmole. The reduction in total number of binding sites by PCMB explains the apparent inhibition of cortisol binding.

Discussion

The method for the isolation of transcortin reported in the present study controls methodological variations more effectively than any previously reported procedures. The unique desorption of transcortin from hydroxylapatite at low concentration of phosphate buffer is the essential step of all purification procedures for transcortin from human (Seal and Doe, 1962b; Slaunwhite *et al.*, 1966; Muldoon and Westphal, 1967) rabbit, and rat (Chader and Westphal, 1968a,b) plasmas. The present modification requires the use of only one hydroxylapatite column, which improves the efficiency and reproducibility of the entire procedure.

The amino acid and carbohydrate composition of human transcortin reported in the present study compares favorably with the values of Muldoon and Westphal (Muldoon and Westphal, 1967). Improved values for the content of carbohydrate residues were obtained in the present study. This can be attributed to the increased reliability of the gas–liquid chromatography assays used in place of colorimetric assays (Seal and Doe, 1963; Muldoon and Westphal, 1967).

A comparison of the properties of the two transcortins isolated in the present study reveals a high degree of similarity in chemical composition and physiochemical characteristics. Winzler (1960) has suggested that distinctive molar ratios of carbohydrate components of human plasma glycoproteins are indicative of probable unique structural features of the oligosaccharides attached to the polypeptide. The significance of such differences in the carbohydrate composition is emphasized by the human blood group specific substances whose immunological specificity is known to be a consequence of their carbohydrate content (Pusztai and Morgan, 1964). The amino acid compositions of these substances are, on the contrary, very similar to one another (Winzler, 1960) and to those of the submaxillary glycoproteins from ovine, bovine, and canine sources (Gottschalk and Simmonds, 1960). Comparison of the compositions of corticosteroid-binding globulins which have been isolated from the plasma of four different species indicates that the probable differences in the nature and structure of the carbohydrate portions of the molecules are not pertinent to steroid binding. The molar ratios of galactose:mannose:hexosamine:sialic acid per 10^5 g of glycoprotein are 1:1:1:8:0.6 for human transcortin and 1:1:2:1.4:1.5 for cavian transcortin. This marked difference indicates that there are probable structural differences in the carbohydrate moiety of the two proteins. If this is true, it is improbable that a unique structure of the carbohydrate is required for interaction of cortisol with transcortin.

The content of *N*-acetylneuraminic acid is approximately two to three times greater in cavian, rat, and rabbit CBG's than in human transcortin. This difference does not seem significant in view of the findings of Muldoon and Westphal (1967) that full cortisol-binding activity remained after enzymatic removal of sialic acid from human transcortin. A similar finding was reported (Ganguly and Westphal, 1968) for the association of progesterone with sialic acid free acid glycoprotein.

The tabulation of the amino acid compositions of transcortins from different species (Table III) demonstrates the similarity in composition of the proteins from four different animals. It is evident that human and cavian transcortin have low half-cystine contents, typical of human plasma glycoproteins (Heimburger *et al.*, 1964). The rat and rabbit proteins have a markedly higher half-cystine content. Examination of a substantial number of human plasma glycoproteins (Heimburger *et al.*, 1964), however, does not substantiate the contention of Chader and Westphal (1968b) that similarities in amino acid composition indicate the existence of a unique class of proteins in plasma which bind hormones with high affinity. All plasma glycoproteins, including transcortin, have very similar amino acid compositions.

The k_{assoc} for formation of a complex between cortisol and pure human transcortin has been calculated in two other laboratories (Seal and Doe, 1966; Muldoon and Westphal, 1967). All of the determinations have been done by the method of equilibrium dialysis of transcortin which contained close to saturation amounts of cortisol. As a result, equilibration with very small (100 ng) increments of cortisol was necessary. The consequent narrow range of $\bar{\nu}$ examined by Muldoon and Westphal (1967) was from 0.87 to 0.95 and in the present study was from 0.88 to 0.99. The value of k_{assoc} of 1.7×10^8 M^{-1} calculated in the present studies is lower than that of Muldoon and Westphal (5.2×10^8 M^{-1}) but is reasonable when the experimental error involved is considered. In analysis of the data, the slope of the line used to determine k_{assoc} is very sensitive to small changes in the values for the total cortisol concentration. Small errors in determination of the endogenous

cortisol bound to transcortin (determined in our laboratory by a protein-binding method) would produce the differences reported for k_{assoc} for human transcortin and cortisol.

The extensive use in this study of polyacrylamide gel electrophoresis to examine cavian transcortin provides a sensitive means of observing the aggregation of the protein. The rate of migration of a protein in polyacrylamide gels has been shown to be a function of the molecular weight and radius (Marinis and Ott, 1964) as well as the charge. The distance of migration of any band seen in Figures 4 and 5 relative to the band immediately preceding it is a constant value of *ca.* 1.5 which indicates that a higher molecular weight species of the same size relative to the presumed monomer is present in each substance. The appearance of additional bands, as well as the increase in the slower bands, indicates the formation of polymeric forms of cavian transcortin. Changes in the number and intensity of the bands seen in the presence of PCMB suggests that this reagent induces aggregation. The involvement of sulfhydryl groups is not probable since such groups can be presumed to have reacted with the 10-fold molar excess of PCMB used. A sulfhydryl-independent dimerization of human and bovine plasma albumins has been reported (Hartley *et al.*, 1962; Green and Ray, 1963).

The complexities involved in the determination of k_{assoc} for an aggregating system necessitates restraint in the interpretation of binding data from equilibrium dialysis experiments. The value of k_{assoc} for the cavian transcortin–cortisol equilibrium is only an estimate of the true value. While the mechanism of aggregation is unknown, the decrease in the extent of inhibition of cortisol binding caused by PCMB in the presence of cortisol is of interest. The simplest interpretation of the decrease in cortisol binding in the presence of PCMB is based upon the electrophoretic evidence that the reagent promotes self-association. Preequilibration of cortisol and transcortin reduces the aggregation effect of PCMB observed in electrophoresis, but when PCMB is added first, the cortisol is ineffective. In equilibrium dialysis experiments with PCMB, the reduction of the amount of cortisol bound is essentially the same for each concentration of cortisol used. This would be expected if a fixed fraction of the total binding sites had been lost. The inhibition is not affected by increasing concentrations of cortisol. This implies that cortisol cannot reverse the binding inhibition. However, an apparent protective effect of the steroid is manifested by a decrease in the extent of the inhibition when cortisol was preequilibrated with cavian transcortin. A loss of binding sites is induced by PCMB as the direct consequence of the formation of a higher molecular weight inactive species. The spontaneous, rapid aggregation of cavian transcortin in the absence of cortisol and the accelerated aggregation in the presence of PCMB is similar to the polymorphism of rat corticosteroid-binding globulin (Chader and Westphal, 1968b). This aggregation, however, was reversed by corticosterone; the polymeric species associated with the steroid.

None of the phenomena observed for the cavian and rat (Chader and Westphal, 1968b) steroid-binding globulins has been demonstrated for the association of human transcortin and cortisol. Studies done on the human protein, however, have not been on steroid-free protein since removal of cortisol has been found to inactivate the human protein (Seal and Doe, 1962a,b; Muldoon and Westphal, 1967). Investigation of this inactivation is necessary before the significance of the observed steroid-sensitive aggregation of the rat protein and the steroid-insensitive aggregation of the cavian protein can be assessed. The elucidation of the role of steroids in the formation of such aggregates is essential for those interested in the mechanism of steroid–protein interactions.

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