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DIRECT EFFECT OF PLASMA SEX HORMONE BINDING GLOBULIN (SHBG) ON THE METABOLIC CLEARANCE RATE OF 17β-ESTRADIOL IN THE PRIMATE

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Summary—Sex hormone binding globulin (SHBG) has been shown to be a major determinant of testosterone clearance in the primate. It has also been suggested that SHBG would also be a determinant of estradiol clearance (MCR-E_2). However, published studies have suggested that the MCR-E_2 do not always vary with changes in the level of SHBG. Therefore, the present study was undertaken to address this issue. The baseline MCR-E_2 was determined in adult male pigtail macaques (*Macaca nemestrina*). Following the baseline determination of MCR-E_2 the animals were infused with either purified human (h)SHBG or antibody against hSHBG, which also has a high degree of cross-reactivity with primate SHBG. Following the infusions of either hSHBG or anti-SHBG, MCR-E_2 was again determined. In addition, luteinizing hormone (LH) was measured using a mouse Leydig cell bioassay. Following the infusion of hSHBG, a marked increase in serum SHBG was noted and the MCR-E_2 decreased. Associated with the increase in SHBG, the SHBG bound T levels decreased and LH increased. Following the infusion of antibody, serum SHBG decreased, and the MCR-E_2 also decreased. With the decrease in SHBG following the antibody infusion, non-SHBG bound T increased and serum LH fell. This study demonstrates that an increase in the serum SHBG levels is associated with a decrease in MCR-E_2, however, an acute decrease in serum SHBG also decreases the MCR-E_2. This later result demonstrates that factors in addition to serum SHBG binding may be important in determining the MCR-E_2.

INTRODUCTION

Although biochemical characterization of the sex hormone binding globulin of plasma (SHBG) has been achieved to a significant degree, its physiological role remains unclear. Shortly after its discovery in the mid-1960s [1–3], a number of published studies in humans suggested an inverse relationship between SHBG plasma level and the metabolic clearance rate of testosterone (MCR-T) in normal and in various physiological and pathological states [4–6]. More recently, an inverse relationship between SHBG and MCR-T was directly demonstrated by infusing pure SHBG and its antibodies into cycling female rhesus monkeys [7]. However, the effect of SHBG on the metabolic clearance of 17β-estradiol, MCR-E_2, remained unknown. Existing data on the MCR-E_2 in humans and sub-human primates have demonstrated that the MCR maybe related to a number of factors including the endogenous level of SHBG and serum estradiol levels and that the MCR-E_2 does not always change with a change in serum SHBG levels [8]. In addition, the decreased binding affinity of E_2 for SHBG as compared to T ($K_dT = 5.97 \times 10^8\ M^{-1}$, $K_dE_2 = 3.14 \times 10^8\ M^{-1}$, 37°C) [9] would be expected to affect the relationship of SHBG to MCR-E_2 differently. The present study was undertaken to study this effect and to determine a quantitative relationship between SHBG and MCR-E_2. The approach was to infuse pure human SHBG and its antibodies into male macaque monkeys, and measure the influence on MCR-E_2. These experiments were feasible because the biochemical, immunological, and steroid-binding properties of human SHBG are essentially the same as those of macaque SHBGs [10].

EXPERIMENTAL

Animals

Five adult male pigtail macaques (*Macaca nemestrina*) were used for this study. The animals weighed between 10 and 15 kg. All procedures were approved by the animal use committee of Madigan Army Medical Center. The animal facility is approved by the American Association of Laboratory Animal Scientists. Prior to the start of each study the animals were sedated with ketamine. Catheters were placed in a femoral artery and vein of one leg and the popliteal vein of the opposite leg. Polyvinyl tubing was used in the femoral artery and vein lines and

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polyethylene tubing in the popliteal line. Following the insertion of the catheters, the animals were allowed to recover from the sedation under light restraint by handlers who were known to the animals on a daily basis for six months. The animals were kept in a supine position during the studies.

Purification of human SHBG and polyclonal antibodies

Human SHBG was prepared as previously described [11] with added modifications [12]. Purity was checked by SDS–PAGE [13]. The SHBG concentration in stock solutions (1–3 mg/ml) was measured spectrophotometrically using $\epsilon 280$ of $1.14 \times 10^5\,cm^{-1}\,M^{-1}$ [13] and a dimeric molecular weight of 93,400 [14].

Polyclonal antibodies were prepared in rabbits and goats with pure human SHBG antigen and purified as previously described [15]. The antibodies were further purified by affinity chromatography on a column of SHBG covalently-linked to agarose [16]. The antibody titre was measured by immunoelectrophoresis [15] and protein concentration according to Lowry *et al.*[17]. For the rabbit antibody the titre was 869 μg SHBG/ml antibody and the protein concentration 1.54 mg/ml, respectively, and for the goat antibody they were 176 μg SHBG/ml antibody and 0.86 mg/ml, respectively.

Determination of metabolic clearance

The metabolic clearance rate of estradiol, MCR-E_2, was determined by a constant infusion technique originally described by Tait[18]. [6,7^{3}H]17β-estradiol (New England Nuclear, Boston, Mass: sp. act. of 40 Ci/mmol) was found to be greater than 98% pure by C18 reversed-phase HPLC. Estradiol was infused in physiological saline at a final concentration of 3 μCi/ml. A bolus of 2.4 ml of the [^{3}H]E_2 solution was infused through the femoral vein catheter for a period of 60 s using a glass syringe attached to a Harvard infusion pump (Harvard Apparatus Company Inc., South Natick, Mass). Initial [^{3}H]E_2 flow rate was determined by drawing blood samples (1 ml) from the arterial catheter every 10–15 min into heparinized tubes. The plasma was removed and the red blood cells were reinfused through the popliteal catheter with a volume of normal saline equal to the volume of plasma removed. The rate of infusion was considered constant when three consecutive measurements of [^{3}H]E_2 plasma levels were within 10% coefficient of variation. Metabolic clearance rate of E_2 was determined by the following formula originally described by Tait: MCR-E_2 (l/day) = ([^{3}H]E_2 infused dpm/min) ([^{3}H]E_2 concentration in the blood). MCR-E_2 was determined during the initial control period and 60 min after the infusion of either human SHBG or anti-hSHBG. Blood samples (3 ml) were also drawn daily for five days following the completion of the infusion portion of the study. Two animals were used as controls and had MCR-E_2 determined over an 8-h period at 4 and 8 h after the start of the E_2 infusion. No marked difference was noted in the MCR-E_2 over the period of infusion.

SHBG and antibody infusion

SHBG was diluted to a concentration of 1 mg/ml in normal saline, mixed with dextran-coated charcoal at a 1:1 ratio of SHBG to charcoal for 5 min to strip excess steroid, and filtered through a 0.22 μm Millipore filter (Millipore Corp., Bedford, Mass). After baseline MCR-E_2 was reached, the SHBG solution, 5 mg in 20 ml saline, was infused over 20 min through the popliteal catheter. The amount of hSHBG infused was adjusted to be at least double the animal's endogenous SHBG concentration which varies from 30 to 60 nM in male monkeys. 5 ml of antibody solution was prepared and infused similarly except that the dextran-coated charcoal step was omitted.

Steroid, luteinizing hormone, and SHBG measurements

Serum T and E_2 measurements were performed as previously described [19]. Serum SHBG concentrations were measured by dextran-coated charcoal saturation analysis with dihydrotestosterone as the ligand [19]. Serum SHBG was also measured by a radioimmunometric assay from Farmos Diagnostica (Ounsulu, Finland). A 90% reactivity with *Macaca nemestrina* SHBG was demonstrated by this assay. The intra- and inter-coefficients of variation for the immunoassay were 3.5 and 5% respectively. Luteinizing hormone (LH) was measured by a bio-assay based on T response on Leydig cells isolated from immature Swiss-Webster mice [20].

RESULTS

The results of SHBG infusion are shown in Fig. 1 and Table 1. As expected, the plasma SHBG level of the infused animals increased markedly following administration of the pure protein. The SHBG measurements were made by both the charcoal assay, which reflects the steroid-binding activity of SHBG, and by IRMA which measures its presence as protein. The charcoal assay data confirms previous conclusions that the pure protein is active *in vivo* when infused into animals, and therefore the purification process does not alter its biological properties [7]. After the SHBG levels had reached its new plateau after a 4-h infusion, a new measurement of MCR-E_2 revealed a marked decrease as compared to basal level as shown in Table 1. The data collected in the two animals further indicate that the higher the SHBG level the more the decrease in MCR-E_2. Although both T and E_2 increased with the SHBG infusion (data not shown), because SHBG rose the T/SHBG ratio fell markedly following infusion (Fig. 2b). This fall in the T/SHBG ratio, and thus an increase in SHBG bound T, was associated with a rise in LH. The marked rise in LH noted in Fig. 2a on day 3 and in Fig. 4 on day 2 may not be related to changes

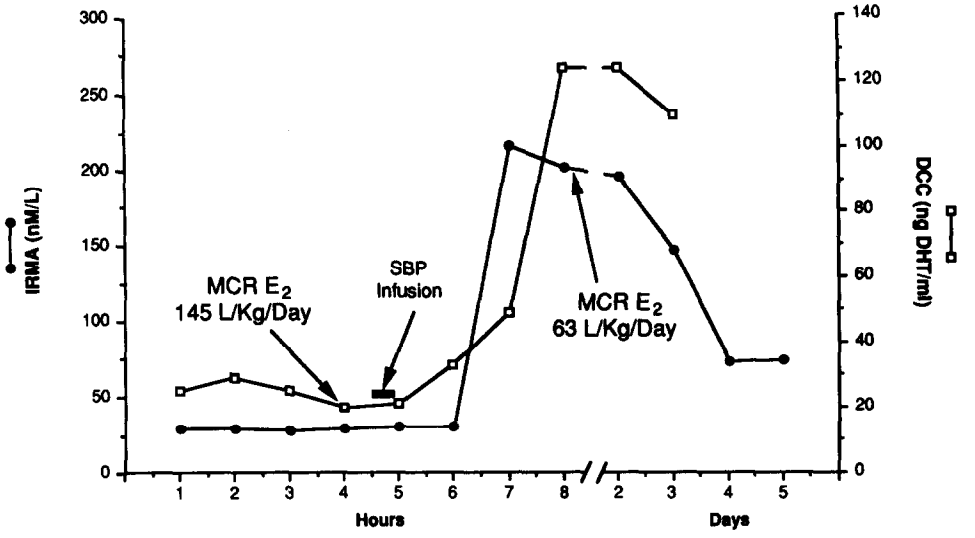

Fig. 1. Changes in SHBG levels in primate No. 1 as measured by the DCC assay (□) and IRMA (●). Note the decrease in MCR-E_2 following the injection of SHBG and subsequent increase in SHBG levels.

in free or bound testosterone since they are single samples and a spontaneous LH pulse may have occurred. On the other hand, the changes in LH occluding during the day of infusion are based on frequent sampling and would be expected to reflect changes in free or bound steroid. Finally, the rate of SHBG elimination from plasma after infusion is consistent with slower component of the biphasic curve of SHBG half-life of 3.95 days [21].

The results of the antibody infusions are demonstrated in Table 1 and Fig. 3. As can be seen by both the binding assay and immunoassay, a marked decrease in serum SHBG occurred following antibody infusion. This decrease included both functional mass as measured by the immunoassay and decrease in steroid binding activity as measured by the binding assay. However, instead of the expected increase in MCR-E_2 as a result of a lowering in SHBG level, a decrease was observed (Table 1). Also, bioactive LH declined during day 1 in response to the increasing levels of free T as shown by the increasing T/SHBG ratio following antibody infusion (Figs 4a,b). In addition, the E_2 serum levels rose during antibody infusion in contrast to a decrease in total T.

DISCUSSION

The results of this study demonstrate that SHBG may in part influence the metabolic clearance rate of E_2 and provides the first direct experimental proof for such a function. This finding, coupled with that previously determined for T [7], is consistent with the idea that one of the physiological roles of SHBG is to regulate the clearance of sex steroids in plasma. This study also demonstrates that when the SHBG level is increased to a greater degree than T, thereby reducing the free T and E_2 fractions, a change in tissue availability of the steroid is evident by the elevation of luteinizing hormone. This is also consistent with the changes previously described [22]. The second part of the study involving the infusion of anti-SHBG, however, appears at first to be inconsistent with the interpretation stated above since a decreasing level of endogenous SHBG, caused by presence of anti-SHBG in the circulation, should have increased MCT-E_2 rather than lowering it. This result differs from those previously described on effects on T clearance where infusion of anti-SHBG markedly increased the MCR-T [7]. One possible explanation could be that the SHBG-antibody complex binds E_2 and therefore its formation would not contribute to an increase in free E_2 and no increase in MCR-E_2 would be observed. This proposal is very unlikely since it was already shown that the binding affinity of T to the immune-complex is greatly reduced [16]. Certainly one would expect the same to apply to E_2. Furthermore, as indicated in Fig. 4, serum levels of T decreased dramatically following

Table 1. Sex hormone binding globulin (SHBG) levels measured by the DCC binding assay before and after hSHBG or antibody infusion and MCR-E_2 before and after each infusion

Animal No.	Infusion	SHBG Concentration (mol/l) Before	SHBG Concentration (mol/l) After	MCR-E_2 (l/day/kg) Before	MCR-E_2 (l/day/kg) After
1	SHBG	29	216	145	63.4
2	SHBG	44	132	144	118
3	Anti-SHBG	65	12.8	74.5	30
4	Anti-SHBG	89	38	155	102
5	Anti-SHBG	36	30	115	107

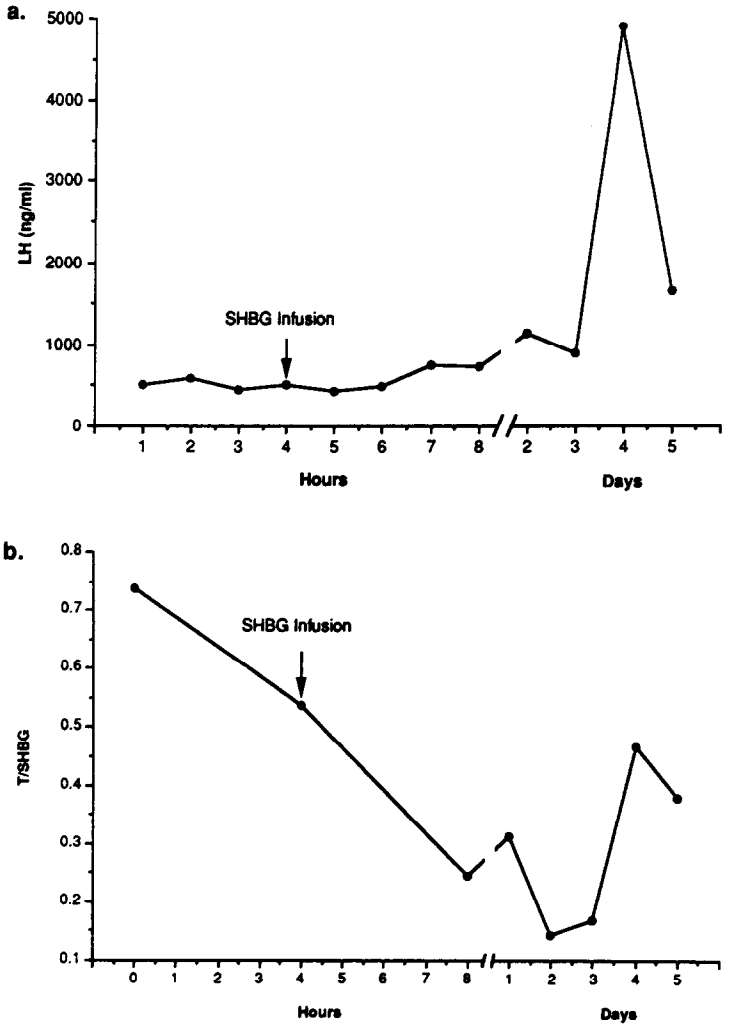

Fig. 2. (a) This figure demonstrates the increase in bioactive serum LH following the infusion of SHBG and a decrease in T/SHBG ratio in primate No. 1. (b) The decrease in the T/SHBG ratio following the infusion in primate No. 1 is noted in this figure.

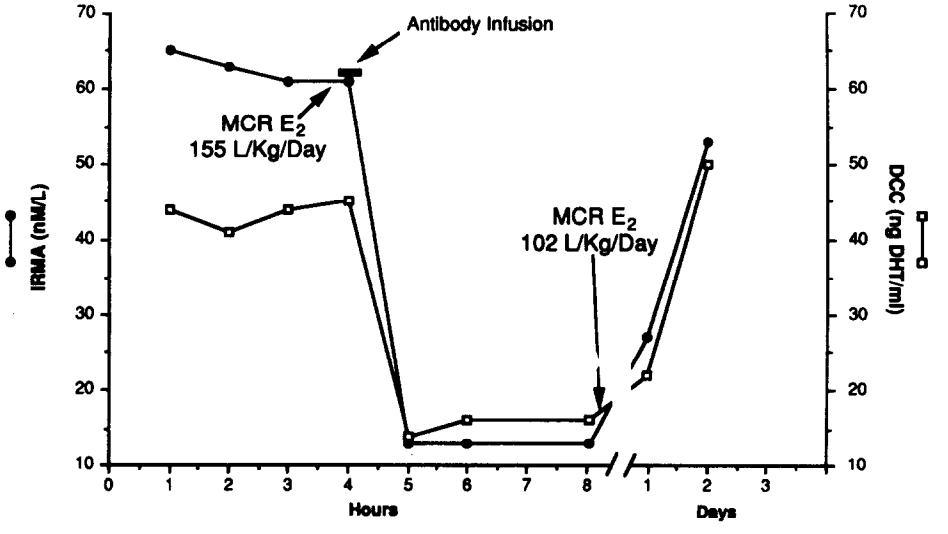

Fig. 3. This figure demonstrates the decline in SHBG following the infusion of SHBG antibody in primate No. 3. The MCR-E_2 declines following the antibody infusion.

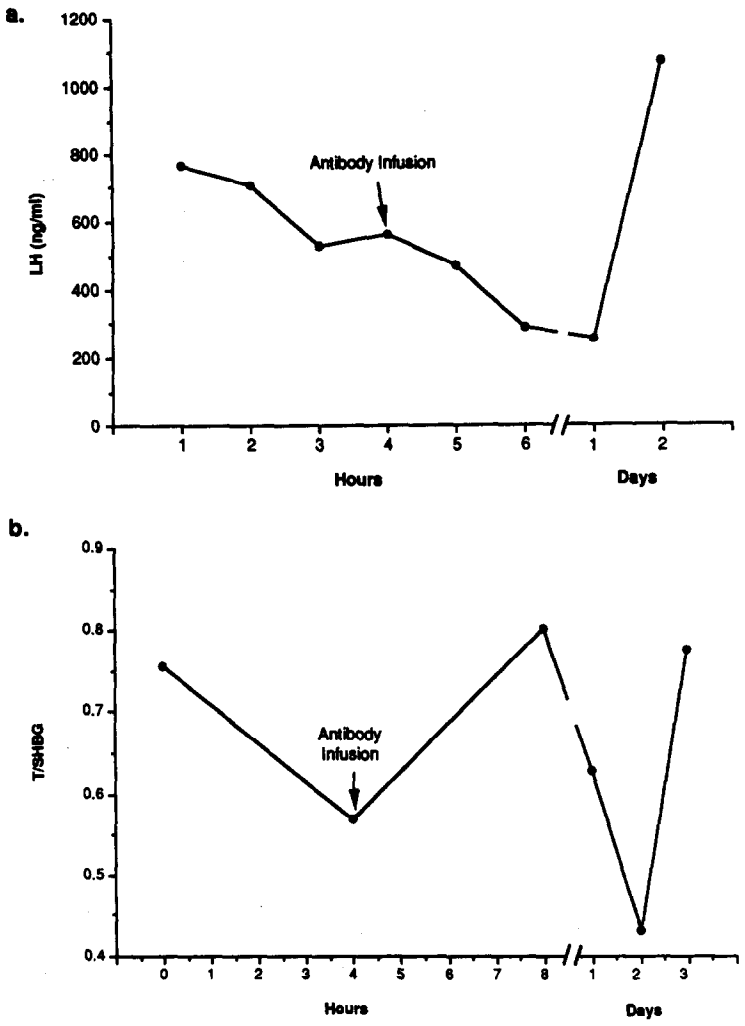

Fig. 4. (a) This figure demonstrates the decrease in bioactive serum LH following the antibody infusion and subsequent increase in T/SHBG ratio in primate No. 3. (b) The increase in T/SHBG ratio following the antibody infusion in primate No. 3 is noted in this figure.

antibody infusion. Since the animals used in this study are males, a better explanation is that an increase in free T, expected to occur as a result of antibody infusion, could directly decrease the clearance of E_2. Such an effect of T on MCR-E_2 has been recently proposed [23]. Therefore, the expected effect of an increase in MCR-E_2 as a result of anti-SHBG infusion could be masked by the increase in free T which in turn would result in a net lowering of MCR-E_2. Also, the free T fraction could be converted to E_2 by aromatase and thus compete with [^{3}H]E_2 being infused for clearance rate measurements. This hypothesis is in part supported by the work of Reed *et al.*[23] who administered androgen and labelled estradiol and showed a decrease in the clearance rate of E_2. Since most of the monkey's circulating T is released from plasma after administration of the antibody, the free steroid concentration increased to greater than 100-fold in a short period of time, and one could expect any effect of free T to be maximal. This was demonstrated by the decrease in LH seen after the infusion of antibody.

Although these and previous data strongly suggest that SHBG plays a role in controlling the clearance rate of sex steroids, one must emphasize that this may not be the only biological role for this protein. Published data describing the possible existence of an SHBG membrane receptor [16, 24–28] suggest that SHBG may in fact have a dual functional role as previously stated [27], one for controlling the plasma levels of T and E_2 and the other to assist in the specific incorporation of sex steroids into specialized tissues such as the prostate and, in the case of ABP, the epididymis. Since human SHBG and human ABP have the same amino acid sequence [29, 30] and are therefore coded by the same gene, they very likely play identical roles in these two different tissues. Because SHBG does not cross the blood–testis barrier, SHBG gene expression must also take place in testis to provide protein for sex steroid transport.

It should also be pointed out that SHBG may not be the only regulator of the clearance rate of sex steroids. Other studies in primates where the endogenous levels of SHBG were altered by either thyroxine or dexamethasone did not show a difference in MCR-E_2. Additionally, Bourget *et al.*[31] have found differences in hepatic extraction of E_2 compared to T. These and other studies suggest that multiple factors besides the serum binding of E_2 maybe be important in determining its clearance. An additional explanation for the results noted in the macaque is that SHBG physiology may not be similar to that of humans. Certainly the regulation of serum SHBG levels is different in macaques vs humans, e.g. SHBG decreases in macaques during pregnancy whereas it increases in humans [32]. Therefore, the effects on clearance of bound steroids could also be different.

In conclusion, the results of the infusion of SHBG on the MCR-E_2 demonstrate that serum high affinity binding proteins may affect the clearance of the bound steroids. However, lowering serum SHBG levels by the infusion of antibody to SHBG demonstrates that the clearance of individual steroids, e.g. T vs E_2, is influenced by factors in addition to steroid binding proteins and may depend on the relative concentrations of the steroids as well as the sex of the animal in which the clearance studies are being performed.

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REFERENCES

1. Mercier-Bodard C., Alfsen A. and Baulieu E.-E.: Sex steroid binding plasma protein (Sbp). *Acta Endocr. (Copenh.)* **147** (1966) 204–224.
2. Rosenbaum W., Christy N. P. and Kelly W. G.: Electrophoretic evidence for the presence of an estrogen-binding b-globulin in human plasma. *J. Clin. Endocr. Metab.* **26** (1966) 1399–1403.
3. Pearlman W. H. and Crepy O.: Steroid-protein interaction with particular reference to testosterone-binding by human serum. *2nd Int. Congr. Hormonal Steroids*, Milan, May. Excerpta Med. Intern. Congr. Ser. No. 111 (1966) 48.
4. 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.
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 thyroidiennes. *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. Petra P. H., Stanczyk F. Z., Namkung P. C., Fritz M. A. and Novy M. J.: Direct influence of the sex steroid-binding protein (SBP) of plasma on the metabolic clearance rate of testosterone. *J. Steroid Biochem.* **22** (1985) 739–746.
8. Hotchkiss J.: The metabolic clearance rate and the production rate of estradiol in sexually immature and adult female rhesus monkeys. *J. Clin. Endocr Metab.* **56** (1983) 979–984.
9. 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.
10. Turner E., Ross J. B. A., Namkung P. C. and Petra P. H.: Purification and characterization of the sex steroid-binding protein from macaque serum. Comparison with the human protein. *Biochemistry* **23** (1984) 492–497.
11. Petra P. H. and Lewis J.: Modification in the purification of the sex steroid-binding protein of human serum by affinity chromatography. *Analyt. Biochem.* **105** (1980) 165–169.
12. Griffith P. R., Kumar S., Shabanowitz J., Charbonneau H., Namkung P. C., Walsh K. A., Hunt D. F. and Petra P. H.: The amino acid sequence of the sex steroid-binding protein of rabbit serum. *J. Biol. Chem.* **264** (1989) 19066–19075.
13. Petra P. H., Namkung P. C., Senear D. F., McCrae D. A., Rousslang K. W., Teller D. C. and Ross J. B. A.: Molecular characterization of the sex steroid-binding protein (SBP) of plasma. Re-examination of rabbit SBP and comparison with the human, macaque, and baboon proteins. *J. Steroid Biochem.* **25** (1986) 191–200.
14. Petra P. H., Namkung P. C., Titani K. and Walsh K. A.: Characterization of the plasma sex steroid-binding protein. In *Binding Proteins of Steroid Hormones* (Edited by M. G. Forest and M. Pugeat). Colloque/INSERM/Libbey, London/Paris, Vol. 149 (1986) pp. 15–30.
15. Bordin S., Lewis J. and Petra P. H.: The preparation and cross-reactivity of a monospecific antibody to the sex steroid-binding protein of human and rabbit serum. *Biochem. Biophys. Res. Commun.* **85** (1978) 391–401.
16. Bordin S. and Petra P. H.: Immunocytochemical localization of the plasma sex steroid-binding protein (SBP) in tissues of the adult male monkey, *Macaca nemestrina*. *Proc. Natn. Acad. Sci. U.S.A.* **77** (1980) 5678–5682.
17. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
18. Tait J. F.: Review: The use of isotopic steroids for the measurement of production rates *in vitro*. *J. Clin. Endocr. Metab* **23** (1963) 1285–1297.
19. Plymate S. R., Fariss B. L., Bassett M. L. Matej L. and Garrison M. J.: Obesity and its role in polycystic ovary syndrome. *J. Clin. Endocr. Metab.* **52** (1981) 1246–1248.
20. Plymate S. R., Vaughan G. M., Mason A. D. and Pruitt B. A.: Central hypogonadism in burned men. *Horm. Res.* **27** (1987) 152–158.
21. Namkung P. C., Stancyzk F. Z., Cook M. J., Novy M. J. and Petra P. H.: Half-life of plasma sex steroid-binding protein (SBP) in the Primate. *J. Steroid Biochem.* **32** (1989) 675–680.
22. Garden G. A., Hannan C. J., Spencer C. A. and Plymate S. R.: The effect of sex hormone binding globulin (SHBG) on the pituitary testicular axis in hyperthyroid men. *Steroids* **52** (1988) 385–386.
23. Reed M. J., Lai L. C., Ghilchik M. W. and James V. H. T.: The effects of androgens and cortisol on the *in vivo* metabolism of estradiol. *J. Steroid Biochem.* **30** (1988) 489–492.
24. Tardivel-Lacombe J., Egloff M., Mazabrand A. and Degrelle H.: Immunocytochemical detection of the sex steroid-binding protein in human mammary carcinoma

cells. *Biochem. Biophys. Res. Commun.* **118** (1984) 488–494.
25. Robel P., Eychenne B., Blondeau J. P., Jung-Testas I., Groyer M. T., Mercier-Board C., Hechter O., Roux C. and Dadoune J. P.: Androgen receptors in rat and human prostate. *Horm. Res.* **18** (1983) 28–36.
26. Strel'chyonok O. A., Avvakumov G. V. and Survilo L. I.: A recognition system for sex-hormone-binding protein-estradiol complex in human decidual endometrium plasma membranes. *Biochem. Biophys. Acta* **802** (1984) 459–466.
27. Stanczyk F. Z., Namkung P. C., Fritz M. A., Novy M. J. and Petra P. H.: In *Binding Proteins of Steroid Hormones* (Edited by M. G. Forest and M. Pugeat). Colloque/INSERM/Libbey, London/Paris, Vol. 149 (1986) pp. 555–563.
28. Hryb D. J., Khan M. S., Romas N. A. and Rosner W.: Solubilization and partial characterization of the sex hormone-binding globulin receptor from human prostate. *J. Biol. Chem.* **264** (1989) 5378–5383.
29. Walsh K. A., Titani K., Kumar S., Hayes R. and Petra P. H.: Amino acid sequence of the sex steroid-binding protein (SBP) of human blood plasma. *Biochemistry* **25** (1986) 7584–7590.
30. Gershagen S.: PhD. thesis, University of Malmo, Sweden (1989).
31. Bourget C., Femino A., Franz C. and Longcope C.: Estrogen and androgen dynamics in the cynomologus monkey. *Endocrinology* **122** (1988) 202–206.
32. Kottler M. L., Tardivel-Lacombe J., Domingo M., Genier J., Dang C. D. and Degrelle H.: Regulation of plasma sex-steroid-binding protein in adult cynomolgus monkeys (*Macaca fascicularis*) during different reproductive states. *Biol. Reprod.* **39** (1988) 97–103.