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Steroid–Protein Interactions. Human Corticosteroid-Binding Globulin: Characterization of Dimer and Electrophoretic Variants†

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ABSTRACT: Human corticosteroid-binding globulin (CBG) forms a dimer that was isolated by gel filtration, has full binding affinity and capacity, and can be dissociated to the monomer. Monomeric CBG consists of two distinct molecular variants, which were detected by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. The two monomeric CBG species were separated by preparative gel electrophoresis and were found to bind cortisol, as well as progesterone, with equal affinity. They have one steroid binding site per CBG molecule. Amino acid and carbohydrate analyses are essentially the same for both of the CBG variants. Removal of sialic acid or 90% of the carbohydrate did not affect the existence of the two molecular forms. The two CBG species were isolated from each of the sera from five individual donors, indicating that the observed heterogeneity does not result from pooling genetic variants. The two species are immunologically identical. A possible explanation for the existence of the two electrophoretic variants is a difference in amidation.

More than 20 years have past since the first reports on the corticosteroid-binding globulin (CBG)[1] of human blood serum were published (Daughaday, 1956; Bush, 1957; Sandberg & Slaunwhite, 1958). CBG has been extensively studied in the last 5 years since the protein became more readily available following the use of affinity chromatography for its isolation (Rosner & Bradlow, 1971, 1975; Trapp et al., 1971; Le Gaillard et al., 1974). CBG consists of one single polypeptide chain containing about 30% carbohydrate and having a molecular weight of about 52 000 (Westphal, 1971; Le Gaillard et al., 1975). One molecule of CBG binds one molecule of cortisol or progesterone with high affinity ($K_a \sim 10^9$ M^{-1}, 4 °C). Recently, human CBG was reported to form a dimer with full binding activity (Stroupe et al., 1977) and to consist of two molecular variants separable by electrophoresis in acrylamide gels of low porosity (Harding et al., 1978a,b). This paper reports the results of binding and compositional analyses of the various species of human CBG.

Materials and Methods

Term human pregnancy serum was obtained from Louisville's Norton-Children's Hospital (residual samples from routine testing) and University Hospital (placental blood). Outdated human blood was obtained from the American Red Cross (Louisville, KY). Amberlite XAD-2 and XAD-4 was from Rohm and Haas; it was washed 10 times with equal volumes of methanol and then in a similar way with distilled water at room temperature. Radiolabeled steroids were purchased from New England Nuclear. The radiopurity was checked by thin-layer chromatography. Endogenous steroids were removed by gently shaking 1 L of serum with 200 mL (settled volume) of Amberlite XAD-2 resin for 8 h at room temperature. The serum was then filtered through a fritted disk funnel (coarse), dialyzed for 40 h against 50 mM phosphate–0.5 M KCl (pH 9.0, 4 °C, 0.02% sodium azide), centrifuged for 30 min at 12000*g*, and filtered through a Whatman no. 2 filter paper.

Purification of human CBG was achieved by affinity chromatography with agarose to which 11β-hydroxy-3-oxo-4-androstene-17β-carboxylic acid was coupled through an

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[1] Abbreviations: CBG, corticosteroid-binding globulin; K_a, equilibrium association constant; n, number of binding sites per molecule of protein; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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amide bond formed with the primary amino group present in the coupling product of 1,2-diaminoethane with the oxirane that originated from coupling the bisoxirane, 1,4-butanediol diglycidyl ether, to agarose (Mickelson & Westphal, 1979). The structure is shown under Results. The steroid-free pregnancy or nonpregnancy serum (1 L) was passed at a flow rate of 150 mL/h at 4 °C through a column (2.2 × 25 cm) containing the affinity resin, which had been equilibrated with 50 mM sodium phosphate–0.5 M KCl, pH 9.0, 4 °C. The column was washed with the same buffer until the A_{280} absorbance was negligible (<0.05). The column was then placed at room temperature and after 1 h washed with a slightly oversaturated solution of cortisol (280 μg/mL) in 50 mM sodium phosphate–0.1 M NaCl buffer, pH 7.4, at room temperature. The protein fractions were pooled and concentrated to 30 mL by ultrafiltration with an Amicon PM-30 membrane. The 30 mL of protein solution was dialyzed twice for 48 h against 2 L of 5 mM sodium phosphate buffer (pH 6.8, 4 °C) containing 10 mg of cortisol. The dialyzed protein solution was passed at a flow rate of about 20 mL/h through a column (2.2 × 7 cm) containing hydroxylapatite (Bio-Rad) equilibrated with the 5 mM phosphate buffer (pH 6.8, 4 °C). The protein fractions were either concentrated in an Amicon-stirred filtration cell or pooled, dialyzed against 50 mM phosphate buffer containing 1 mM EDTA and 0.02% sodium azide (pH 7.4), and stored at −80 °C. The yield of pure protein was generally 55 mg/L of pregnancy serum.

Removal of Cortisol from CBG. Pure CBG was stripped of steroid by a modification of the method described by Chan & Slaunwhite (1977). A 30-mL solution of CBG (5.0 μg/mL of 50 mM phosphate buffer containing 0.1% gelatin, pH 7.4) was shaken with 6 mL of settled Amberlite XAD-4 for 4 h at 23 °C. The protein solution was then filtered through a Millipore filter (0.2 μm).

Equilibrium dialysis was performed essentially as previously described (Westphal, 1969; Mickelson & Westphal, 1980) in order to determine the affinity constants for the binding of radioactive steroids to CBG. Two milliliters of a CBG solution (9.2×10^{-9} M) was the inside volume, and 20 mL of 50 mM sodium phosphate buffer (pH 7.4, 4 °C) containing 20 mM mercaptoethanol, 1 mM EDTA, and 0.02% sodium azide was the outside volume. The dialysis flasks were gently shaken for 48 h at 4 °C. Triplicate samples of the inside and outside solutions were taken for scintillation counting, and the triplicate values were averaged. Scatchard plots consisting of eight points were analyzed by a computer program that gave a least-squares fit of the data.

Preparative polyacrylamide gel electrophoresis was performed with a Canalco "prep disc" apparatus equipped with a PD 2/320 gel holder. A 10 cm long gel [10% acrylamide–0.3% bis(acrylamide)] was cast at room temperature. A 1-mL solution of pure CBG (2 mg) in 5 mM sodium phosphate buffer (pH 7.4) containing 20% glycerol was fractionated in the gel at a constant current of 15 mA. The temperature was maintained at 18 °C with a Lauda K-2/RD circulating cooler. After 15 h the gel was removed from the tube and frozen at −80 °C. Liquid nitrogen was slowly poured over the frozen gel, and the protein was detected by its phosphorescence after excitation for 10 s with a UV lamp as described by Isenberg et al. (1975). No separation of the electrophoretic variants of CBG was seen because of the relatively large quantity of CBG fractionated. The area of protein phosphorescence was cut into three sections after the gel was thawed to facilitate slicing. Each gel section was homogenized with a glass tissue homogenizer in 50 mL of 50 mM sodium phosphate buffer (pH 7.4, 23 °C) containing 0.1 M NaCl, 0.02% sodium azide, and 14 mg of cortisol. The homogenized gel mixture was placed in a dialysis bag and dialyzed for 72 h at 4 °C against 4 L of 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.02% sodium azide, and 20 mg of cortisol. The gel solutions were then centrifuged for 20 min at 12000*g*, and the supernatants were passed through a Millipore filter (0.2 μm). The protein solutions were concentrated to 5–7 mL in an Amicon filtration apparatus using a PM-30 membrane and dialyzed against 2 L of 1 mM sodium phosphate buffer (pH 6.8, 4 °C) containing 10 mg of cortisol. The dialyzed protein was adsorbed to a column of hydroxylapatite at 1 mM sodium phosphate and eluted with 5 mM sodium phosphate buffer at a flow rate of about 20 mL/h essentially as previously described (Le Gaillard et al., 1974). The protein fractions were pooled and lyophilized. The lyophilized protein was dissolved in 1 mL of 50 mM phosphate buffer and centrifuged for 30 min at 12000*g*. The protein solution was stored at −80 °C.

Isoelectric focusing was done on a 5% polyacrylamide slab, 1.5 mm thick, 12.5 × 20 cm, containing 1.9% (v/v) of LKB pH 3.5–10 ampholines at 2 °C on a LKB Multiphor apparatus. The anode wick was saturated with 0.5 M citric acid and the cathode wick with 1.0 M NaOH. Human red blood cells were washed with normal saline to remove serum proteins and lysed with 2 volumes of distilled water per volume of sedimented erythrocytes. Five microliters of human erythrocyte lysate was placed near both cathode and anode in the same lane. Solutions of 10 μg of CBG were placed near the center of the slab. Current was applied at 200 V (approximately 15 mA) until the hemoglobin bands converged at which time the voltage was increased to 500 V and focusing continued until the amperage stabilized. The proteins were stained with Coomassie brilliant blue as described by Allen et al. (1974).

Determination of Amino Acid Composition. Pure protein (0.5 mg) was hydrolyzed in 1 mL of 6 N HCl (Sequanol grade from Pierce Chemical Co.) at 110 °C for 24 h in evacuated, sealed tubes. Cystine and cysteine were determined as cysteic acid after performic acid oxidation by the method of Moore (1963). Amino acid analyses were performed with an automatic single-column amino acid analyzer (Benson, 1973). Tryptophan and tyrosine content were determined by the method of Edelhoch (1967).

Carbohydrate Analysis. Standard colorimetric methods were used for the measurement of hexose (Roe, 1955), hexosamine (Winzler, 1955), fucose (Dische & Shettles, 1948), and sialic acid (Warren, 1959). Reagent volumes were scaled down by a factor of 10.

Determination of Optical Absorptivity. Human CBG (0.2 mg/mL of 5 mM sodium phosphate, pH 6.0) was extensively (72 h) dialyzed against water at 4 °C, lyophilized, and further dried in a vacuum desiccator over phosphorus pentoxide. An extinction coefficient of $E_{1cm}^{1\%} = 6.5$ was determined at 279 nm (maximum absorption) in a Zeiss PMQ II spectrophotometer with triplicate protein solutions (1.0 mg of CBG/mL of 50 mM phosphate buffer, pH 7.4, 23 °C). Correction was made for a water content of 8%, determined by drying triplicate samples to a constant weight at 100 °C.

Preparation of Deglycosylated CBG. A solution of CBG (2.0 mg) in 4 mL of 0.1 M sodium acetate (pH 5.0) was incubated at 37 °C for 24 h with a mixture of the following glycosidases: neuraminidase (*Clostridium perfringens*, Sigma Type VI); β-galactosidase (*Aspergillus niger*, Sigma); β-*N*-acetylglucosaminidase (*Aspergillus niger*, Sigma); α-mannosidase (jack bean: *Canavalid ensiformis*, Sigma Type III). All enzyme concentrations were adjusted to 0.05 unit/mL

except α-mannosidase which was adjusted to 0.25 unit/mL. Hydrolyzed carbohydrate was removed by dialysis at room temperature for 48 h against 1 L of 50 mM sodium phosphate, 1 mM EDTA, and 0.02% sodium azide, pH 7.4. The protein was diluted 1:10 with water and passed through a column of hydroxylapatite to remove the glycosidases.

Analytical Polyacrylamide Gel Electrophoresis Procedures. Disc gel electrophoresis was performed at room temperature in 10% polyacrylamide–0.3% bis(acrylamide) gels according to the method of Davis (1964). For measurement of binding activity CBG was incubated with [^{3}H]cortisol and electrophoresis was performed in an ice bath. The areas of the frozen gels (−80 °C) corresponding to stained protein on duplicate gels were imbedded in Ames O.C.T. tissue embedding medium, sliced at 500 μm with a freezing microtome, and counted for radioactivity. The method described by Weber et al. (1972) was used for $NaDodSO_4$ gel electrophoresis. Protein samples were denatured in 1% $NaDodSO_4$–1% 2-mercaptoethanol at 100 °C for 10 min. Samples containing 5–10 μg of protein in 5–10 μL of solution were applied per gel.

Results

Purification of Human CBG. Human CBG was initially purified to homogeneity from term pregnancy serum and nonpregnancy serum by the method described by Le Gaillard et al. (1974). More recently, a number of modifications of their procedure were introduced. Instead of attaching the spacer via the cyanogen bromide activated agarose method (Axēn et al., 1967), a very long spacer was attached to the agarose matrix via a more stable ether bond (Mickelson & Westphal, 1979): agarose–$OCH_2CH(OH)CH_2O$-$(CH_2)_4OCH_2CH(OH)CH_2NHCH_2CH_2NHCO$–C(17) of 11β-hydroxy-3-oxo-4-androstenyl. This modification results in a more stable affinity column. The serum was also adsorbed at pH 9.0 to the affinity resin since the affinity constant of CBG to steroids is greater at higher pH values (Mickelson et al., 1980). Protein from the affinity column was passed through a column of hydroxylapatite at 5 mM phosphate which retains contaminating proteins but not CBG. The quality of CBG obtained by this procedure appears to be the same as that obtained by the previously published procedure of Le Gaillard et al. (1974).

Dimer Formation. Polyacrylamide gel electrophoresis revealed the presence of a slower migrating band in pure human CBG preparations that had been stored in solution, frozen, or lyophilized (Figure 1, no. 1). Freezing and thawing of the CBG solutions, repeated up to 10 times, increased the amount of the slower band (Figure 1, no. 2). Determination of the molecular weights of these two species on a calibrated gel-filtration column (Sephadex G-200, 80 × 1.5 cm) gave 110000 and 230000 daltons for the fast and slow component, respectively. The discrepancy of the lower value from the molecular weight of 52000 obtained by sedimentation methods results from the "carbohydrate error" (Andrews, 1970), which can present apparent molecular weights of glycoproteins twice their actual values. The observed weight ratio of 2.1:1 shows clearly the presence of a dimeric form if we assume for monomer and dimer about the same abnormal behavior of the structure expanded by hydration. The monomeric and dimeric CBG were separated by gel filtration over Sephadex G-200. Polyacrylamide gel electrophoresis of the separated monomer shows absence of dimer (not shown), but the isolated dimer spontaneously reforms some monomer (Figure 1, no. 3).

Figure 2 shows that the dimer retains full steroid-binding activity as seen in the polyacrylamide electrophoresis of the CBG-corticosterone complex. This was confirmed by Sephadex G-200 gel filtration. Analysis of the eluents from the column by which the molecular weights were determined showed coincidence of protein and radiolabeled corticosterone for both monomer and dimer (not shown).

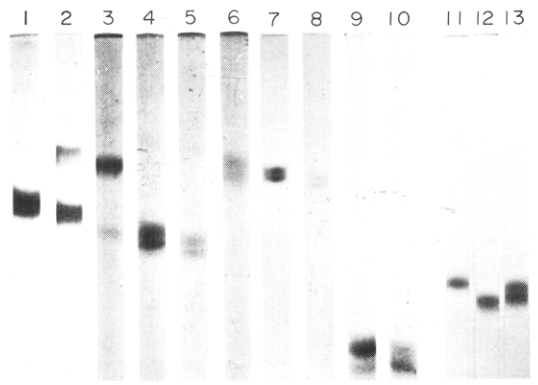

FIGURE 1: Polyacrylamide gel electrophoresis of human CBG: (1) pure CBG (20 μg/gel), concentrated after hydroxylapatite filtration, kept at 4 °C for 10 weeks with 0.02% azide added (a small amount of dimer is visible); (2) pure CBG (20 μg/gel), prepared as no. 1, 10 times frozen and thawed at room temperature (formation of dimer); (3) pure CBG (same preparation as no. 1) frozen and thawed as no. 2, after isolation of the dimer by three filtrations over Sephadex G-200 (some reformation of monomer); (4) pure CBG, standard polyacrylamide gel electrophoresis; (5) gel electrophoresis of the same preparation as no. 4 in the presence of $NaDodSO_4$ and mercaptoethanol; (6) pure CBG (7 μg/gel) after neuraminidase treatment; (7) pure CBG (7 μg/gel), standard polyacrylamide gel electrophoresis; (8) same preparation as no. 7 after deglycosylation; (9) pure CBG, higher concentration of slow component; (10) pure CBG, higher concentration of fast component; (11) 5 μg of pure CBG slow component; (12) 5 μg of pure CBG fast component; (13) mixture of 5 μg each of no. 11 and 12.

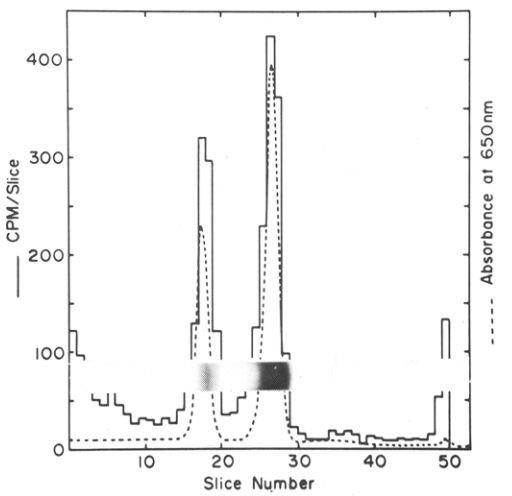

FIGURE 2: Polyacrylamide gel electrophoresis (0–4 °C) of purified human CBG–radiocorticosterone complex. The ratio of radiolabeled steroid to protein stain indicates about equal binding activity of dimer and monomer. Anodic migration is from left to right. The radioactivity of slices no. 49 and 50 originates from association of corticosterone with the dye band (bromophenol blue).

Equilibrium dialysis (Figure 3) also indicated retention of full binding activity by the dimer. It is not known whether or not the dimer structure remains intact throughout the dialysis; we have observed frequently that monomer is spontaneously reformed from dimer.

Demonstration of Two Electrophoretic Variants of Human CBG. Our previous report (Stroupe et al., 1977) showed that monomer and dimer of human CBG migrated as broad bands in polyacrylamide gel electrophoresis [see Figure 3 in Westphal (1980)]. We have later found that the broad bands (Figure 1, no. 1 and 3) appear only when the gels are overloaded (20

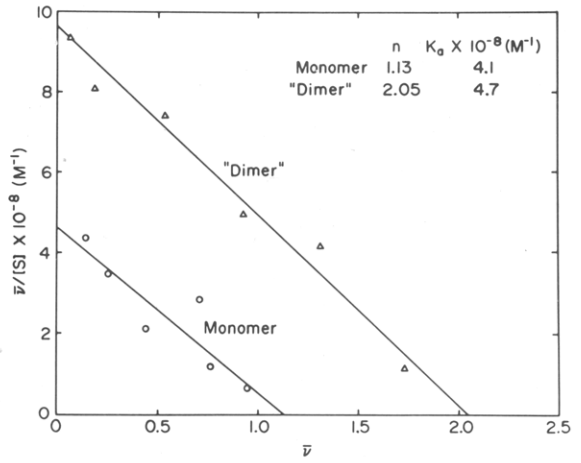

FIGURE 3: Equilibrium dialysis of XAD-stripped human CBG monomer and dimer in the presence of tritiated corticosterone. Scatchard analysis gave $K_a = 4.1 \times 10^8$ M^{-1} and 4.7×10^8 M^{-1} for the monomer and dimer, respectively. The apparent n values were 1.13 for the monomer and 2.05 for the dimer.

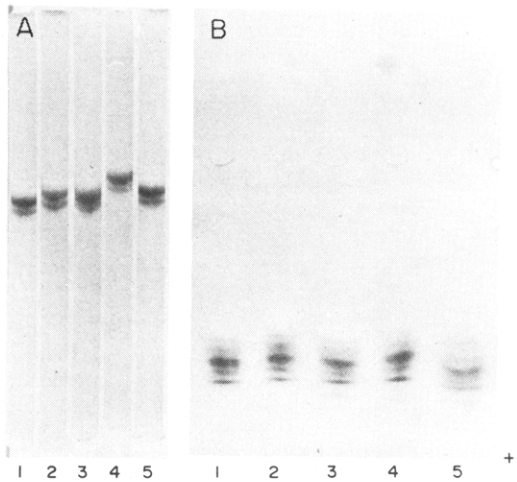

FIGURE 4: (A) Polyacrylamide gel (10%) electrophoresis of affinity-purified CBG preparations (5 μg/gel) from five individual units of outdated human blood obtained from the American Red Cross. (B) Isoelectric focusing of the CBG preparations shown in (A) on a 5% acrylamide slab gel containing 1.9% of pH 3.5–10 ampholines. Citric acid (0.5 M) was used at the anode and NaOH (1 M) at the cathode.

μg or more). When pure CBG was examined by disc gel electrophoresis with gels of low porosity (7.5 or 10%), extended migration time (approximately 4 h) at about 3 mA/tube, and less than 10 μg of protein, two distinct bands were observed (Figure 1, no. 4). Preelectrophoresis of the gels, addition of a reducing agent such as thioglycolic acid, incubation of CBG with 10 mM dithiothreitol, or 200 mM 2-mercaptoethanol did not affect the appearance of two bands. Gel electrophoresis in the presence of $NaDodSO_4$ and mercaptoethanol also yielded two bands (Figure 1, no. 5). Determination of molecular size by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ as previously described (Chader et al., 1972) gave apparent molecular weights of 65 000 and 71 000 for the two bands. Removal of sialic acid by neuraminidase (Figure 1, no. 6) or removal of 90% of the carbohydrate by glycosidases (Figure 1, no. 7 and 8) did not eliminate the doublet. The two variants were detected in freshly drawn blood plasma, blood serum, and also in outdated serum by [^{3}H]-cortisol binding as described for pure CBG, indicating that purification of CBG did not produce the appearance of a doublet. Figure 4A shows that the doublet exists in five individual plasma samples and is not the result of pooling samples. Isoelectric focusing indicates greater heterogeneity (Figure 4B); however, no differences are apparent among the samples from five individual donors.

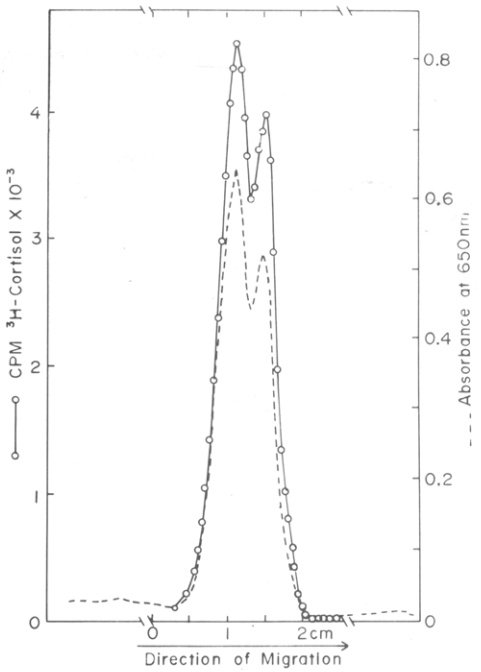

FIGURE 5: Polyacrylamide gel electrophoresis of human CBG–[^{3}H]cortisol complex for 7 h at 4 °C, 300 mW/tube. Localization of protein with Coomassie blue. The protein region of a frozen companion gel was sectioned in a freezing microtome, and the slices were counted in a scintillation spectrometer. The ratio of cpm/protein absorbance was the same for the monomeric and the dimeric form.

Table I: Association Constants of Human CBG Components at 4 °C in the Presence of Mercaptoethanol

component	K_a (M^{-1} X 10^{-9}) (±SD) cortisol	progesterone
fast	0.41 ± 0.05	0.32 ± 0.05
slow	0.52 ± 0.08	0.31 ± 0.02
unfractionated	0.58 ± 0.06	0.35 ± 0.06

Isolation of the Fast and Slow Components. The two electrophoretic variants were separated by preparative gel electrophoresis. Each isolated component remained distinct after isolation (Figure 1, no. 11 and 12); no conversion from one to the other was detected within at least 6 weeks. Polyacrylamide gel electrophoresis of the mixture of the slow and fast component resulted again in separation (Figure 1, no. 13). Whereas in most cases the slower component seemed to be in excess of the faster one (Figure 1, no. 9; Figures 4 and 5), the opposite relationship was also observed (Figure 1, no. 10).

Binding Analysis of the Two Variants. Gel electrophoresis of the CBG–[^{3}H]cortisol complex with subsequent slicing of the gel indicated that both bands bound steroid (Figure 5). Table I indicates that no significant difference in binding affinity for cortisol and progesterone was detected for either the fast, slow, or unfractionated CBG.

Chemical Composition of the Two Variants. Amino acid analysis of each species did not indicate any significant difference in composition (Table II). The squared correlation coefficients were >0.99 for the three compositions (unfractionated, fast, slow). The glycine values for the separated components are not reported since they were very high and variable due to the presence of glycine in the electrophoresis

Table II: Amino Acid Composition of Human CBG Components

amino acid	no. of residues[a]/mol[b] of protein (±SD) unfractionated	fast	slow
Lys	14.7 ± 0.4	15.0 ± 0.1	14.9 ± 0.1
Arg	8.7 ± 0.1	8.4 ± 0.3	8.7 ± 0.3
Asp	34.9 ± 1.9	35.7 ± 0.3	35.7 ± 0.6
Glu	29.3 ± 0.4	31.4 ± 0.4	31.6 ± 0.2
Thr	21.8 ± 0.7	21.9 ± 0.5	21.7 ± 0.6
Ser	28.9 ± 0.6	29.9 ± 0.6	30.3 ± 1.1
Ala	23.3 ± 0.3	23.4 ± 0.5	23.4 ± 0.4
Val	23.4 ± 0.6	23.7 ± 0.6	23.6 ± 0.9
Ile	15.7 ± 0.4	15.6 ± 0.6	14.8 ± 0.3
Leu	37.4 ± 0.4	37.0 ± 0.6	36.8 ± 0.3
Met	11.2 ± 0.7	10.8 ± 0.6	10.9 ± 0.3
Pro	10.6 ± 0.2	10.8 ± 1.5	10.5 ± 0.3
His	9.5 ± 0.2	9.6 ± 0.1	9.2 ± 0.2
Phe	19.6 ± 0.4	19.0 ± 0.4	18.9 ± 0.3
Gly	21.0 ± 0.7		
$^1/_2$-Cys	2.0[c]	2.1[d]	
Trp	4.1	3.8	3.9
Tyr	10.2	10.1	9.9

[a] Average ± SD of three determinations. Trp and Tyr values are the average of two determinations. [b] Based on a molecular weight of 52 000 of which 30% is carbohydrate. [c] Determined as cysteic acid by AAA Laboratories, Mercer Island, WA. [d] Determined as cysteic acid.

Table III: Carbohydrate Composition of Human CBG Components Expressed as % ± SD

carbohydrate	component unfractionated	fast	slow
hexose	11.4 ± 0.4	11.2 ± 0.3	11.1 ± 0.1
hexosamine	10.6 ± 0.4	10.0 ± 1.2	9.8 ± 0.2
sialic acid	7.5 ± 0.9	7.7 ± 0.4	7.9 ± 0.1
fucose	0.9 ± 0.3	0.9 ± 0.3	0.8 ± 0.2
total	30.4	29.8	29.6

buffer used in isolating the two species. Dialysis did not eliminate the elevated glycine content, indicating possible interaction between CBG and glycine. Substitution of alanine for glycine in the electrophoresis buffer resulted in an elevated alanine peak, suggesting that the presumed interaction is nonspecific in nature. Possibly, a similar interaction with ampholines may be the cause of the microheterogeneity seen in isoelectrofocusing experiments of CBG [Figure 4; see also Van Baelen et al. (1972)].

Carbohydrate analysis of each species indicates that the existence of the doublet is not due to differences in carbohydrate composition (Table III). Further support of this was seen in the finding that enzymatic removal of 90% of the carbohydrate did not affect the appearance of a doublet. This deglycosylation had no effect on the n value and binding affinity for cortisol. Scatchard binding analysis by equilibrium dialysis at 4 °C in the presence of mercaptoethanol gave K_a = (0.58 ± 0.06) × 10^9 M^{-1} (average n = 0.95) and (0.56 ± 0.12) × 10^9 M^{-1} (average n = 1.00) for control and deglycosylated human CBG, respectively (Figure 6).

Antigenicity of the Two Variants. Ouchterlony immunodiffusion of unfractionated CBG, the fast species, and the slow species with rabbit anti-human CBG is shown in Figure 7. Lines of identity are seen between all three fractions, indicating that the difference between the variants does not alter the antigenicity of CBG.

Discussion

Human CBG was first purified to apparent homogeneity by Seal & Doe (1962) using conventional chromatographic

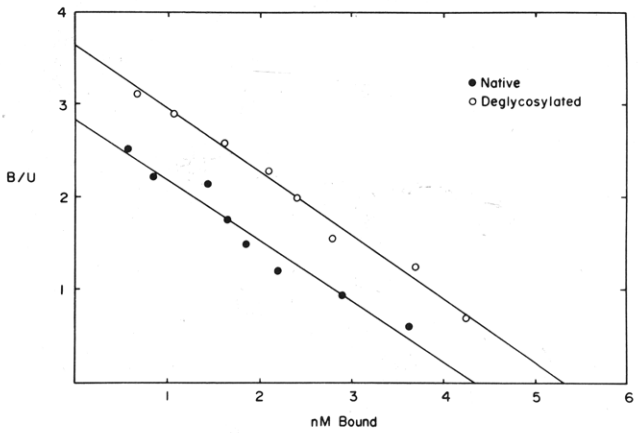

FIGURE 6: Equilibrium dialysis of purified control and deglycosylated human CBG against tritiated cortisol in the presence of mercaptoethanol: native CBG, K_a = (5.8 ± 0.6) × 10^8 M^{-1}, average n = 0.95; deglycosylated CBG, K_a = (5.6 ± 1.2) × 10^8 M^{-1}, average n = 1.00.

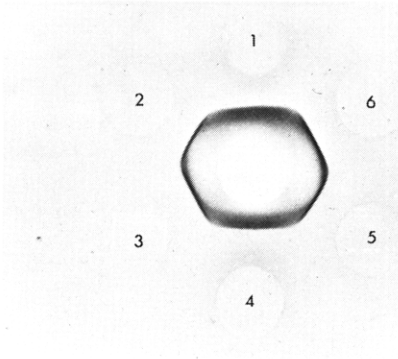

FIGURE 7: Ouchterlony immunodiffusion of purified human CBG pool, fast, and slow components against rabbit anti-human CBG. Central well contained 8 μL of rabbit antiserum against purified human CBG; wells 1 and 4, 8 μL of CBG pool (0.6 mg/mL); wells 2 and 5 and wells 3 and 6, about the same amount of CBG slow and CBG fast, respectively.

methods. Rosner & Bradlow (1971) introduced affinity chromatography as a method for purifying CBG, which drastically reduced the time involved in the isolation. Le Gaillard et al. (1974) improved the affinity resin by utilizing a relatively stable amide bond to connect the steroid to the spacer moiety of the agarose resin. In this study, we present further improvements of the affinity chromatographic procedure. First, the spacer group is linked to the agarose via an ether bond, which is more stable than the isourea bond formed by the cyanogen bromide activated agarose method. The resulting affinity resin is very stable and has been used about 30 times with no detectable loss in its capacity to remove CBG from serum. A second improvement is the passage of serum at high pH values (pH 9) through the affinity column. Complex formation between CBG and steroid is maximal between pH 8 and 11 (Mickelson et al., 1980); thus, more CBG is retained by the affinity resin at elevated pH values. Yields greater than 80% are attainable by using the conditions applied in this study.

The dimer formation observed in our laboratory (Stroupe et al., 1977) and described in this paper is different from the polymerization reactions reported by other investigators. Polymerization of human CBG, reversible by $NaDodSO_4$, has been described earlier (Rosner, 1972). Affinity-purified human CBG polymerizes when heated for 15 min at 60 °C, treated with urea (Le Gaillard et al., 1975), or exposed to pH 4 (Le Gaillard et al., 1976). These polymerizations lead to

inactivation in all cases. Subsequent reaction with $NaDodSO_4$ and dithiothreitol results in re-formation of monomeric inactive CBG. The dimer observed by Wolf & Rosner (1979) has not been tested for binding activity. In contrast to all polymeric forms described in the literature, the dimer obtained in our work retains full corticosteroid-binding activity (Figures 2 and 3).

Even though pure human CBG has been available for almost 2 decades, its existence as two electrophoretic variants has only recently been reported (Harding et al., 1978a,b). Human CBG separates into two distinct bands on electrophoresis in low porosity polyacrylamide gels. Similar electrophoretic variants have been reported for rabbit CBG [see Figure 8 in Chader et al. (1972)] and also for the estrogen-binding protein α_1-fetoprotein in rat plasma [for review, see Smith & Kelleher (1980)]. As found for rat α_1-fetoprotein, the two variants of human CBG have essentially identical chemical composition with respect to amino acid and carbohydrate residues. Sequence analysis of the first eight N-terminal amino acid residues of CBG (Le Gaillard et al., 1975) indicates that the two variants possess the same amino terminal sequence. We also found only methionine as the N-terminal residue (unpublished data). The possibility of a small difference between the two CBG variants in amino acid sequence and/or arrangement of carbohydrate cannot be excluded at this time. It appears unlikely that the existence of the observed electrophoretic variants is due to the carbohydrate moiety. Removal of 90% of the carbohydrate did not eliminate the existence of the two species.

As discussed by Dixon & Webb (1979), multiple forms of enzymes which may occur in a single species have been classified into seven groups on the basis of genetic origin and variation of their properties. Since the two electrophoretic variants of human CBG were observed in the presence of $NaDodSO_4$, the heterogeneity is not the result of polymer formation but lies in the single polypeptide chain. Allelic genetic variants may also be excluded as the reason for the observed variation since the CBG isolated from five individual donors showed the presence of the two electrophoretic variants in all five individuals (Figure 4). Isoelectric focusing resulted in the appearance of five easily recognizable components, again in the same way for the CBG preparations from the five donors. The p*I* values for three of the five components measured were 4.16, 4.36, and 4.46. Bernutz et al. (1979) also observed five bands in isoelectric focusing of human CBG; their p*I* range was 3.7–4.2. Only two components at approximately pH 6 were seen after neuraminidase treatment. Van Baelen et al. (1972) reported two isoelectric components (p*I* 5.4 and 5.6) for desialylated human CBG. A difference in conformation between the two variants appears unlikely since the binding properties are essentially the same and denaturation by $NaDodSO_4$ does not eliminate the existence of variants. This conclusion is also supported by the immunochemical studies that show complete identity between the two variants and unfractionated CBG (Figure 7).

A possible explanation for the presence of two electrophoretic variants is a difference in amidation. Since acid hydrolysis converts asparagine and glutamine residues to their acid form, a difference in amidation cannot be excluded. Microheterogeneity of rabbit-muscle aldolase has been shown to result from in vivo deamidation of an asparagine residue near the C terminus (Midelfort & Mehler, 1972). The existence of two bands on $NaDodSO_4$ gel electrophoresis does not support the idea of charge heterogeneity since the negatively charged $NaDodSO_4$ should mask a difference. deJong et al. (1978), however, have shown that a single amino acid substitution can alter the electrophoretic mobility of $NaDodSO_4$–protein complexes.

No significant differences were detected in the binding of cortisol and progesterone to the isolated variants of CBG, indicating similar binding sites. An important observation is that deglycosylation (90%) of human CBG had no effect on the affinity constant or binding capacity, supporting the idea that the carbohydrate moiety of CBG is not involved in the binding process. Further research is required to elucidate the basis of the difference between the observed electrophoretic variants.

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Comparison of the Binding of Cholera and *Escherichia coli* Enterotoxins to Y1 Adrenal Cells†

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ABSTRACT: The binding of iodinated cholera and *Escherichia coli* (LT) enterotoxins to Y1 mouse adrenal cells was studied by using saturation analysis (Scatchard). Each toxin bound to Y1 cells with similar affinity [$K_A = (1.5\text{-}2.0) \times 10^9\ M^{-1}$], but there appeared to be twice as many receptor sites per cell for *E. coli* toxin ($\sim 4 \times 10^5$). Despite the increased binding of *E. coli* toxin, Y1 cells respond sooner to, and to smaller concentrations of, cholera toxin. The binding of each toxin was inhibited competitively by both toxins, although twice as much *E. coli* toxin was required to inhibit 50% of the binding of cholera toxin as was needed for either homologous inhibition or the inhibition of *E. coli* toxin binding by cholera toxin. The B subunits of both toxins were equally effective in competing for the binding of both iodinated toxins. Whereas the A subunits of both toxins had little or no effect on the binding of *E. coli* toxin, they consistently inhibited 20-40% of the binding of cholera toxin to cells. These results suggest that there are receptor loci on cells for the A subunit and that conformational differences exist between the two toxins that might explain the greater sensitivity of Y1 cells to cholera toxin. A model is suggested in which cholera toxin exhibits a greater degree of multivalent ligand binding than does the *E. coli* toxin, resulting in a more favorable situation for apposition of the A subunit to its receptor or for its insertion into the membrane.

The heat-labile enterotoxins of *Vibrio cholerae* and *Escherichia coli* resemble each other immunochemically and in their mechanisms of action. Both toxins are NAD glycohydrolases (Moss et al., 1979c; Moss et al., 1979b), effect ADP-ribosylation of membrane proteins (Moss et al., 1979b; Gill & Meren, 1978), and activate adenylate cyclase. They share antigenic determinants but possess unique determinants as well (Clements et al., 1980; Kunkel & Robertson, 1979). Their subunit structures and amino acid sequences show similarities, especially between the "binding" (B) subunits (Clements & Finkelstein, 1979; Robertson et al., 1979).

There is good evidence that cholera enterotoxin (CT) binds to GM_1 gangliosides present in cell membranes (Holmgren et al., 1973; King & van Heyningen, 1975; Moss et al., 1976; Fishman et al., 1979). The evidence that this ganglioside also serves as a receptor for the *E. coli* toxin (LT) has been indirect (Donta & Viner, 1975; Moss et al., 1979a), and there are conflicting reports regarding the similarity of the receptors for the two toxins (Holmgren, 1973; Pierce, 1973; Guerrant & Brunton, 1977). With the successful purification of LT, it has become possible to directly analyze and compare their binding properties.

Experimental Procedures

Tissue Culture. Y1 mouse adrenal cells were derived from those used in previous studies (Donta & Viner, 1975). The cells were maintained and propagated in Ham's nutrient mixture F-10 (GiBCO) supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Assessment of biologic activity (changes in morphology; cyclic AMP levels) utilized previously described methods (Donta & Haddow, 1978).

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