Purification and Properties of Squirrel Monkey (*Saimiri sciureus*) Corticosteroid Binding Globulin†

Robert W. Kuhn,* Christel VestWeber, and Pentti K. Siiteri

Department of Obstetrics/Gynecology and Reproductive Sciences, University of California, San Francisco, San Francisco, California 94143

Received August 7, 1987; Revised Manuscript Received November 19, 1987

ABSTRACT: Corticosteroid binding globulin (CBG), a serum glycoprotein which binds glucocorticoids and progestins with high affinity, is widely distributed throughout the animal world. Although its charge and size characteristics have largely been conserved across species, we found the behavior of CBG in squirrel monkey (*Saimiri sciureus*) serum during fractionation by polyacrylamide gel electrophoresis or Sephadex chromatography was consistent with a molecule about twice the size of that found in most species. To more fully understand the basis for this difference, we purified the protein by sequential affinity and DEAE-Sepharose chromatographies. The final product was obtained in greater than 60% yield and was found to migrate as a single homogeneous band when examined by electrophoresis at pH 8.3 in polyacrylamide gels varying total acrylamide concentration or under conditions of severe protein overload. The steroid binding specificity of the purified protein was identical with that of the protein in the starting serum. The ultraviolet absorption spectrum of the isolated CBG–steroid complexes revealed that the protein had no pyridine nucleotide cofactor or nucleic acid. Amino acid analyses showed that the composition of the squirrel monkey protein is quite similar to that of CBG molecules from other species but distinct from albumins, hemoglobin, or rabbit progesterone receptor. In contrast to the single protein band observed following electrophoresis under normal conditions, separations in the presence of sodium dodecyl sulfate (SDS) resolved the pure protein into two bands: one at 54 000 daltons and one at 57 000 daltons. Following treatment of the purified material with the reversible cross-linking agents methyl 4-mercaptobutyrimidate or dimethyl dithiobis-(propionimidate), a band migrating at 110 000 daltons was detected on SDS gels in the absence of reducing agents. This band was eliminated by treatment with reducing agents prior to electrophoresis. This shows that unlike other species, squirrel monkey CBG exists as a dimer in its native state. Antibodies were generated against the purified material and tested for cross-reactivity against the sera from other species by both radioimmunodiffusion and radioimmunoassay techniques. Only serum from titi monkeys was observed to cross-react when examined by radioimmunoassay. Taken together, our results suggest that New World monkey CBG's are distinct from those of other species in both size and immunologic characteristics.

Corticosteroid binding globulin (CBG) is a serum glycoprotein that binds natural glucocorticoids and progesterone with high affinity. While it is generally believed that the physiologic role of CBG is regulation of free cortisol levels in blood and thus its availability to target cell receptors, other functions including intracellular transport of hormone have been suggested (Siiteri et al., 1982). The biologic importance of this protein is attested to by its presence in virtually every species examined although its plasma concentration varies widely (Seal & Doe, 1966).

In an attempt to better understand the role of CBG in transport and the action of steroids in primates, we recently examined the distribution and binding of cortisol in sera from various primates (Klosterman et al., 1986). The distribution of cortisol between free, CBG-bound, and albumin-bound fractions and CBG properties (binding affinity, relative size, and temperature dependence of steroid binding) were similar in sera from humans, Old World monkeys, and prosimians. In contrast, several New World monkeys including the squirrel monkey were found to have extremely high concentrations of free cortisol in serum since (1) the total cortisol levels greatly exceeded those of CBG and (2) CBG in these species has unusually low affinity for cortisol when measured at physiologic temperatures. In addition, several physicochemical characteristics of New World CBG appeared to differ from those of CBG found in other species. Analysis of serum by polyacrylamide gel electrophoresis and sucrose density gradient centrifugation suggested that the CBG from New World monkeys is considerably larger than that found in other species including the rat and Old World primates (Klosterman et al., 1986).

The squirrel monkey (*Saimiri sciureus*) is the best studied of the New World species. They have recently been described as resistant to cortisol (Chrousos et al., 1982, 1984) because they apparently function normally despite the highly elevated plasma total and free cortisol levels. The same is true of other steroid hormones including vitamin D_3 (Chrousos et al., 1984; Mendoza et al., 1978; Shinki et al., 1983). Studies of glucocorticoid receptor binding in cells from the squirrel monkey revealed low-affinity glucocorticoid receptors and suggested that the high cortisol levels in blood are the consequence of high rates of cortisol secretion due to altered feedback (Chrousos et al., 1982). However, we have suggested that the situation is more complex since reduced clearance due to inefficient metabolism of cortisol and other steriods also appears to contribute to the high plasma steroid levels (Siiteri, 1986). In order to clarify these issues further, we have isolated CBG from the serum of squirrel monkeys to more carefully examine

†Supported by National Institutes of Health Grant HD10328 (to R.W.K.) and National Cancer Institutes Grant CA39825A (to P.K.S.).

* Address correspondence to this author at the Reproductive Endocrinology Center, Box 0556, HSW 1656, University of California, San Francisco, San Francisco, CA 94143.

0006-2960/88/0427-2579$01.50/0

its physicochemical properties. We now report its purification to homogeneity and the preparation of polyclonal antibodies.

Experimental Procedures

All chemicals were reagent grade and obtained from J. T. Baker (Phillipsburg, NJ), except tris(hydroxymethyl)aminomethane (Tris) (Sigma Chemical Co., St. Louis, MO) and all electrophoretic-quality chemicals which were from Bio-Rad (Richmond, CA). Sephacryl S-200 was from Pharmacia Fine Chemicals (Piscataway, NJ). Radioactive cortisol (97 Ci/mmol) and $Na^{125}I$ (100 mCi/mL) were obtained from New England Nuclear Corp. (Boston, MA) while all radioinert steroids were from Steraloids (Wilton, NH).

Synthesis of Affinity Resin. The affinity resin 11-hydroxy-3-oxo-4-androstene-17-carboxylic acid–(aminoethyl)amine–1,4-butanediol diglycidyl ether–Sepharose was synthesized as described by Mickelson and Westphal (1979).

Purification by Affinity Chromatography. In the squirrel monkey, plasma CBG levels increase 50–100-fold during pregnancy or following estrogen treatment (Coe et al., 1986). Therefore, pregnancy serum was used as the source for isolation. Pregnant squirrel monkey serum (16 mL) was added to a charcoal pellet which was obtained by centrifugation of 5 mL of a suspension of 2.5% charcoal–0.25% dextran and removal of the supernatant. The serum–charcoal mixture was stirred for 1 h at room temperature. The charcoal was removed by centrifugation at 3000 rpm for 30 min in a Beckman J6B centrifuge followed by filtration through a Millipore filter (Millipore type SM, 5.0-μm pore size). The serum was passed at a flow rate of 16 mL/h through a column (2 × 25 cm) containing 60 mL of the affinity resin. Prior to use, the affinity column was preequilibrated with buffer consisting of 50 mM Tris, 0.4 M NaCl, and 5 mM $CaCl_2$, pH 7.4. Unless otherwise stated, this and all other chromatographic steps were performed at 4 °C. After application of the sample, the column was washed first with 5 column volumes of the equilibration buffer followed by the same buffer containing 10% ethanol until no UV-absorbing material could be detected in the eluate. The absorbed CBG was eluted from the column with buffer–ethanol containing 200 μg/mL cortisol at a flow rate of 16 mL/h. Fractions (8 mL) were collected and analyzed for protein according to the method of Bradford (1976) and for cortisol binding activity. Fractions containing CBG were pooled and dialyzed against several changes of 10 mM Tris–5 mM $CaCl_2$, pH 7.4, buffer containing 2 μM cortisol.

DEAE-Sepharose Chromatography. The affinity-purified squirrel monkey CBG solution was loaded onto a column (0.8 × 10 cm) of DEAE-Sepharose Cl 6B (preequilibrated with 10 mM Tris–5 mM $CaCl_2$, pH 7.5) at a flow rate of 6 mL/h. The column was washed with 2 column volumes of starting buffer prior to elution with a linear salt gradient (300 mL) from 0 to 400 mM NaCl in 10 mM Tris–5 mM $CaCl_2$, pH 7.5. The column was developed at a flow rate of 60 mL/h, and fractions (5 mL) were collected and analyzed for protein and cortisol binding activity. Fractions containing CBG were pooled and dialyzed overnight at 4 °C against 10 mM Tris–5 mM $CaCl_2$ (pH 7.5) buffer containing 2 μM cortisol. The purity of the resulting preparation was assessed by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate.

Sucrose Gradient Centrifugation. Linear gradients of 10–40% sucrose in 10 mM Tris buffer (pH 7.4) with or without 0.4 M KCl were prepared in polyallomer tubes (Beckman Instruments, Palo Alto, CA). The gradients were centrifuged for 16 h at 50 000 rpm in a Spinco SW 60 rotor (Beckman Instruments, Mt. View, CA) at 0–2 °C. Gradients were fractionated on an Isco gradient fractionator, and 0.1-mL samples were collected and counted. ^{14}C-labeled bovine serum albumin (4.6 S) was included in each sample as an internal marker.

Gel Electrophoresis in a Discontinuous Buffer System Containing Sodium Dodecyl Sulfate. Electrophoresis in 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate was performed according to the method of Laemmli (1970). Samples were dissolved in 3% sodium dodecyl sulfate containing 5% β-mercaptoethanol and 20% glycerol and were boiled for 3–5 min to accomplish complete solubilization. After electrophoresis, the gels were fixed for 18 h in 40% methanol–7% acetic acid and stained in 0.05% Coomassie brilliant blue for 2 h and then destained in the same solution as that utilized for fixation.

Nondenaturing Polyacrylamide Electrophoresis. Electrophoresis was carried out in 7.5% acrylamide gels at pH 8.3 as described by Ornstein (1964). Gels were electrophoresed at 4 mA/tube until the bromphenol blue marker was within 0.5 cm of the bottom of the gel tube. The gels were removed, severed at the dye marker, and stained according to the procedure of Blakesley and Boezi (1977).

Measurement of Steroid Binding Activity. Samples to be analyzed for steroid binding activity were freed of cortisol by treatment with charcoal at 22 °C for 1 h, essentially as described by Heyns et al. (1967). Ovalbumin (3.33%) was added to purified fractions prior to the stripping procedure in order to avoid losses of CBG due to absorption to the charcoal. Preliminary studies using radiolabeled steroids demonstrated that 98–100% of the cortisol was removed by this treatment. CBG binding activity was determined using [^3H]cortisol as the ligand by the filter dilution assay as described by Siiteri et al. (1982). Aliquots (300 μL) of sequential serial dilutions were incubated for 1 h at 0 °C with 10 nM [^3H]cortisol in 0.01 M Tris, pH 7.4. Duplicate 100-μL samples were applied to DEAE filters (2.4 cm; Whatman DE81). After 10 1-mL washes with buffer, the filters were removed and placed in scintillation vials, 10 mL of scintillation fluid (Scintiverse) was added, and radioactivity was determined at 32% efficiency in a Packard Model 3385 scintillation counter (Packard, Downers Grove, IL). Duplicate samples containing 1000-fold molar excess of radioinert cortisol were analyzed to determine nonspecific binding.

Absorption Spectra. Absorption spectra were obtained between 400 and 220 nm with a Perkin-Elmer Lambda 3A recording UV–vis spectrophotometer. The protein solution was first subjected to Millipore filtration (HAWP filter) to remove any light-scattering particles.

Treatment with Bifunctional Cross-Linking Agents. Squirrel monkey CBG was reacted with bifunctional cross-linking reagents essentially as described by Birnbaumer et al. (1979). To squirrel monkey CBG (200 μL of a 0.2 mg/mL solution in 10 mM Tris, pH 7.5, containing 5 mM $CaCl_2$) was added 175 μL of water, 25 μL of 1 M triethylamine (pH 8.0), 2 μL of 500 mM magnesium acetate, and sufficient monothioglycerol to obtain a final concentration of 2 mM. After cooling in an ice–water bath, methyl 4-mercaptobutyrimidate (100 μL of a 10 mg/mL solution in water) was added. The resulting mixture was stirred for 20 min at 0 °C. Excess reagents were removed by dialysis for 1.5 h against three changes of 100 mL each of 20 mM triethylamine, pH 8.0, buffer containing 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Final cross-linking was achieved by the addition of 10 μL of 30% H_2O_2 and allowing the mixture to stir at room temperature for 20 min.

The resulting solution was dialyzed against PBS (0.01 M sodium phosphate, pH 7.0, buffer containing 0.15 M NaCl) overnight at 4 °C prior to analysis by sodium dodecyl sulfate–polyacrylamide electrophoresis.

Squirrel monkey CBG was also cross-linked with dimethyl dithiobis(propionimidate) (DDP). CBG (200 μL of a 0.2 mg/mL solution in 10 mM Tris buffer, pH 7.5, containing 5 mM $CaCl_2$) was diluted with 275 μL of water and 25 μL of 1 M triethylamine buffer, pH 8.0, and 2 μL of 500 mM magnesium acetate was added. The solution was then cooled in an ice–water bath, DDP (150 μL of a 10 mg/mL solution in water) was added, and the solution was stirred at 0 °C for 30 min. The product was dialyzed at 4 °C against PBS prior to analysis by SDS–polyacrylamide gel electrophoresis.

Sephadex G-200 Chromatography. Samples (0.5 mL) of plasma preequilibrated with [^{3}H]cortisol were applied to a column (1.6 × 90 cm) of Sephadex G-200 equilibrated with 10 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl at 4 °C. The column was developed with the same buffer at a constant flow rate of 30 mL/h (maintained with an LKB peristaltic pump; LKB Instruments, Gaithersburg, MD). Dextran blue, [^{14}C]albumin, and [^{14}C]valine were added to each sample before chromatography in order to determine the void volume, relative elution position, and internal volume, respectively. Fractions (1 mL) were collected and analyzed for protein and radioactivity.

Amino Acid Composition. Amino acid analyses were performed following hydrolysis of samples with 6 N HCl in vacuo for 24 h at 110 °C (Moore & Stein, 1963). Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, on 24-h acid hydrolysates of a performic acid oxidized sample (Hirs, 1967).

Antibody Production. Intact, adult male New Zealand White rabbits were used for antiserum production according to the method of Vaitukaitus et al. (1971). Purified squirrel monkey CBG (300 μg in 1 mL of 0.15 M NaCl)was emulsified with 1 mL of Freund's complete adjuvant (Gibco, Grand Island, NY) and injected into multiple intradermal sites on the back of two rabbits. Half of the above amount of CBG diluted in normal saline and emulsified in Freund's incomplete adjuvant (Gibco) was used for booster injections into both intradermal and intramusclular sites at biweekly intervals. Blood was collected from the marginal ear veins at intervals of 1 week after each booster injection, allowed to clot, and centrifuged. The serum was collected and stored frozen at −70 °C until use.

Results

Purification of CBG. Affinity chromatography proved to be a highly effective technique for the isolation of CBG from pregnant squirrel monkey serum. Virtually all of the cortisol binding activity was retained by the affinity column and was subsequently eluted by the addition of cortisol to the elution buffer. When this eluted material was examined by electrophoresis, it was found to be more than 96% pure. To achieve final purification, the fractions containing binding activity were applied to a DEAE-Sepharose column. Development of the column with a linear NaCl gradient resulted in the elution of a single protein peak which coincided with cortisol binding activity. Similar results were obtained on four separate occasions, and a summary of the isolation procedure is shown in Table I.

The purity of the final product was examined by electrophoresis. Purified CBG (110 μg) was incubated overnight with 10 nM [^{3}H]cortisol at 4 °C, and duplicate samples (50 μg) were then electrophoresed in gels which contained 1 nM labeled cortisol to minimize losses of bound hormone during the separation procedure. Following electrophoresis, one gel was stained for protein while the second gel was frozen and sliced into 2-mm slices, and the amount of radioactive steroid present in each slice was determined. As shown in Figure 1, only a single band of staining material was observed despite the fairly large amount of protein applied to the gel. A single band of protein was also observed when 20-μg samples were separated on gels varying in total acrylamide concentrations from 5% to 15% at pH 8.3 (data not shown). Figure 1 also shows that all of the cortisol binding activity coincided with the single band of stained material. Taken together, these results indicate that the isolated material was homogeneous since a contamination of less than 3% could have been detected on the basis of the amount of protein applied to the gels and the sensitivity of the staining procedure utilized for detection.

Table I: Purification of Squirrel Monkey CBG

step	total protein (mg)	total binding sites (pmol)	sp act. (pmol/mg)	% yield	purification (x-fold)
plasma	652.5	381700	585	100	1×
affinity eluate	18.6	247800	13322	66	22.8
DEAE-Sepharose	11	232600	21145	61	36.15

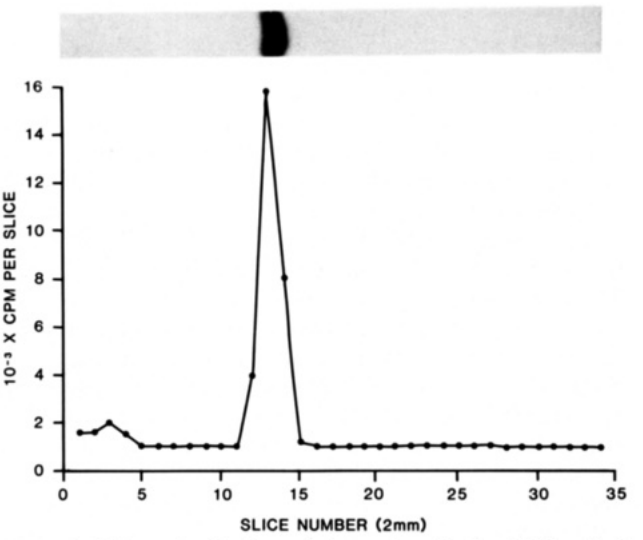

FIGURE 1: Polyacrylamide disc gel electrophoresis of purified squirrel monkey CBG. Samples of purified CBG (50 μg each) were allowed to incubate overnight at 4 °C with 10 nM [^{3}H]cortisol. The samples were then electrophoresed at 4 °C in gels containing 1 nM [^{3}H]cortisol with the discontinuous buffer system of Davis (1964). At the end of the separation, one gel was stained for protein while the second was frozen and cut into 2-mm slices. The slices were placed into counting vials, scintillation fluid was added, and the distribution of radioactivity was determined.

Ultraviolet Absorption Spectrum. The ultraviolet absorption spectrum of freshly isolated squirrel monkey CBG was determined. The spectrum showed the expected peak absorbance at 280 nm and no evidence of nucleotide or pyridine nucleotide cofactors. The spectrum is more flat than expected in the region of 240–280 nm. This is probably the result of bound cortisol (A_{max} = 240 nm) since the spectrum was determined with steroid–protein complex rather than with the apoprotein. Similar results have been observed with isolated hen oviduct progesterone receptor–steroid complexes (Kuhn et al., 1977).

Amino Acid Composition. The amino acid composition of pure squirrel monkey CBG is given in Table II. The protein

Table II: Amino Acid Composition of Purified Squirrel Monkey CBG

residue	nmol	g/100 g[a]	residues/100 000 g[a]
Asx	15.20	10.93	95.0
Thr	9.16	5.79	57.2
Ser	12.04	6.55	75.2
Glx	12.89	10.40	80.5
Pro	5.00	3.03	31.2
Gly	9.37	3.34	58.6
Ala	10.28	4.57	64.2
Val	9.77	6.05	61.1
Ile	7.01	4.96	43.8
Leu	16.93	11.98	105.8
Tyr	5.11	5.21	32.0
Phe	9.89	9.10	61.8
His	4.87	4.17	30.4
Lys	6.80	5.45	42.5
Arg	4.68	4.57	29.3
Met[b]	3.92	3.21	24.2
$^1/_2$-Cys[b]	1.00	0.70	6.3

[a] Calculated relative to the polypeptide portion of the molecule. [b] Half-cysteine and methionine were determined after performic acid oxidation.

contains high levels of acidic (aspartic and glutamic acids) compared to basic amino acids residues (arginine and lysine). This is in agreement with the protein's behavior on ion-exchange resins and during polyacrylamide gel electrophoresis. In addition, the protein contains a high content of leucine residues.

A comparison of the amino acid composition of squirrel monkey CBG with that of other proteins is shown in Table III. The data have been expressed as the mole percent of each amino acid to allow comparison without the complication of differing molecular weights. As can be seen, the compositions of corticosteroid binding globulin from rat, human, guinea pig, and squirrel monkey sera are remarkably similar; they all contain high levels of aspartic and glutamic acids and leucine. The amino acid composition of CBG is distinct from that of other steroid binding proteins. Of particular interest is its difference from that of rabbit progesterone receptor (another high-affinity binding protein) and that of the two albumins examined which also bind steroids although with low affinity.

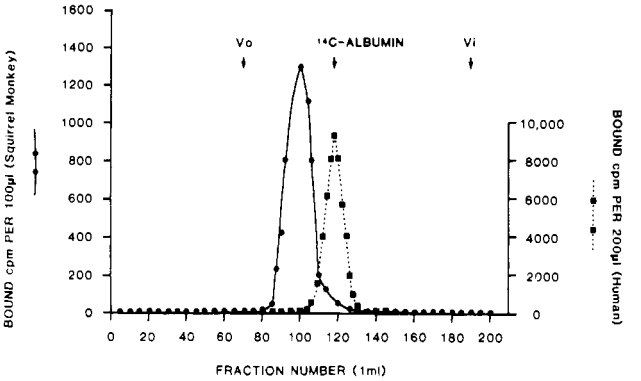

FIGURE 2: Separation of cortisol binding activities by gel filtration on Sephadex G-200. Samples (0.5 mL) of plasma were preequilibrated with 10 nM [^{3}H]cortisol. Dextran blue, [^{14}C]albumin, and [^{14}C]valine were added to each sample to determine V_o, the relative elution position, and V_i, respectively. Separations were performed at 4 °C on a column (1.6 × 90 cm) of Sephadex G-200 which had been equilibrated with 10 mM sodium phosphate, pH 7.4, buffer containing 0.1 M NaCl. Fractions (1 mL) were collected and analyzed for radioactivity. Samples were squirrel monkey serum (closed circles, solid line) and human serum (closed squares, dotted line).

Determination of Molecular Size. Several lines of evidence suggest that squirrel monkey CBG may be larger than those found in other species. First, our previous studies (Klosterman et al., 1986) revealed that squirrel monkey CBG sedimented significantly faster in sucrose gradients (5.3 S) than CBG from either humans (3.7 S) or cynomolgus monkeys (4.0 S). We also found that squirrel monkey CBG migrates slower than other CBG's in polyacrylamide gel electrophoresis. The results of gel exclusion chromatography also are consistent with this interpretation. Squirrel monkey or human serum was equilibrated with [^{3}H]cortisol and chromatographed together with [^{14}C]albumin on Sephadex G-200. As can be seen in Figure 2, squirrel monkey CBG migrated in a position consistent with a size considerably larger than that of human CBG. The elution position of both proteins was unchanged if the serum was labeled prior to chromatography or whether binding activities were determined following separation. In addition, the relative elution position was not altered when the chroma-

Table III: Mole Percent of Amino Acids in CBG and Other Proteins[a]

residue	sm CBG	r CBG[b]	h CBG[c]	gp CBG[d]	rabbit prog receptor[i]	mouse HbA[e]	HSA[g]	BSA[h]	hGH[f]
Asx	10.56	11.82	9.61	11.27	6.28	8.57	9.08	9.20	10.70
Thr	6.36	6.97	6.61	7.17	3.25	5.00	4.62	5.90	5.35
Ser	8.37	8.18	6.61	8.45	10.61	9.29	4.11	4.86	9.62
Glx	8.96	12.73	11.41	8.95	10.50	3.57	14.38	13.54	13.90
Pro	3.47	5.15	6.61	5.00	10.82	4.29	4.11	4.86	4.28
Gly	6.51	6.36	5.71	4.96	8.23	7.89	2.05	2.60	4.27
Ala	7.14	6.97	6.91	7.63	10.28	14.29	10.79	7.99	3.74
Val	6.79	6.06	7.81	3.85	5.52	7.14	6.68	6.25	3.74
Ile	4.87	3.94	4.80	5.30	3.25	1.43	1.37	2.40	4.28
Leu	11.76	10.30	12.01	14.02	11.15	12.14	10.45	10.59	13.37
Tyr	3.55	3.03	3.00	2.00	2.27	2.14	3.08	3.30	4.28
Phe	6.87	4.85	5.71	6.00	3.35	5.0	5.31	4.51	6.95
His	3.38	2.12	2.70	3.28	1.52	7.86	2.74	2.95	1.60
Lys	4.73	6.06	4.50	3.71	4.44	7.86	10.10	10.24	4.81
Arg	3.25	3.03	3.00	4.14	4.33	2.14	4.11	3.99	5.35
Met	2.72	2.42	3.00	2.67	1.73	0.71	1.02	0.69	1.60
$^1/_2$-Cys	0.70	N.D.	N.D.	1.03	2.27	0.71	5.99	6.08	2.14
Glx + Asx	19.52	24.55	21.02	20.22	16.78	12.14	23.46	22.74	24.60
His + Lys + Arg	11.36	11.21	10.20	11.13	10.29	17.89	16.95	17.18	11.76
Met + $^1/_2$-Cys	3.42			3.67	4.00	1.42	7.01	6.77	3.74

[a] Abbreviations: sm CBG, squirrel monkey CBG; r CBG, rat CBG; h CBG, human CBG; gp CBG, guinea pig CBG; rabbit prog receptor, rabbit progesterone receptor; mouse HbA, mouse hemoglobin; HSA, human serum albumin; BSA, bovine serum albumin; hGH, human growth hormone; N.D., not determined. [b] Chader & Westphal (1968). [c] Muldoon & Westphal (1967). [d] Schneider & Slaunwhite (1971). [e] Popp (1965). [f] Li et al. (1966). [g] Meloun et al. (1975). [h] Brown (1975). [i] Loosfelt et al. (1986).

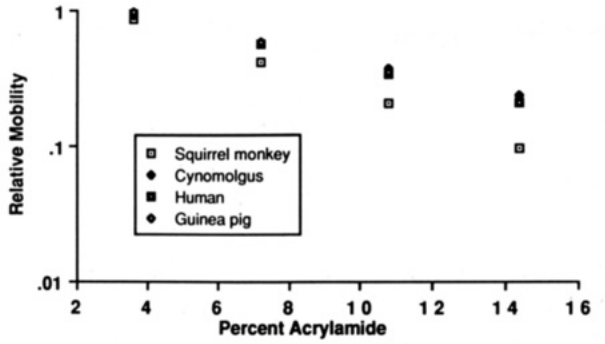

FIGURE 3: Effect of varying total acrylamide concentration on the relative mobility of CBG. Serum samples (20 μL) were equilibrated with 10 nM [^{3}H]cortisol and then separated on polyacrylamide gels of various total acrylamide concentrations in the discontinuous system of Davis (1964). In all cases, the gels contained 2.6% cross-linker. Separations were performed at 4 °C. At the conclusion of each run, the gels were removed, frozen, and cut into 2-mm slices. The slices were placed in vials, scintillation fluid was added, and the distribution of radioactivity was determined. The migration of cortisol binding is expressed relative to that of the ion front. The serum samples employed were as follows: guinea pigs (open diamonds), human (closed squares), squirrel monkey (open squares), and cynomolgus monkey (closed diamonds).

tography was performed at 4, 25, or 37 °C (data not shown). Regardless of the conditions employed, squirrel monkey CBG eluted at a position consistent with its being about twice the size of the human protein.

The molecular weight of squirrel monkey CBG in serum was also estimated by altering the total concentration of acrylamide in electrophoretic gels as described by Ferguson (1964). Figure 3 shows a plot of the total acrylamide concentration versus the log of the mobility of cortisol binding activity relative to the ion front. As can be seen, the slopes of the line for human, cynomolgus monkey, and rat are all similar. Squirrel monkey CBG, however, is characterized by a steeper slope which is indicative of a greater molecular size. When the molecular weights of the CBG's were estimated from plots of molecular weight vs K_R as described by Rodbard and Chrambach (1971), it was found that guinea pig, human, and cynomolgus monkey CBG had values of about 50 000 while the molecular weight of squirrel monkey was approximately 110 000. Like the gel filtration experiments, these results suggested that squirrel monkey CBG is about twice the size of that of any previously described CBG.

To examine the possible subunit structure of squirrel monkey CBG, the purified protein was subjected to electrophoresis in the presence of sodium dodecyl sulfate (SDS). As can be seen, the purified protein separated into two bands: one at 54 000 daltons and one at 57 000 daltons (Figure 4A, lane 2). The size of each of these bands is about the same as that found for CBG's of other species. Since only a single protein band was found following electrophoresis of the purified material under nondenaturing conditions and since other techniques suggested that the native squirrel monkey protein was about twice the size of previously described CBG's, the possibility that the native molecule was a dimer containing two subunits was examined following reaction of the purified protein with reversible cross-linking agents. When the reaction products formed with either methyl 4–mercaptobutyrimidate or DDP were separated on SDS gels in the absence of 2-mercaptoethanol, two areas of staining were observed (Figure 4B, lane 1). One was in the region of 50 000 daltons and likely represents unreacted subunits. The second migrated at a position consistent with a molecular size of 110 000 daltons or roughly equal to that of the purified native protein. If electrophoresis

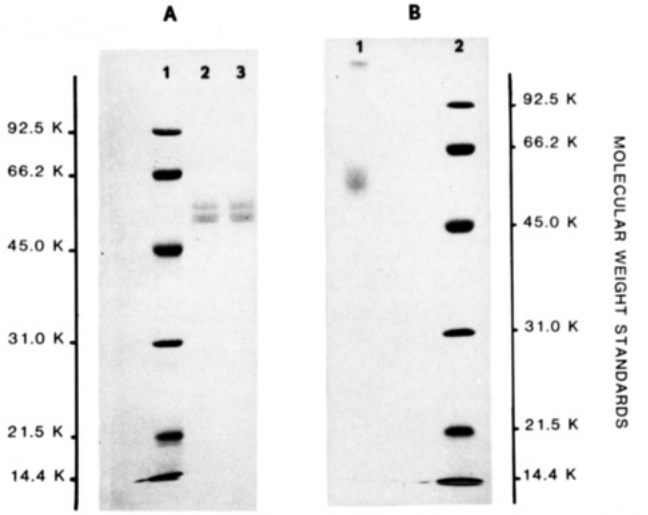

FIGURE 4: Molecular weight determination of native and cross-linked purified squirrel monkey CBG by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Separations were performed on slab gels using the discontinuous system of Laemmli (1970). The separations shown on panel A were performed after treatment of the samples with 0.1% 2-mercaptoethanol: lane 1, molecular weight standards; lane 2, unreacted purified squirrel monkey CBG; lane 3, squirrel monkey CBG previously reacted with the cross-linking agent dimethyl dithiobis(propionimidate) (DDP) as described under Experimental Procedures. (Panel B) Separations were performed without treating the samples with 2-mercaptoethanol: lane 1, pure squirrel monkey CBG reacted with the cross-linking reagent DDP; lane 2, molecular weight standards.

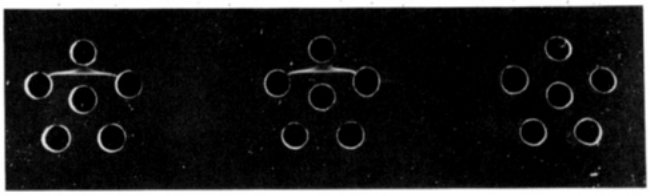

FIGURE 5: Cross-reactivity between antisera prepared against squirrel monkey CBG and sera from other animal species. The immunodiffusion technique of Ouchterlony (1949) was used. Samples (6 μL) of antisera were placed in each of the three center wells. Samples (6 μL) of various sera were placed in each of the outer wells. The samples examined are (starting from the top and proceeding clockwise) as follows: (A) pure squirrel monkey CBG, a pool of human pregnancy serum, female rhesus monkey serum, male titi serum, and male brown marmoset serum; (B) squirrel monkey serum, female guinea pig serum, male rat serum, male baboon serum, and male cynomolgus serum; (C) antiserum diluted 1:10 in the center and male titi monkey serum, male brown marmoset serum, human pregnancy plasma pool, female rhesus monkey serum, and female guinea pig serum.

was performed after treatment with 2-mercaptoethanol (a condition which reverses the effects of cross-linking with the agents employed), only the two subunits were observed (Figure 4A, lane 3). Similar results were found when squirrel monkey serum was cross-linked and the products examined by Western blotting techniques (data not shown).

Antibody Studies. To examine the antigenic relatedness of squirrel monkey CBG and those from other species and to develop a radioimmunoassay for the measurement of this protein, antibodies were produced in rabbits according to the technique of Vaitukaitis et al., (1971) as described under Experimental Procedures. The ability of the resulting antiserum to interact with CBG from other species was examined by the technique of Ouchterlony (1949). As shown in Figure 5, only a single protein in squirrel monkey serum was recognized by the antiserum. No cross-reactivity was observed between antiserum to pure squirrel monkey CBG and other sera using this technique. To test for immunologic cross-re-

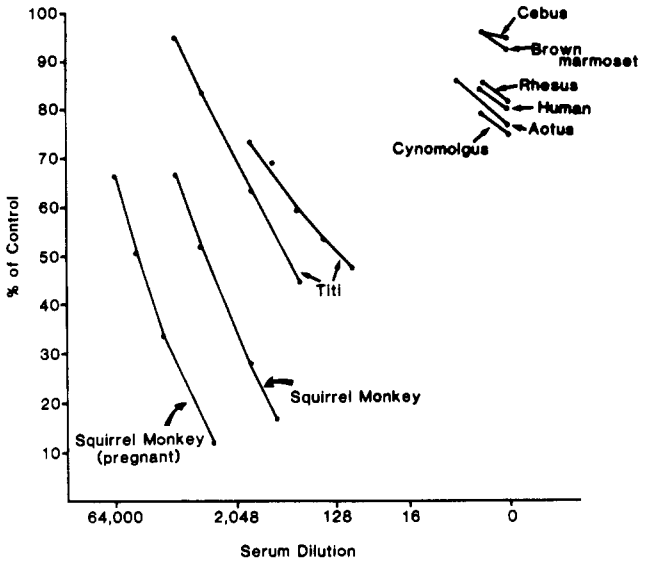

FIGURE 6: Cross-reactivity of sera from various primates in a radioimmunoassay for squirrel monkey CBG.

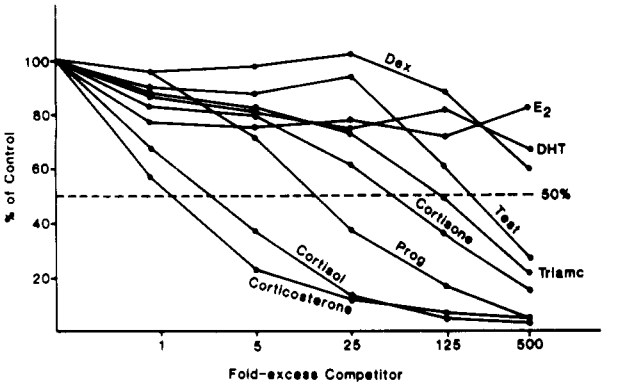

FIGURE 7: Binding specificity of squirrel monkey CBG. Dilute (1:20) squirrel monkey serum was incubated with 20 nM [^{3}H]cortisol with or without various concentrations of nonradioactive competing steroids at 4 °C for 18 h. Bound radioactivity was then measured by the DEAE filter assay. Results are expressed as percent of control, i.e., no competing steroid (12 300 cpm).

activity of the polyclonal antibody prepared against squirrel monkey CBG in a more sensitive manner, the ability of sera from a variety of species to compete with ^{125}I-labeled squirrel monkey CBG for binding to the antibody was examined (Figure 6). Interestingly, no cross-reactivity was observed with human or rhesus monkey CBG, and of the New World primate sera tested, only serum from titi monkeys competed for squirrel monkey CBG binding.

Binding Specificity. In view of the unusual physical and immunological properties of squirrel monkey CBG, it was of interest to determine its binding specificity. This was especially important since our previous studies with squirrel monkey serum revealed that unlike other CBG's a linear van't Hoff plot was obtained when the affinity for cortisol was measured at different temperatures (Klosterman et al., 1986). The results of competition experiments shown in Figure 7 demonstrate that the binding preference for a variety of steroids is very similar to that of CBG from many other species (Westphal, 1971).

Discussion

Our previous studies (Klosterman et al., 1986) suggested that the molecular size of CBG from New World primates is greater than that of CBG found in the serum of Old World primates when examined both by sucrose density gradient centrifugation and by polyacrylamide gel electrophoresis. In contrast, Pugeat et al. (1984) concluded that there was essentially no difference in the size of CBG from Old and New World monkeys although they also observed low-affinity cortisol binding to New World monkey CBG. This apparent discrepancy led us to examine the properties of sqiurrel monkey CBG more closely. Gel filtration studies clearly demonstrated that squirrel monkey CBG is larger than the corresponding molecule in either cynomolgus monkey or human sera. A similar result was obtained when patterns of migration of cortisol binding activity on polyacrylamide gels of varying total acrylamide concentration were compared for guinea pig, human, cynomolgus monkey, and squirrel monkey serum. To further substantiate these results, we decided to prepare pure squirrel monkey CBG.

The use of an affinity column followed by ion-exchange chromatography allowed the rapid and efficient purification of CBG from squirrel monkey serum. The final product was found to be homogeneous when examined by electrophoresis at pH 8.3 in polyacrylamide gels with varying (5–15%) total acrylamide concentrations. The single band of stained protein correspond to a single peak of cortisol binding activity. The high yield of purified CBG (61%) together with its properties being concordant with those observed in serum indicates that the isolated CBG is representative of the major circulating species.

The amino acid composition of squirrel monkey CBG bears remarkable similarities to those of CBG's from the rat, human, and guinea pig when compared on a mole percent basis. All are characterized by relatively high acidic (Asx plus Glx) versus basic (Arg plus Lys plus His) residues. This is consistent with our observation (data not presented) that the isoelectric points of all these CBG molecules are less than pH 5.0. In addition, these proteins contain a high proportion of leucine residues. In contrast, rabbit progesterone receptor contains a much higher proportion of proline than found for the serum binding proteins. The amino acid compositions of the CBG's are also clearly distinct from those of either human or bovine serum albumin.

The availability of purified squirrel monkey CBG allowed us to determine its molecular weight directly, using polyacrylamdie gel electrophoresis in the presence of sodium dodecyl sulfate. SDS gel electrophoresis revealed the presence of two protein bands: one at 54 000 daltons and a second at 57 000 daltons. The staining intensity of both bands was nearly identical, suggesting that the native molecule contains equal amounts of both species. The molecular weight of each of these two bands is in the normal range found for CBG's from a variety of species (Westphal, 1971). The presence of a single protein band following electrophoresis in nondenaturing gels and two bands following separation in gels containing SDS suggested that the native structure of squirrel monkey CBG was a dimer containing two subunits of slightly different molecular weight. The two bands were routinely observed whether or not the sample was treated with 2-mercaptoethanol prior to electrophoresis. Furthermore, cortisol binding activity in squirrel monkey serum could not be shifted to a smaller molecular weight species by treatment with reducing agents prior to G-200 chromatography (data not shown). Together these results suggest that disulfide bond formation is not responsible for generation of the dimer.

The cross-linking experiments were performed to confirm that squirrel monkey CBG is in fact a dimer. Dimethyl dithiobis(propionimidate) and 4-mercaptobutyrimidate were selected as cross-linking reagents because their effects could

be reversed by treatment with thiol reagents. The cross-linking experiments were also conducted at low protein concentrations to ensure that nonspecific cross-linking would be minimized. When similar experiments were carried out with ovalbumin at a protein concentration 100 times that used for squirrel monkey CBG, no evidence for the generation of higher molecular weight species was found. The molecular weights of the two subunits, their identical staining intensities following polyacrylamide gel electrophoresis, and the molecular weight of the cross-linked moiety suggest that the native molecule is a heterodimer consisting of 54K and 57K subunits. The present data, however, do not allow us to rule out the possible existence of homodimers consisting of identical subunits. Whether or not both subunits bind steroid remains to be determined. Preliminary attempts to determine the stoichiometry of cortisol binding to pure CBG have been inconclusive because of its unknown carbohydrate content.

On the basis of our earlier work with serum (Klosterman et al., 1986), it is possible that CBG's from other New World primates such as the titi and marmoset also are dimers. The presence of higher molecular weight aggregates of CBG has been described in pure preparations from rat, rabbit, human, and guinea pig (Westphal, 1986). However, with the single exception of a report indicating the presence of dimeric CBG in human serum (Mueller, 1986), no evidence for CBG dimers has been found in either serum or plasma of other species. The formation of higher molecular weight aggregates of CBG from these other species appears to occur only when CBG is separated from other serum proteins. The presence of dimeric CBG in serum appears to be the norm only in New World primates. The relationship between the dimeric structure of squirrel monkey CBG and its biologic function is not currently understood. It seems probable, however, that it contributes to such unique properties as the unusual temperature dependence of steroid binding which is observed with New World primate cortisol binding activity (Klosterman et al., 1986).

The polyclonal antibodies prepared against pure squirrel monkey CBG showed no cross-reactivity when human or rhesus monkey serum was added to a system containing iodinated squirrel monkey CBG and antisera. Previous studies indicated that CBG antisera prepared against human CBG do not cross-react with squirrel monkey CBG (Robinson et al., 1985). Together these results indicate that there may be substantial differences in the amino acid sequence and/or carbohydrate moieties present in human and squirrel monkey CBG. Somewhat surprising is the fact that CBG in only one (titi) of four New World primate sera tested competed with iodinated squirrel monkey CBG for binding to the antibody. However, further studies are needed to determine whether this reflects differences in CBG structure or simply very low plasma levels of CBG.

Further investigation of the difference in structure and properties of CBG from various species may provide important clues concerning the physiologic role of serum steroid binding proteins. Although the free fraction of steroid in blood has been considered to be the biologically active component for more than 30 years, recent studies have demonstrated that CBG and sex hormone binding globulin (SHBG) can bind to target cell plasma membranes (Strel'chyonok & Avvakumov, 1983; Hryb et al., 1985, 1986; Rosner et al., 1986; Hsu et al., 1986) and they also have been detected within cells (Bordin & Petra, 1980; Perrot-Applanat et al., 1981, 1984; Egloff et al., 1982; Tardinal-Laucombe et al., 1984; Kuhn et al., 1986). We have previously speculated that both CBG and SHBG may serve as intracellular carriers and thereby protect steroid hormones from the ubiquitous steroid metabolizing enzymes (Siiteri et al., 1982). In squirrel monkeys, free cortisol levels are far higher than might be predicted from the modestly reduced affinity of the glucocorticoid receptor, suggesting that CBG may play an even more essential role in steroid hormone action. The high cortisol levels together with the low affinity of CBG may be indicative of an important interaction of the cortisol–CBG complex with target cell plasma membranes. This possibility is supported by a recent report demonstrating that the glucocorticoid receptor is located in the plasma membrane of glucocorticoid-responsive S49 mouse lymphoma cells (Gametchu, 1987).

References

Birnbaumer, M. E., Schrader, W. T., & O'Malley, B. W. (1979) *Biochem. J. 181*, 201–213.

Blakesley, R. W., & Boezi, J. A. (1977) *Anal. Biochem. 82*, 580–582.

Bordin, S., & Petra, P. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5678–5682.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brown, J. R. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 591.

Chader, G. J., & Westphal, U. (1968) *Biochemistry 7*, 4272–4282.

Chrousos, G. P., Renquist, D., Brandon, D., Eil, C., Pugeat, M., Vigersky, R., Cutler, G. B., Jr., Loriaux, D. L., & Lipsett, M. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2036–2040.

Chrousos, G. P., Loriaux, D. L., Brandon, D., Shull, J., Renquist, D., Hogan, W., Tomita, M., & Lipsett, M. B. (1984) *Endocrinology (Baltimore) 115*, 25–32.

Coe, C. L., Murai, J. T., Wiener, S. G., Levine, S., & Siiteri, P. K. (1986) *Endocrinology (Baltimore) 118*, 435–440.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Egloff, M., Vendrely, E., Tardinal-Laacombe, J., Dadoune, J. P., & Degrelle, H. (1982) *C. R. Seances Acad. Sci., Ser. 3 292*, 107–111.

Ferguson, K. A. (1964) *Metab., Clin. Exp. 13*, 985–100.

Gametchu, B. (1987) *Science (Washington, D.C.) 236*, 456–461.

Heyns, W., Van Baelen, H., & De Moor, P. (1967) *Clin. Chim. Acta 18*, 361–370.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59–62.

Hsu, B. R.-S., Siiteri, P. K., & Kuhn, R. W. (1986) in *Binding Proteins of Steroid Hormones* (Forest, M. G., & Pugeat, M., Eds.) pp 577–591, John Libbey & Co., London.

Hyrb, D., Khan, M., & Rosner, W. (1985) *Biochem. Biophys. Res. Commun. 128*, 432–440.

Hyrb, D., Khan, M., Romas, N., & Rosner, W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3253–3256.

Klosterman, L. L., Murai, J. T., & Siiteri, P. K. (1986) *Endocrinology (Baltimore) 118*, 424–434.

Kuhn, R. W., Schrader, W. T., Coty, W. A., Conn, P. M., & O'Malley, B. W. (1977) *J. Biol. Chem. 252*, 308–317.

Kuhn, R. W., Green, A. L., Raymoure, W. J., & Siiteri, P. K. (1986) *J. Endocrinol. 108*, 31–36.

Laemmli, U. K. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 305–312.

Li, C. H., Liu, A. K., & Dixon, J. S. (1966) *J. Am. Chem. Soc. 88*, 2050–2051.

Loosfelt, H., Atger, M., Misrahi, M., Guichon-Mantel, A., Meriel, C., Logeat, F., Benarous, R., & Milgrom, E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9045–9049.

Meloun, B., Morave, K. L., & Kostka, V. (1975) *FEBS Lett. 58*, 134–137.

Mendoza, S. P., Lowe, E. L., Davidson, J. M., & Levine, S. (1978) *Horm. Behav. 11*, 295-303.
Mickelson, K. E., & Westphal, U. (1979) *Biochemistry 18*, 2685-2690.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819-831.
Mueller, U. W., & Potter, J. M. (1984) *J. Steroid Biochem. 20*, 1261-1266.
Muldoon, T. G., & Westphal, U. (1967) *J. Biol. Chem. 242*, 5636-5643.
Ornstein, L., (1964) *Ann. N.Y. Acad. Sci. 121*, 404-427.
Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand. 40*, 95-134.
Perrot-Applanat, M., David-Ferreira, J. F., & David-Ferreira, K. L. (1981) *Endocrinology (Baltimore) 109*, 1625-1633.
Perrot-Applanat, M., Racadot, O., & Milgrom, E. (1984) *Endocrinology (Baltimore) 115*, 559-569.
Popp, R. A. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 1252-1257.
Pugeat, M. M., Chrousos, G. P., Nisula, B. C., Loriaux, D. L., Brandon, D., & Lipsett, M. B. (1984) *Endocrinology (Baltimore) 115*, 357-361.
Robinson, P. A., Hawkey, C., & Hammond, G. L. (1985) *J. Endocrinol. 104*, 251-257.
Rodbard, D., & Chrambach, A. (1971) *Anal. Biochem. 40*, 95-134.
Rosner, W., Khan, M. S., Romas, N. A., & Hyrb, D. J. (1986) in *Binding Proteins of Steroid Hormones* (Forest, M. G., & Pugeat, M., Eds.) pp 567-575, John Libbey & Co., London.
Schneider, S. H., & Slaunwhite, W. R., Jr. (1971) *Biochemistry 10*, 2086-2093.
Seal, U. S., & Doe, R. P. (1965) *Steroids 5*, 827-841.
Shinki, T., Shiina, Y., Takahashi, N., Tanioka, Y., Koizumi, H., & Suda, T. (1983) *Biochem. Biophys. Res. Commun. 114*, 452-457.
Siiteri, P. K. (1986) in *Mechanisms and Clinical Aspects of Steroid Hormone Resistance* (Chrousos, G. P., Loriaux, D. L., & Lipsett, M. L., Eds.) pp 279-289, Plenum, New York.
Siiteri, P. K., Murai, J. T., Hammond, G. L., Nisker, J. A., Raymoure, W. J., & Kuhn, R. W. (1982) *Recent Prog. Horm. Res. 38*, 457-510.
Strel'chyonok, O, & Avvakumov, G. (1983) *Biochim. Biophys. Acta 755*, 514-517.
Tardival-Laacombe, J., Egloff, M., Mazabraud, A., & Degrelle, H. (1984) *Biochem. Biophys. Res. Commun. 118*, 488-494.
Westphal, U. (1971) in *Steroid-Protein Interactions*, pp 334-347, Springer-Verlag, New York.
Westphal, U. (1986) in *Steroid-Protein Interactions II*, pp 116-119, Springer-Verlag, New York.

Biphasic Stimulation of Cellular Calcium Concentration by 3,5,3′-Triiodothyronine in Rat Thymocytes†

Joseph Segal*

Charles A. Dana Research Institute and Harvard-Thorndike Laboratories of Beth Israel Hospital of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215, and Hubert H. Humphrey Center for Experimental Medicine and Cancer Research, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel

Received July 31, 1987; Revised Manuscript Received October 28, 1987

ABSTRACT: 3,5,3′-Triiodothyronine (T_3) produced a rapid and transient increase in ^{45}Ca uptake and cytoplasmic free calcium concentration in rat thymocytes, which is the most rapid effect of T_3 in this system. This effect was manifested in cells suspended in medium containing 1 mM calcium. The T_3 effect on ^{45}Ca uptake was evident at 15-30 s, reached maximum at 30-60 s, and returned to control values at 5 min. The T_3 effect on cytoplasmic free calcium concentration was seen after 30 s, reached maximum at 7 min, and returned to control values after 24 min. In cells suspended in Ca^{2+}-free medium, T_3 produced a similar rapid increase in ^{45}Ca uptake, which was sustained for at least 60 min, but T_3 failed to change cytoplasmic free calcium concentration. Alprenolol (10 μM) blocked the stimulatory effects of T_3 on these two functions in a similar fashion. From these results, I suggest that in rat thymocytes T_3 influences cellular calcium economy through a biphasic mechanism in which T_3 first increases calcium uptake which, in turn, is followed by a release of calcium from intracellular pool(s), resulting in a further increase in cytoplasmic free calcium concentration and the activation of Ca^{2+}-regulated systems. Moreover, the present study provides further support for the postulate that in the rat thymocyte calcium serves as the first messenger for the plasma membrane-mediated stimultory effects of T_3 on several metabolic functions.

In several tissues, such as the heart, it has been postulated that agents exert their calcium-mediated functions through a two-step process. First, the agent acts to increase calcium uptake and thereby cytoplasmic free calcium concentration; such an increase, which is too small to activate the system itself, triggers the release of calcium from intracellular pool(s), primarily the endoplasmic reticulum, which results in a further increase in cytoplasmic free calcium concentration and the activation of the biologic system (Berridge, 1985; Langer, 1976, 1979).

We have shown previously in rat thymocytes that thyroid hormone requires calcium to exert its plasma membrane-

† This work was supported in part by Grant AM-18416 from the NIAMDD, NIH, Bethesda, MD, and by a grant from the Center of Absorption in Science, Ministry of Absorption, Israel.

* Address correspondence to this author at the Hubert H. Humphrey Center for Experimental Medicine and Cancer Research.

0006-2960/88/0427-2586$01.50/0