J. steroid Biochem. Vol. 22, No. 6, pp. 739–746, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

DIRECT EFFECT OF SEX STEROID-BINDING PROTEIN (SBP) OF PLASMA ON THE METABOLIC CLEARANCE RATE OF TESTOSTERONE IN THE RHESUS MACAQUE‡

PHILIP H. PÉTRA*, FRANK Z. STANCZYK†, PEARL C. NAMKUNG*,
MARC A. FRITZ† and MILES J. NOVY†
*Departments of Ob/Gyn and Biochemistry, University of Washington, Seattle, WA 98195 and
†The Oregon Regional Primate Research Center, Beaverton, Department of Ob/Gyn,
The Oregon Health Sciences University, Portland, OR, U.S.A.

(*Received* 31 *July* 1984)

Summary—We report direct evidence for the effect of the sex steroid-binding protein (SBP) on the metabolic clearance rate of testosterone (MCR^T). Pure rhesus SBP or human SBP was infused intravenously into three different cycling female rhesus monkeys. MCR^T was measured before and after SBP had reached 150–300% of basal levels. A decrease in MCR^T was observed in all cases. The effect of SBP on MCR^T was tested further in four additional cycling females by infusing immunoaffinity-purified monospecific human SBP antibodies known to cross-react with rhesus SBP. SBP dropped to 54, 40, 4 and 2% of basal levels with a concomitant increase of 118, 190, 320 and 640% of basal MCR^T. In one of these animals, pure rabbit SBP was administered after the anti-human SBP infusion resulting in a decrease in MCR^T. The magnitude of the SBP effect on MCR^T is related to the distribution of testosterone (T) bound to SBP and albumin in the plasma. Calculations show that as long as the percent of T bound to SBP is equal or higher than the percent of T bound to albumin, the influence on MCR^T is small. However, if SBP is reduced to the extent that T is bound mostly to albumin, the redistribution of T is associated with a dramatic increase in MCR^T. We conclude that under normal conditions each animal has an optimum concentration of plasma SBP which binds a maximum amount of T. If SBP increases above this level, little effect on MCR^T will result. However, a drop below the optimum level, as is the case in certain physiological or clinical conditions, will produce a large increase in the clearance of T.

INTRODUCTION

For the past fifteen years many investigators have reported possible roles for the sex steroid-binding protein, SBP[1], in plasma. Early studies on measurements of the protein in humans revealed a sex difference in SBP levels, with normal adult females having about twice as much as normal adult males [1–4]. Vermeulen and co-workers compared the metabolic clearance rate of testosterone [MCR^T] in normal human females and males and found that (at comparable plasma T concentrations) MCR^T is always higher in the latter [3]. In hyperthyroidism, where SBP is elevated, a decrease in MCR^T was found [5]. An increase in MCR^T in hirsutism [6] appears to be associated with low SBP levels normally found in these patients [3, 7–10]. Although these studies suggest an inverse relationship between SBP and MCR^T, they do not prove, as is generally accepted, that SBP has a direct effect on MCR^T. This is evident because the relationship between SBP and MCR^T found in those studies was deduced indirectly by comparing MCR^T in males and females where SBP levels are known to be different. There are endocrine differences in normal males and females, as well as in hirsute and hyperthyroid patients, which could account not only for the changes in MCR^T but also for regulating those changes.

In order to provide direct evidence on whether or not SBP has a direct effect on MCR^T, we infused pure SBP and its antibodies in rhesus monkeys and measured their effect on MCR^T. These experiments were possible because of the availability of pure human SBP [11], rhesus SBP [12], rabbit SBP [13], as well as anti-human SBP [14]. We have published a preliminary report which was presented at the VIth

[1] This nomenclature was first adopted at the IVth meeting of the International Study Group for Steroid Hormones, Rome, 1969. Subsequently, other terms have been introduced in the literature. These are: steroid-binding β-globulin, SBβG; sex hormone-binding globulin, SHBG; testosterone-binding globulin, TBG; estradiol-binding protein, EBP; testosterone-estradiol-binding globulin, TeBG. The latter three terms are somewhat misleading because the protein actually binds 5α-dihydrotestosterone better than testosterone or 17β-estradiol. In rabbits and other non-primate species an insignificant amount of 17β-estradiol appears to be bound significantly in plasma at physiological conditions.

Abbreviations: T, testosterone; DHT, 5α-dihydrotestosterone; SDS, Sodium dodecyl sulfate.

‡Supported by NIH grants HD13956 (P.H.P.) and HD00163 (M.J.N.).

International Congress on Hormonal Steroids [15] consisting of data obtained from four animals, two infused with SBP and two others with anti-SBP. These preliminary data, however, were insufficient to convincingly establish the relationship between SBP and MCR^T, also the detailed methodology was not presented. Here we formally describe the entire work including the complete methodology as well as additional experiments including infusion of pure rabbit SBP which does not cross-react with anti-human SBP. This last experiment not previously reported is crucial to the final interpretation of the data.

EXPERIMENTAL

Animals

Seven cycling female rhesus monkeys (*Macaca mulatta*) from the Oregon Regional Primate Research Center (ORPRC) colony were used in this study. The animals weighed between 4.6 and 7.6 kg. Each animal was pre-conditioned in a primate restraining chair for 36–48 h before the study and was then implanted with indwelling catheters in surgery under ketamine anesthesia. Polyethylene tubing was inserted into the left femoral vein and polyvinyl tubing into the right femoral artery and vein. At surgery each animal was transfused with 50–75 ml of whole rhesus blood. The animal was then fitted with a specially tailored vest to protect the catheters, placed in a primate restraining chair, and allowed to recover for 24–48 h. During the study, blood pressure of the animal was monitored continuously.

Determination of the metabolic clearance rate of testosterone

The MCR^T was determined by the continuous infusion method of Tait[16] in control and experimental periods during which 1α,2α-[^{3}H]testosterone (New England Nuclear, Boston, MA: sp. act., 40 Ci/mmol) was administered to each animal for 6 h. A preliminary experiment in an untreated animal showed that the concentration of plasma [^{3}H]T did not change significantly during that length of time. The [^{3}H]T used for infusion was checked for radiochemical purity by reverse isotope dilution and was found to be greater than 97% pure. The infusion consisted of [^{3}H]T dissolved in a solution of 4% (v/v) ethanol in saline. A priming dose containing 7.2 μCi of the labeled steroid in 2.4 ml of solution was administered as a bolus prior to constant infusion of the substrate at a rate of 6 μCi/2 ml/h by means of a Harvard infusion pump (Harvard Apparatus Company, Inc., South Natick, MA) fitted with a 20-ml glass syringe. Infusion of the substrate was carried out through the polyethylene tubing inserted in the femoral vein. It was shown that there was no significant adsorption of T by the polyethylene tubing (unpublished data). Isotopic equilibrium (mean ±10%) in the plasma concentration of [^{3}H]T was attained within 30 min in the control periods.

Blood sampling

All blood samples (2–3 ml) were collected into heparinized syringes, and the plasma was separated and stored at −20°C until it was processed. In the control period, blood was obtained via the femoral artery at 0, 30, 40, 50 and 60 min following the onset of continuous infusion. The schedule of blood sampling during the experimental periods is described below.

Infusion of pure SBP and anti-SBP

Immediately following the control period, either pure human or rhesus monkey SBP or purified monospecific antibodies prepared against human SBP were infused continuously through the polyvinyl tubing in the femoral vein by means of a Harvard infusion pump. Rhesus SBP was prepared as recently described [12]. Because of the scarcity of rhesus plasma we could only isolate enough rhesus SBP to perform one experiment. However, the immunochemical and biochemical similarities of rhesus and human SBP [12] allowed us to use the pure human protein which is available in larger quantities. Both proteins were found to be pure by native and SDS gel electrophoresis. The proteins were dissolved in 0.01 M Tris–HCl buffer, pH 7.4, containing 0.1 M sodium chloride, 0.005 M calcium chloride, 10% (v/v) glycerol, and 0.02 mM 5α-dihydrotestosterone (DHT); the SBP concentration varied between 1.4–2.5 mg/ml. Immediately prior to infusion, the SBP was mixed with a suspension of 0.5% (w/v) charcoal (Norit A) and 0.005% (w/v) Dextran T 70 in 0.01 M Tris–HCl buffer, pH 7.4 and shaken at 0°C for 15 s to remove excess DHT. Following centrifugation of the mixture, the supernatant was filtered through a 0.22 μm Millex®-GS filter unit (Millipore Corp., Bedford, MA). The SBP was then infused at a rate of 0.08–0.12 ml/min for 1 h. Human SBP was infused in two animals and rhesus monkey SBP in 1 animal. Blood samples were collected for 5 h at intervals of 15 min following onset of protein infusion.

Monospecific antibodies against pure human SBP were raised in rabbits and purified by immunoaffinity chromatography using homogeneous human SBP covalently-linked to agarose as previously described [14, 17]. These antibodies cross-react with rhesus monkey SBP [14]. Prior to infusion, the antibodies were dissolved in phosphate buffered-saline (PBS) consisting of 0.006 M monobasic potassium phosphate, 0.079 M dibasic sodium phosphate, 0.15 M sodium chloride and 0.011 M potassium chloride, pH 7.4; the solution contained 4 mg of protein per ml of PBS. This solution was passed through a 0.22 μm Millex-GS filter and infused immediately after the control period in animals either as a bolus or continuously at a rate of 0.2 ml/min for 30 min. During that time, blood samples were collected at 0, 5, 10, 20 and 30 min following the onset of infusion and at

15-min intervals thereafter for $4\frac{1}{2}$ h. In one of the animals, pure rabbit SBP [13], which does not cross-react with the antibodies raised against human SBP [14], was infused $2\frac{1}{2}$ h after the onset of anti-SBP administration to determine whether or not SBP depletion in the plasma is restored. Rabbit SBP was dissolved in the same Tris–HCl buffer used for human and rhesus monkey SBP at a concentration of 1.15 mg/ml, and was treated in the same manner prior to infusion. The rate of infusion was 0.2 ml/min for 15 min.

Quantitation of testosterone and dihydrotestosterone

Plasma concentrations of [^{3}H]T were measured following the addition of 4-[^{14}C]T (New England Nuclear; sp. act. 51.9 mCi/mmol) as internal standard to monitor procedural losses. The steroid was first extracted with diethyl ether and then purified by LH-20 column chromatography in the solvent system, hexane–benzene–methanol (85:15:5, by vol). Using that method, the radiochemical purity of [^{3}H]T isolated from plasma was shown to be greater than 90% by reverse isotope dilution.

Endogenous levels of DHT and T in plasma were quantitated by RIA, following extraction of the steroids with diethyl ether and separation by LH-20 column chromatography. Measurement of DHT was carried out at the ORPRC RIA Core Laboratory using the method and assay reliability criteria described previously [18]. Testosterone was assayed in our laboratory using the procedure described by Resko *et al.*[19], with the exception that testosterone-3-(*O*-carboxymethyl) oximino-[^{125}I]iodohistamine (Cambridge Medical Diagnostics, Inc., Billerico, MA; sp. act., 4060 Ci/mmol) was used as radioligand instead of [^{3}H]T. An iodinated tracer was required for the T RIA because of the presence of significant amount of [^{3}H]T in the plasma samples. Accuracy of the RIA was assessed in a recovery experiment in which a known amount of T, ranging from 60 to 2000 pg, was added to individual 1.0 ml aliquots of rhesus monkey plasma stripped of steroid with charcoal and 0.3 ml aliquots of the "spiked" plasma were assayed in duplicate. Linear regression analysis of the T concentration measured (y) vs the T concentration added (x) yielded the following equation and correlation coefficient: $y = 0.95x + 2.21$; $r^2 = 0.911$ ($n = 12$). Parallelism in the T RIA was tested by measurement of the steroid in serially diluted aliquots of rhesus monkey plasma. The resultant T values yielded a line which was parallel to the corresponding standard curve. The intra-assay and inter-assay coefficients of variation are 8.7 and 13.7%, respectively.

Quantitation of SBP and albumin

SBP was assayed as described previously by Mickelson and Petra[20]. Aliquots of plasma were diluted 50-fold with 10 mM Tris–HCl, pH 7.4 and incubated with 20 nM 1,2-[^{3}H]5α-dihydrotestosterone (New England Nuclear; sp. act. 50 Ci/mmol) in the presence or absence of 100-fold molar excess of radioinert DHT. The SBP–DHT complex was then measured by the DEAE-filter assay [20].

Siiteri *et al.*[21] have recently claimed that it is unnecessary to correct for loss of specific binding during the performance of the filter assay as we had previously found [20, 22]. These investigators therefore assume that 98–100% of the specific steroid–protein complexes originally present in the diluted serum sample incubated with saturated radiolabeled steroid are retained by the DEAE-filter during the application and washing procedures. This assumption is incorrect. The amount of specific steroid–protein complexes which remain bound to the filter during application depends upon the charge capacity and hydration of the filter. Using published procedures [20, 22] we have determined that between 55 and 90% of the specific complexes present in the diluted serum adsorb to the filter during application of the sample. The values therefore need to be corrected to account for the loss of complexes. This "efficiency" correction is determined under conditions where the tritiated steroid is at least 98% bound to the specific protein (SBP or CBG) as calculated by mass action on two equilibria for the specific protein and albumin in the diluted serum. Highest retention of the steroid–protein complex (85–90%) is obtained by using 50 μl samples applied on dry DEAE-filters [23, and our unpublished results], whereas the lowest (55–80%) is obtained by applying 100 μl samples on wet DEAE-filters placed on a pad of Whatman No. 1 paper. Percent retention also depends on the animal species. Once the specific complexes have adsorbed to the filter, steroid dissociation from the specific steroid–protein complex does not occur [20, 22]. We therefore urge those laboratories using this assay to continue estimating their own filter "efficiencies" and to correct for losses as previously described [20, 22]. Failure to do so will result in underestimated values. Albumin was quantitated colorimetrically [24].

Calculation of percent of testosterone bound to SBP and albumin

The percentage of T bound to SBP and albumin at equilibrium and 37°C was calculated by solving the two equations describing each equilibrium according to our published procedure [25]. The mass action relationships are:

$$K_{SBP} = \frac{[ST]}{[S][T]}, \quad K_{Alb} = \frac{[AT]}{[A][T]}$$

where:

$K_{SBP} = 1.37 \times 10^8\ M^{-1}$, equilibrium constant of association of T macaque SBP at 37°C [26]
[ST] = Concentration of T bound to SBP
[S] = Concentration of unbound SBP
[T] = Concentration of unbound T

$K_{alb} = 2.02 \times 10^4\ M^{-1}$, equilibrium constant of association of T to human albumin at 37°C [27]
[AT] = Concentration of T bound to albumin
[A] = Concentration of unbound albumin

Percent of T bound to SBP was calculated using $K_{SBP} = 1.37 \times 10^8\ M^{-1}$ at 37°C. This value was estimated by taking $\frac{1}{3}$ of $K_{SBP} = 0.41 \times 10^9\ M^{-1}$ at 4°C for macaque SBP [26]. We have consistently found about a 3-fold decrease in K_a for human and rabbit SBP when raising the temperature from 4 to 37°C which agrees well with published results [28]. The same constant was used for calculating percent of T bound to SBP after human SBP infusion because there is no significant difference in T binding between macaque and human SBP [26]. In the calculation of percent T bound to rhesus albumin, we have used $K_{Alb} = 2.02 \times 10^4\ M^{-1}$ at 37°C for human albumin [27] because the K_{Alb} value for T binding to rhesus albumin is not available. We assume that there is no significant difference in T binding between human and macaque albumin. When pure rabbit SBP was infused, we used $K_{SBP} = 4.2 \times 10^7\ M^{-1}$ for the equilibrium constant of association of T to rabbit SBP at 37°C [28] to calculate the percent of T bound to SBP.

The conservation relationships at equilibrium used to substitute in the above equations are:

$$[S] = [S]_t - [ST]$$

$$[A] = [A]_t - [AT]$$

$$[T] = [T]_t - [ST] - [AT]$$

where $[S]_t$, $[A]_t$ and $[T]_t$ are the total concentrations of SBP, albumin, and T, respectively, determined experimentally in each animal before and after infusion of SBP and anti-SBP. [ST] is calculated by solving the following polynomial using a Hewlett–Packard model 67 programmed calculator:

$$A[ST]^3 + B[ST]^2 + C[ST] + D = 0$$

where A, B, C and D are constants described previously [25]. Once [ST] is known, [AT] is calculated by substitution [25].

RESULTS

Effect of rhesus and human SBP on MCR^T

The first three experiments in Table 1 describe the effect of SBP infusion on MCR^T. Figure 1A illustrates experiment 3 in more detail. As expected, the plasma SBP levels increased as a result of infusion of the pure protein, which indicates that the purification procedure did not alter the *in vivo* steroid-binding properties of SBP. Since SBP is purified as the SBP–DHT complex [11], both protein and steroid are infused together. The bound-DHT could not be removed by either charcoal adsorption or Sephadex gel filtration prior to infusion because of the combined effects of high affinity and high concentration of SBP in the infusate. The increase of DHT in the plasma as a result of infusion is shown in Fig. 1A. As expected, the rise in DHT coincided with that of SBP. Because of the large increase in dilution as the infusate mixes with blood, DHT begins to dissociate rapidly as the steady-state equilibrium is disturbed and the steroid is cleared rapidly. This is shown by comparing the concentrations of both SBP and DHT at the DHT "peak" in Fig. 1A. Because of the 1:1 stoichiometry of steroid-binding to SBP [12] one would expect to find equal concentrations of SBP and DHT at the DHT "peak" if dissociation did not occur. Instead, one finds concentrations of 250 nM SBP and 40 nM DHT followed by rapid clearance of the steroid. In order to eliminate any possible interference of DHT on MCR^T, plasma samples used for the calculation of MCR^T were taken after most of the DHT had cleared (Fig. 1A). The same approach was used when infusing rabbit SBP in experiment 7 (Fig. 1C) because the rabbit protein is also prepared as a SBP–DHT complex.

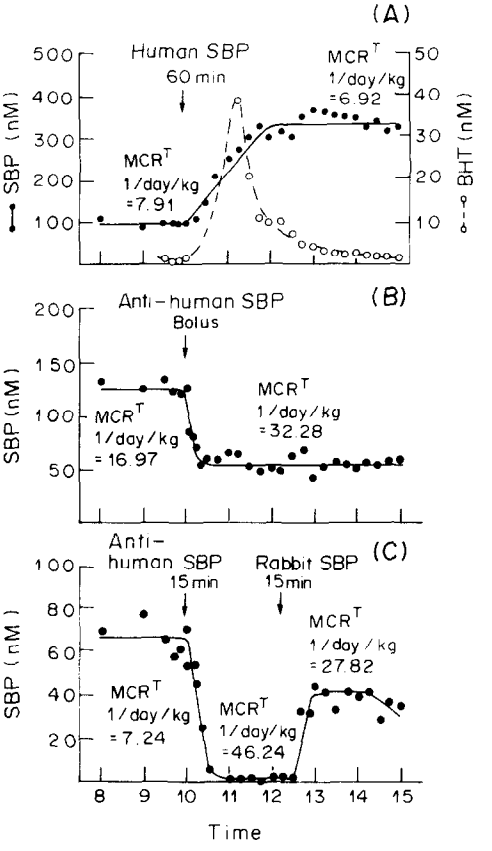

Fig. 1. Infusion of SBP and anti-human SBP in the rhesus macaque as described in the Experimental section. A, human SBP infusion; B, anti-human SBP infusion; C, anti-human SBP infusion followed by rabbit SBP infusion. A, B and C correspond to the third, fourth and seventh experiments described in Table 1, respectively.

The first three experiments in Table 1 demonstrate an inverse effect of SBP on MCR^T. Values obtained for MCR^T in control animals (animals not infused

Table 1. Influence of SBP on the metabolic clearance rate of testosterone

	Type of infusion	Before infusion SBP (nM)	Before infusion MCR^T (l/day /kg)	Before infusion %T Bound to SBP	Before infusion %T Bound to Alb	After infusion SBP (nM)	After infusion MCR^T (l/day /kg)	After infusion %T Bound to SBP	After infusion %T Bound to Alb
1.	Rhesus SBP	120	7.88	63	33	170	6.92	71	26
2.	Human SBP	175	5.94	71	26	525	5.54	88	11
3.	Human SBP	89	7.91	52	44	324	6.92	80	18
4.	Anti-human SBP	125	16.97	64	32	50	32.28	41	53
5.	Anti-human SBP	256	6.56	78	20	139	7.73	66	30
6.	Anti-human SBP	85	8.54	54	41	3.02	27.33	4.1	86
7.	Anti-human SBP	63	7.24	47	48	1.24	46.24	1.7	88
	+ Rabbit SBP	1.24	46.24	1.7	88	40.8	27.82	14	76

with SBP) agreed very well with previously published data [29]. The decrease in MCR^T was not as dramatic as we had expected; the reasons for this will become clear as discussed below. The calculated percentage of T bound to SBP before infusion was always higher than the percent T bound to albumin; this agrees with calculated values obtained from human female plasma [30, 31, 32]. After SBP infusion this difference was more pronounced as a result of increased plasma SBP concentrations. It should be pointed out that the one-ligand with two binding equilibria used to calculate the percent T bound to SBP and albumin does not take into consideration the contribution of other androgen metabolites which may be present in rhesus plasma (DHT; 5α-androstan-3α,17β-diol; 5-androstene-3β,17β-diol). Södergard *et al.*[32] have recently found, however, that in the case of human plasma the free, SBP-bound, and albumin bound T is affected very little by the low level of these androgen metabolites. The same values were obtained whether or not the androgen metabolites were omitted from the calculations. We assume that the same result should be obtained for rhesus plasma.

Effect of anti-human SBP on MCR^T

The relationship between SBP and MCR^T was further studied in 4 additional cycling female rhesus macaques by infusing monospecific human SBP antibodies known to cross-react with rhesus SBP. The data are presented as experiments 4, 5, 6 and 7 in Table 1, and Figs 1B and 1C which describe experiments 4 and 7 in more detail, respectively. Different amounts of antibodies were infused in order to reduce SBP levels to two different levels: (a) 40–54% of the endogenous concentrations as in experiments 4 and 5; (b) 2–4% of the endogenous concentrations as in experiments 6 and 7. In the case of experiments 4, 6 and 7, the increase in MCR^T was quite dramatic. In experiment 5, however, only a slight increase in MCR^T was observed, although SBP levels had been reduced by 46%. The animal in experiment 4 (Table 1) had unusually high basal MCR^T when compared to the other animals. It was found that animal 4 had been treated with 17β-estradiol benzoate a month earlier which may have effected MCR^T. Nevertheless, infusion of anti-SBP to this animal also resulted in the increase of MCR^T.

Comparing these results with the percent of T bound to SBP and albumin before and after antibody infusion we obtain evidence that the magnitude of the effect of SBP fluctuation on MCR^T depends upon the distribution of T bound to SBP and albumin. For instance, in experiments 4, 6 and 7 the percent of T bound to SBP is always higher or equal to the percent T bound to albumin before anti-SBP infusion. However, after infusion of the antibodies, the reverse is true in all 3 cases. The most dramatic increase in MCR^T is obtained in experiments 6 and 7 where most of the T is bound to albumin after infusion of the antibodies.

The availability of pure rabbit SBP, which does not cross-react with anti-human SBP [14], provided the opportunity for designing experiment 7 (Table 1 and Fig. 1C). Anti-human SBP was infused first, resulting in a complete redistribution of T bound to SBP and albumin and an increase of MCR^T from 7.24 to 46.24 l/day/kg as a result of SBP removal from plasma. Pure rabbit SBP was then infused producing an increase of T bound to SBP and a decrease of T bound to albumin. The result was a decrease in MCR^T from 46.24 to 27.82 l/day/kg. However, for unknown reasons, MCR^T did not return to the control value of 7.24 l/day/kg after the infusion of pure rabbit SBP. Although the K_ds of T binding to human and rabbit SBPs are similar, T dissociates 13 times faster from rabbit SBP at 0°C [28]. At 37°C the dissociation would be faster and could result in higher MCR^T.

Total testosterone concentrations were determined in each animal before and after infusion of SBP and anti-SBP. The values ranged from 0.75 to 1.84 nM. As expected, the levels decreased after infusion of the antibodies as a result of increased MCR^T, but remained unchanged when SBP was infused. The mean (±SD) albumin concentration was found to be $4.6 \pm 0.4 \times 10^{-4}$ M and did not vary significantly between animals.

DISCUSSION

The results presented here provide direct experimental evidence for the role of SBP in the regulation of the metabolic clearance rate of T in plasma. The mechanism of this process appears to involve a

complex steady-state equilibrium controlled by the levels of SBP, albumin and T. Since the albumin concentration remains essentially unchanged under normal conditions, the major controlling factors are SBP and T. The calculated values shown in Table 1 indicate that as long as the percent of T bound to SBP is higher or equal to the percent of T bound to albumin, maximum buffering capacity of T is reached in plasma and a minimum metabolic clearance of the hormone is achieved. As long as this condition persists (and can be demonstrated by calculation at equilibrium as shown in Table 1) there appears to be little effect of additional SBP on the clearance of T *in vivo* no matter how much is infused. This is demonstrated in the first three experiments of Table 1. Hence, under normal conditions, SBP appears to be present at an optimum level and largely in the unbound state in plasma to produce maximum binding capacity for testosterone.

This conclusion is further supported by the results obtained from the anti-SBP infusion experiments. Infusion of antibodies reduces SBP below the optimum level discussed above to the extent where the calculated distribution of the percent of T bound to SBP and albumin is now reversed. This redistribution produces a dramatic increase in MCR^T as shown in experiments 4, 6 and 7. In experiment 5, the final SBP concentration after antibody infusion is 139 nM. Although this represents a 46% drop in SBP, the level is still too high to significantly influence the redistribution of bound T from SBP to albumin. The net result is a minimal increase in MCR^T consistent with the other data.

We also suggest that there may be other factors besides SBP which control MCR^T, otherwise all animals which have higher SBP levels should exhibit lower MCR^T. As shown in Table 1, this was not always the case. For instance, animal 4 had about the same SBP level and percent of T bound to SBP as animal 1 but cleared testosterone twice as rapidly. It was later found that animal 4 had been treated with estradiol benzoate in a previous study 1 month before we undertook our experiments. It is therefore possible that the unusual basal level of MCR^T (16.97 l/d/kg) may be due in part to estrogen induction of steroid metabolizing enzymes in this particular animal. Nevertheless, animal 4 still showed a 2-fold increase in MCR^T after infusion with anti-SBP which is consistent with the other results.

An additional consideration in the anti-SBP infusion studies is the possible formation of a ternary immunocomplex consisting of SBP-T-antiSBP. It could be argued that MCR^T in animals infused with anti-SBP may be artificially enhanced by the elimination of this complex from plasma by the immune system. We have in fact detected the existence of such a complex in sucrose gradients of monkey serum incubated with [^{3}H]DHT and anti-SBP and centrifuged at 4°C for 16 h [17]. The yield of this complex, however, was found to be low at 4°C (< 10%) due primarily to steroid dissociation from the complex during centrifugation most likely caused by the interaction of SBP with anti-SBP. At 37°C, the immunocomplex would be expected to have a very short half-life and therefore should not interfere with normal MCR^T.

We therefore conclude that under normal conditions each individual animal has a unique optimum concentration of plasma SBP which binds a maximum amount of T. This concentration is probably controlled by the plasma sex steroid hormone concentration and will vary from animal to animal. Under certain physiological or clinical situations SBP concentrations may fluctuate producing a change in the percent T bound to SBP in plasma through the establishment of a new steady-state equilibrium. If the particular condition produces an elevation of SBP above the optimum level, more T will be bound but there will be little change in MCR^T. If, on the other hand, there is a decrease in SBP below the optimum plasma level, an increase in MCR^T will occur. A maximum clearance rate of T will be reached when most of the steroid is bound to albumin under steady-state conditions as shown in experiment 7. In addition, other factors such as T metabolizing enzymes present within tissues may also function in concert with SBP to regulate MCR^T.

The conclusions support the hypothesis that the function of specific plasma steroid-binding proteins is to regulate the concentration of unbound steroid which can then diffuse into tissues and therefore suggest that the biological role of these plasma proteins may at last be understood. However, there are recently published data which are not consistent with this interpretation. First, SBP had been found within cells of reproductive tissues of the male macaque monkey [17], the human male, [33, 34], the human female [35], the female rabbit [36] and human breast cancer [37]. Second, specific membrane transport mechanisms have been described for steroids in the case of low-density lipoprotein in mammalian cells [38] and T in bacteria [39].

On the basis of the monkey studies, in 1980 this laboratory first proposed the hypothesis that SBP and other plasma steroid-binding proteins may be involved in the transport of steroid hormones across the plasma membrane of mammalian cells [17]. Subsequently, Siiteri *et al.*[21] have carried out a similar immunofluorescence study on corticosteroid binding globulin (CBG) in the rat. Although interpretation of their results is somewhat open to question because of insufficient experimental documentation concerning the purity of the rat CBG used in that work, their data are for the most part in support of our hypothesis. It is generally, accepted that steroid hormones enter cells by passive diffusion; however, in certain cells there may also exist in addition to that process an active transport mechanism which unlike passive diffusion can be regulated by the cell.

We therefore conclude that specific plasma steroid-binding proteins may have a dual function: (1) to control the plasma metabolic clearance rate of the steroids they bind as demonstrated in this report, and (2) under certain situations to transport the hormones intracellularly through receptor-mediated endocytosis. This latter mechanism would provide the cell with a different "pool" of steroid hormone from that produced by passive diffusion, a "pool" protected from steroid metabolizing enzymes by being sequestered within cytoplasmic organelles. Additional research with cells in culture as well as with animal models will be necessary to test further the validity of this latter proposal.

Acknowledgements—The authors wish to thank Dr Arthur Maley for his invaluable advice in the performance of these expeirments as well as Mr Mike Cook for his excellent technical expertise in the infusion studies.

REFERENCES

1. Pearlman W. H. and Crepy O.: Steroid-protein interaction with particular reference to testosterone binding by human serum. *J. biol. Chem.* **242** (1967) 182–189.
2. Kato T. and Horton R.: Studies of testosterone binding globulin. *J. clin. Endocr. Metab.* **28** (1968) 1160–1168.
3. Vermeulen A. L., Verdonck L., Van der Straeten M. and Orie M.: Capacity of the testosterone binding globulin in human plasma and influence of specific binding of testosterone on its metabolic clearance rate. *J. clin. Endocr. Metab.* **29** (1969) 1470–1480.
4. August G. P., Tkachuk M. and Grumbach N. M.: Plasma testosterone-binding affinity and testosterone in umbilical cord plasma, late pregnancy, prepubertal children, and adults. *J. clin. Endocr. Metab.* **29** (1969) 891–899.
5. Dray F., Sebaoun J., Mowszowics I., Delziant G., Desgrez P. and Dreyfus G.: Facteurs influencant le taux de la testosterone plasmatique chez l'homme: role des hormones thyroïdiennes. *C.r. hebd. Séanc. Acad. Sci., Paris* **264** (1967) 2578–2582.
6. Bardin C. W. and Lipsett M. B.: Testosterone and androstenedione blood production rates in normal women and women with idiopathic hirsutism or polycystic ovaries. *J. clin. Invest.* **46** (1967) 891–902.
7. Dray F., Mowszowicz I., Ledru M. J., Crepy O., Delziant G. and Sebaoun J.: Anomalies de l'affinité de liaison de la testostérone dans le serum des sujets thyrotoxicosiques et dans le virilisme pilaire idiopathique. *Ann. Endocr. (Paris)* **30** (1969) 223–232.
8. Southern A. L., Gordon G. G., Tochimoto S., Olivo J., Sherman D. H. and Pinzon G.: Testosterone and androstenedione metabolism in the polycystic ovary syndrome; studies of the percentage binding of testosterone in plasma. *J. clin. Endocr. Metab.* **29** (1969) 1356–1363.
9. Rosenfield R. L.: Plasma testosterone binding globulin and indexes of the concentration of unbound plasma androgens in normal and hirsute subjects. *J. clin. Endocr. Metab.* **32** (1971) 717–728.
10. Anderson D. C.: Sex-hormone-binding globulin. *Clin. Endocr.* **3** (1974) 69–96.
11. Petra P. H. and Lewis J.: Modification in the purification of the sex steroid-binding protien of human serum by affinity chromatography. *Analyt. Biochem.* **105** (1980) 165–169.
12. Turner E. E., Ross J. B. A., Namkung P. C. and Petra P. H.: Purification and characterization of the sex steroid-binding protein (SBP) from macaque serum. Comparison with the human protein. *Biochemistry* **23** (1984) 492–497.
13. Mickelson K. E. and Petra P. H.: Purification and characterization of the sex steroid-binding protein of rabbit serum. Comparison with the human protein. *J. biol. Chem.* **253** (1978) 5293–5298.
14. Bordin S., Lewis J. and Petra P. H.: Monospecific antibodies of the sex steroid-binding protein (SBP) of human and rabbit sera: cross-reactivity with other species. *Biochem. biophys. Res. Commun.* **85** (1978) 391–401.
15. Pétra P. H., Stanczyk F. Z., Senear D. F., Namkung P. C., Novy M. J., Ross J. B. A., Turner E. E. and Brown J. A.: Current status of the molecular structure and function of the plasma sex steroid-binding protein (SBP). *J. steroid Biochem.* **19** (1983) 699–706.
16. Tait J. F.: The use of isotopic steroids for the measurement of production rates *in vivo*. *J. clin. Endocr. Metab.* **23** (1963) 1285–1297.
17. Bordin S. and Petra P. H.: Immunocytochemical localization of the sex steroid-binding protein of plasma in tissues of the adult monkey, *Macaca nemestrina*. *Proc. natn. Acad. Sci., U.S.A.* **11** (1980) 5678–5682.
18. Resko J. A., Ellinwood W. E., Pasztor L. M. and Buhl A. E.: Sex steroids in the unbilical circulation of fetal rhesus monkeys from the time of gonadal differentiation. *J. clin. Endocr. Metab.* **50** (1980) 900–905.
19. Resko J. A., Malley A., Begley D. and Hess D. L.: Radioimmunoassay of testosterone during fetal development in the rhesus monkey. *Endocrinology* **93** (1973) 156–161.
20. Mickelson K. E. and Petra P. H.: A filter assay for the sex steroid-binding protein (SBP) of human serum. *FEBS Lett.* **44** (1974) 34–38.
21. Siiteri P. K., Murai J. T., Hammond G. L., Nisker J. A., Raymoure W. J. and Kuhn R. W.: The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38** (1982) 457–510.
22. Schiller H. S. and Petra P. H.: A filter assay for the corticosteroid binding globulin of human serum. *J. steroid Biochem.* **7** (1976) 55–59.
23. Lata G. F., Hu H. K., Bagshaw G. and Tucker R. F.: Equilibrium and kinetic characteristics of steorid interactions with human plasma sex steroid-binding protein. *Archs biochem. Biophys.* **199** (1980) 220–227.
24. Doumas B. T. and Biggs H. G.: Determination of serum albumin In *Standard Methods of Clinical Chemistry* (Edited by G. R. Cooper). Academic Press, New York, Vol. 7 (1972) pp. 175–180.
25. Tabei T., Mickelson K. E., Neuhaus S. and Petra P. H.: Sex steroid-binding protein (SBP) in dog plasma. *J. steroid Biochem.* **9** (1978) 983–988.
26. Pétra P. H. and Schiller H. S.: Sex steroid-binding protein in plasma of *Macaca nemestrina*. *J. steroid Biochem.* **8** (1977) 655–661.
27. Vermeulen A. and Verdonck L.: Studies on the binding of testosterone to human plasma. *Steroids* **11** (1968) 609–635.
28. Hansson V., Ritzen E. M., Weddington S. C., McLean W. S., Tindall D. J., Nayfeh S. N. and French F. S.: Preliminary characterization of a binding protein for androgen in rabbit serum. Comparison with the testosterone-binding globulin (TeBG) in human serum. *Endocrinology* **95** (1974) 690–700.
29. Heinrichs W. L., Tabei T., Kuwabara Y., Burry K., Resko J., Petra P. H., Schiller H. and Namkung P. C.: Differentation and regulation of peripheral androgen metabolism in rats and rhesus monkeys. *Am. J. Obstet. Gynec.* **135** (1979) 974–983.
30. Moll G. W., Rosenfield R. L. and Helke J. H.: Estradiol-testosterone binding interactions and free

plasma estradiol under physiological conditions. *J. clin. Endocr. Metab.* **52** (1981) 868–874.
31. Dunn J. F., Nishula B. C. and Rodbard D.: Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. clin. Endocr. Metab.* **53** (1981) 58–68.
32. Sodergard R., Backstrom T., Shanbhag V. and Cartensen H.: Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature. *J. steroid Biochem.* **16** (1982) 801–810.
33. Egloff M., Vendreli E., Tardivel-Lacombe J., Dadoune J.-P. and Degrelle H.: Immunohistochemical localization of a SBP-like antigen in human testis and epididymis. *Sixth International Congress Hormonal Steroids* (1982) Abstr. 143.
34. Robel P., Eychenne B., Blondeau J.-P., Jung-Testas I., Groyer M. T., Mercier-Bodard C., Hechter O., Roux C. and Dadoune J.-P.: Androgen receptors in rat and human prostate. *Horm. Res.* **18** (1983) 28–36.
35. Lo Bue C., Bordin S. and Petra P. H.: Immunocytochemical localization of the six steroid-binding protein (SBP) of human plasma in tissues of the human female reproductive tract. *28th Ann. meeting, Soc. Gynec. Invest.* (1981) Abstr. 81.
36. Brown J. A. and Pétra P. H.: Detection of the sex steroid-binding protein (SBP) in cytosols of various tissues of the female rabbit. *Fedn Proc.* **42** (1983) 1837.
37. Tardivel-Lacombe J., Egloff M., Mazabrand A. and Degrelle H.: Immunohistochemical detection of the sex steroid-binding protein in human mammary carcinoma cells. *Biochem. biophys. Res. Commun.* **118** (1984) 488–494.
38. Goldstein J. L., Anderson R. G. W. and Brown M. S.: Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* **279** (1979) 679–685.
39. Culos D. and Watanabe M.: The effect of carbonyl cyanide m-chlorophenylhydroazone on steroid transport in membrane vesicles of *Pseudomonas testosteroni*. *J. steroid Biochem.* **19** (1983) 1127–1133.