Biochemical Pharmacology, Vol. 45, No. 12, pp. 2411–2416, 1993.
Printed in Great Britain.

0006–2952/93 $6.00 + 0.00

STEROID BINDING TO HUMAN SERUM ALBUMIN AND FRAGMENTS THEREOF

ROLE OF PROTEIN CONFORMATION AND FATTY ACID CONTENT

MARCEL J. E. FISCHER,* OCTAAF J. M. BOS,† RENÉ F. VAN DER LINDEN, JAAP WILTING and LAMBERT H. M. JANSSEN

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, and †Department of Product and Process Development, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

(*Received* 18 *November* 1992; *accepted* 9 *March* 1993)

Abstract—The binding properties of the steroids testosterone and pregnenolone to human serum albumin (HSA) and derived fragments of albumin have been investigated by means of equilibrium dialysis and circular dichroism. The 46 kDa peptic fragment (P46) of HSA comprises domains one and two of the HSA structure, whereas the other fragment, the 45 kDa tryptic fragment (T45) is composed of domains two and three. A comparison of the binding behaviour of the steroid ligands to HSA and its fragments showed that the single primary testosterone binding site in all probability is located in the second domain of the HSA molecule. For pregnenolone it was found that at least two primary binding sites are present, also located in domain two. Both steroids show pH dependent binding profiles in the case of HSA and the P46 fragment. The binding of the steroids to the T45 fragment seems to be pH independent. The same phenomenon was observed with the circular dichroism experiments, indicating a link between the steroid binding properties and the structural behaviour of the proteins. In fact, the binding properties of the steroids can be assigned to the neutral-to-base (N–B) transition. The possible role of fatty acids as modulators in the transport processes of steroids in the body is discussed.

Binding of steroids to plasma proteins is an important contributor to the distribution of these hormones in the body. Steroids were found to bind to several plasma proteins like human serum albumin (HSA‡), testosterone–estradiol binding globulin, corticosteroid binding globulin, α_1-acid glycoprotein and foetal steroid binding protein, all with different affinity or capacity [1]. HSA, in spite of a relatively low steroid binding affinity, binds most of the circulating steroids simply as a result of its high concentration [1–4].

It is commonly accepted that the fraction of hydrophobic ligands, like steroids, which bind with high affinity to plasma proteins, are less easily available to tissues [5]. Of course, the direct availability of a ligand determines the extent of its action. As steroids bind with low affinity to HSA, it is suggested that the HSA-bound fraction of steroids is responsible for most of their availability to tissues [5, 6]. Variables, like temperature, pH, fatty acids, sex and competing ligands, all influence steroid binding to plasma proteins [1, 3]. The physiological significance of the steroid-to-HSA binding can be seen in the phenomenon that free plasma hormone concentrations are more or less constant during strong plasma fluctuations of total steroid [4]. A diminished HSA concentration, e.g. during sickness, is responsible for large increases in the unbound fraction of the steroids in plasma [4]. A rise of the free fatty acid content in plasma also increases the unbound fraction of the steroids by decreasing the steroid-to-HSA binding [5]. Finally, changes in body temperature during inflammatory processes also lead to changes in the binding characteristics of steroid-to-HSA [3]. Elucidation of the nature of steroid binding to HSA could lead to a better understanding of the way by which the unbound fraction of steroid in plasma is affected.

The outlined importance of the binding of steroids-to-HSA, the existence of conformational changes in HSA, sensitive to pH and ionic fluctuations [7–9], and the suggested relationship between ligand transport and HSA conformation around physiological pH [10–12], make a study on the molecular behaviour of the steroid-to-HSA interaction, as a function of the pH, warranted.

In the steroidogenic pathway of Leydig cells, two steroid intermediates account for important phases in the hormone dependent cholesterol side-chain cleavage process [13]. Pregnenolone is the first product and is produced by the mitochondria; testosterone is considered as one of the final products of the microsomes. Both hormones are, therefore, interesting examples for the study of binding phenomena of biologically active steroids-to-HSA.

In this respect we tried to identify the location of the binding site(s) of testosterone and pregnenolone on the HSA molecule and to relate their binding behaviour to the neutral-to-base conformational change (N–B transition) in HSA. For this purpose

* Corresponding author.

‡ Abbreviations: HSA, human serum albumin; T45, the 45 kDa tryptic fragment; P46, the 46 kDa peptic fragment; [Θ], molar ellipticity; N, neutral; B, basic; CD, circular dichroism.

we isolated HSA and a large peptic and a large tryptic fragment thereof. Steroid-to-protein binding was studied with circular dichroism (CD) and equilibrium dialysis, and the role of fatty acid as a competitive ligand was evaluated.

MATERIALS AND METHODS

Reagents. HSA was isolated from pooled human plasma according to the method described by Kremer [14]. Both the 46 kDa peptic fragment (P46) and the 45 kDa tryptic fragment (T45), were isolated from HSA as previously described [7, 15–17]. The P46 fragment contains residues 1–387 and comprises domains one and two of the HSA structure. The tryptic T45 fragment represents residues 198–585 and comprises domains two and three. Protein solutions were deionized before use as described by Bos *et al.* [17]. [7-^{3}H]Pregnenolone (22.6 Ci/mmol) and [1,2,6,7-^{3}H]testosterone (91.7 Ci/mmol) were generous gifts of Dr F. Rommerts, Erasmus University, Rotterdam (from Amersham, the Netherlands; purity more than 98%). All other chemicals were of the highest analytical grade (Pharmacia, Uppsala, Sweden; Janssen Chimica, Beerse, Belgium; the Sigma Chemical Co., St. Louis, MO, U.S.A.).

Equilibrium dialysis. The affinity of HSA and the fragments for testosterone and pregnenolone was measured with equilibrium dialysis as described previously [16, 17]. In all dialysis studies the temperature was set at 25°. Protein concentration was kept at 6×10^{-5} M for both the ratio-dependent binding assay and the pH dependent affinity measurements. Protein solutions were made in phosphate or borate buffer. Ionic strength was always kept at 0.1. The molar ratio of steroid-to-HSA in the ratio dependent equilibrium dialysis studies varied from 0 to 2. Higher ratios are physiologically irrelevant and provoke steroid solubility and/or membrane binding problems. The pH was fixed at 7.4.

In the pH dependent experiments, the pH was varied between 6.0 and 9.5. For these experiments, the molar steroid-to-protein ratio was fixed at the physiological value of 0.02.

In the experiments where the effect of oleate on the binding properties of testosterone to the proteins was studied, the pH was fixed at 7.0 and the molar ratio of testosterone-to-protein was fixed at 0.02. The molar fatty acid-to-protein ratios were raised from 0 to 6.

In all cases the bound and unbound steroids were measured after the equilibrium dialysis experiments by means of liquid scintillation counting. The association constants were calculated assuming that all binding sites on each protein were equivalent and independent [8].

The binding data in the ratio-dependent binding assays were treated with the method described previously [18], with two sets of independent, non-cooperative binding sites. In this way the best fit was obtained between measured and calculated data.

The equation used was:

$$v = \frac{n_1 K_1 c_f}{1 + K_1 c_f} + \frac{n_2 K_2 c_f}{1 + K_2 c_f} \qquad (1)$$

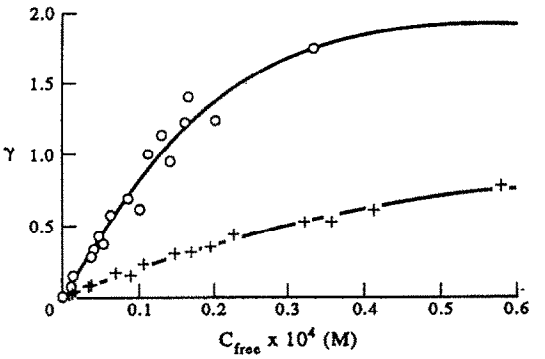

Fig. 1. Binding isotherm for the interaction of testosterone (○) and pregnenolone (+) with HSA at pH 7.4 and 25° in a phosphate buffer (I = 0.1). Plotted is the molar ratio of steroid bound to HSA (v) vs the free steroid concentration (c_{free}). The curve is calculated and drawn according to the parameters estimated by a non-linear least-squares analysis using Eqn 1.

where v is the molar ratio of steroid bound to protein, c_f is the concentration of unbound steroid and n_1 and n_2 are the number of steroid molecules bound to two independent sites on the protein, with affinity constants of K_1 and K_2 respectively. To obtain optimum fits, the iteration was started with a fixed set of values for n_1 and n_2, between 0 and 5.

CD. CD experiments were performed as described previously [16, 17]. In all experiments, the protein concentration was 10^{-4} M and a molar steroid-to-protein ratio of 1.0 was used to obtain reasonable signal-to-noise ratios. The CD-signals of the steroid–protein complexes and the protein alone were measured in 0.15 M KCl and were titrated with KOH over the pH range 6.0–9.5 at 303 nm at 25°. The observed ellipticities (Θ_{obs}) are the differences between the CD-signals of the steroid–protein mixture and of the protein alone at the given wavelength. Molar ellipticities ([Θ]), corrected for titration dilutions, were calculated as described previously [8, 9, 16, 17], using the equation

$$[\Theta] = \frac{\Theta_{obs}}{lc} \text{(degree} \times \text{litre/cm} \times \text{mol)} \qquad (2)$$

where l is the path-length in cm, and c is the molar concentration of the steroid-to-protein complex, as determined by equilibrium dialysis.

Results are presented as mean ± SD of triplicate determinations.

RESULTS

Equilibrium dialysis: binding parameters

In order to be able to calculate binding parameters of the binding of testosterone- and pregnenolone-to-HSA around physiological pH (pH 7.4), the data of the equilibrium dialysis experiments were evaluated. Figure 1 represents a typical binding isotherm. The molar ratio of hormone-to-HSA (v) is plotted versus the unbound hormone concentration

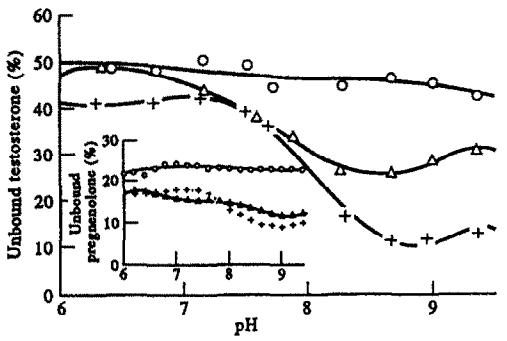

Fig. 2. Equilibrium dialysis data of testosterone and pregnenolone (insert) with HSA (+), the P46 fragment (△) and the T45 fragment (○). The percentage unbound steroid is presented as a function of the pH. Protein concentrations were 6×10^{-5} M and the molar ratio of steroid-to-protein was kept at 0.02. The measurements were performed in phosphate or borate buffers, I = 0.1.

(c_{free}). The bound fraction of testosterone decreases from 60% to 45% and the bound fraction of pregnenolone decreases from 80% to 73%, when the drug-to-HSA ratio is increased from $r = 0.01$ to $r = 2.00$.

From Fig. 1 it can be seen that the ratio-dependent testosterone binding isotherm reaches saturation. The isotherm is best fitted by a model showing the presence of one primary binding site and multiple secondary binding sites. Using Eqn 1, the best fit for the ratio-dependent testosterone binding data is obtained with the binding parameters $n_1 = 1$, $K_1 = (2.0 \pm 0.2) \times 10^4\,M^{-1}$ and $n_2 > 5$, $K_2 < (7.8 \pm 1.2) \times 10^2\,M^{-1}$. The high affinity binding constant agrees well with the reported value of Kragh-Hansen *et al.* [18].

For pregnenolone at least two primary binding sites must be assumed, besides multiple secondary binding sites. A reasonable fit is obtained with $n_1 = 2$, $K_1 = (4.3 \pm 0.6) \times 10^4\,M^{-1}$ and $n_2 > 10$, $K_2 < (2.0 \pm 0.5) \times 10^3\,M^{-1}$. The low affinity binding sites were found to have no influence on n_1 and K_1, as the product of n_2 and K_2 was found to be constant for the various fits with n_2. Such binding behaviour has been found in other studies [1, 2].

Equilibrium dialysis: location of binding sites

To determine the location of the binding sites on the HSA molecule, equilibrium dialysis was performed with HSA and the T45 and P46 fragments. In order to study the possible involvement of N and B or N- and B-like conformations on the steroid-to-protein binding process, the equilibrium dialysis experiments were done over a pH range of 6.0–9.5 and at a fixed molar steroid-to-protein ratio of 0.02. At this low steroid-to-protein ratio, it may be assumed that only the primary binding site(s) is/are occupied (see Fig. 1 for HSA). The results of these experiments are shown in Fig. 2. In contrast to the binding behaviour of drugs like warfarin and diazepam, which are known to bind to the so-called drug binding sites I and II respectively [16, 17], the steroids bind with considerably lower affinity to HSA. As mentioned, at pH 7 and a steroid-to-HSA ratio of 0.02 only about 60% of testosterone and 80% of pregnenolone is bound to HSA compared to 90–99% of both diazepam and warfarin. The increase in steroid binding with increasing pH both for HSA and the P46 fragment is striking, although the pattern is slightly less pronounced in the case of pregnenolone. The pH-dependent binding profiles of steroid-to-HSA and of steroid-to-P46 fragment are similar in shape as the pH-dependent binding profiles of warfarin to the proteins [17]. Neither testosterone nor pregnenolone show a pH-dependent binding profile in the case of the T45 fragment. This is similar to the situation found for the binding of diazepam to the T45 fragment.

The apparent association constants calculated from the equilibrium dialysis experiments are summarized in Table 1. It can be seen that around physiological pH the affinity of the hormones for the fragments is of the same order of magnitude as the affinity for HSA. This indicates that all three proteins contain the primary binding sites for both the hormones. As domain two is the common domain, it is most probable that this domain contains the binding sites.

The pH-dependencies of the apparent association constants for HSA and the P46 fragment point to the direction that one or more conformational changes in the albumin and the P46 fragment molecules play a role in the steroid binding process, since no changes in the physico-chemical properties of testosterone and pregnenolone are known to occur in this pH-region. This observation is quite similar to that found for the binding of, for example, warfarin [17], diazepam [9] and suramin [19] to the proteins.

Table 1. The apparent association constants ($nK \times 10^{-4}\,M^{-1}$) at pH 6 and 9 for the binding of testosterone and pregnenolone to HSA and the fragments thereof

Protein	Testosterone		Pregnenolone	
	pH 6	pH 9	pH 6	pH 9
HSA	1.9 ± 0.6	14.3 ± 3.7	7.8 ± 0.7	17.3 ± 2.8
P46 fragment	2.1 ± 0.1	4.4 ± 1.1	8.1 ± 0.6	12.7 ± 2.3
T45 fragment	1.8 ± 0.4	2.2 ± 0.3	5.9 ± 0.6	5.9 ± 0.1

Results are expressed as mean ± SD of four to six determinations.

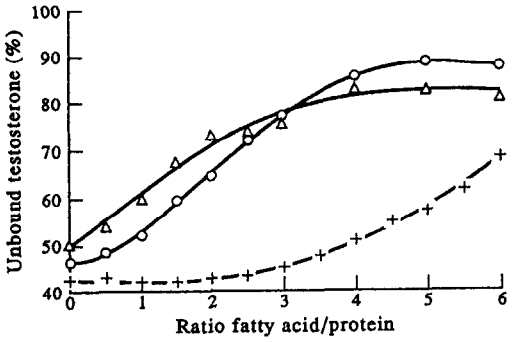

Fig. 3. Equilibrium dialysis performed with testosterone and HSA (+) the P46 fragment (△) and the T45 fragment (○), under the same experimental conditions as outlined in Fig. 2, with the exception that in this case oleic acid was added to the solution at molar fatty acid-to-protein ratios varying from 0 to 6. The figure shows the percentage of unbound testosterone vs the molar ratio of oleic acid-to-protein.

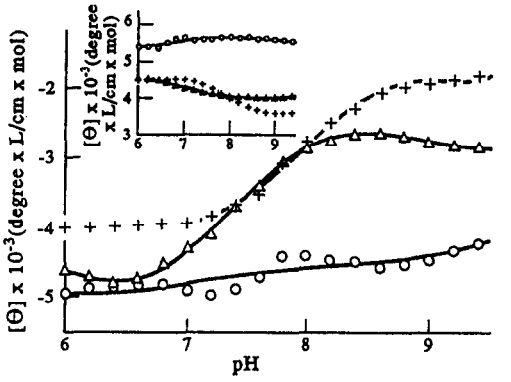

Fig. 4. CD experiments with HSA (+), the P46 fragment (△) and the T45 fragment (○). The testosterone–protein and pregnenolone–protein (insert) complexes are presented as molar ellipticities [Θ] vs pH. The protein concentration was 10^{-4} M and the molar ratio of steroid-to-protein was fixed at 1.0. Ellipticity measurements were performed at 303 nm and 25°. The system was non-buffered and pH was adjusted with KOH. The ionic strength was kept constant at I = 0.15 using KCl.

Equilibrium dialysis: effect of fatty acids

The effect of fatty acids on the binding behaviour of testosterone to HSA and its fragments is presented in Fig. 3. For HSA it can be seen that a significant displacement of the steroid occurs at oleic acid-to-protein ratios of more than 2. For the fragments, testosterone is already displaced at low oleic acid-to-protein ratios. The displacement of testosterone by fatty acid reaches a plateau at molar fatty acid-to-fragment ratios of approximately 4. As the tangent at the inflection point of the curves for all proteins are more or less comparable, one might expect a plateau in the case of HSA too. This would indicate that the displacement mechanism is the same for all three proteins.

CD

The induced extrinsic Cotton effect observed after the binding of testosterone and pregnenolone to the proteins has its maximum at 303 nm (data not shown); this is in accordance with previous work [20]. In Fig. 4, the molar ellipticity is presented. Although a relatively high steroid-to-protein ratio is used, it can be calculated that the observed effects arise mainly from the binding of steroid to the primary binding sites. From the figure, it is evident that the molar ellipticity of the testosterone–HSA and the testosterone–P46 complexes increase with increasing pH. Under the same conditions, a decrease in molar ellipticity with increasing pH can be seen for the pregnenolone–HSA and, although very small, for the pregnenolone–P46 complexes. For both hormones the [Θ] of the steroid–T45 complex is nearly pH independent.

DISCUSSION

HSA contains a large variety of binding sites for ligands. For several drugs the location of the binding site has been established [21]. Also for sex-hormones, plasma binding is largely related to HSA [1–3]. Using natural mutants of HSA, Kragh-Hansen *et al.* [18] have indicated the presence of the high affinity binding site of several steroids to be located in domain two, although it was suggested the sites are not the same for the different steroids. However, so far, the location of the primary binding site(s) for testosterone and pregnenolone has not been published. From the work presented in this article, it may be concluded that the second domain of the HSA molecule contains the primary binding site(s) for both the steroids. The fact that each of the steroids exhibits analogous binding constants for all three proteins at pH 6–7 and induces comparable Cotton effects after binding to the three proteins, supports this statement. The question remains whether or not the two steroids occupy the same binding site(s). For testosterone one primary binding site is measured, whereas pregnenolone seems to occupy two distinct high affinity binding sites. The optical rotation of both steroids is comparable (α_D is +1.09 and +1.18 for testosterone and pregnenolone, respectively [22]), and therefore one might expect the steroid induced [Θ] to be of the same sign. However, the opposite sign of [Θ] and the difference in pH-profile of [Θ] (respectively rising and descending) for both steroid–HSA complexes, makes the existence of a common binding site for these two steroids unlikely. The different signs, however, may also be connected to the reversed positions of −OH and carbonyl groups in the two steroids, in this way inducing a reversed ellipticity in the HSA molecule.

Steroid–HSA interactions seem to be governed by a so-called polarity-rule [1]; affinity decreases with increasing number of polar groups in the steroid molecule. Apparently polar interactions are dominant and hydrophobic interactions play a minor role in the binding of steroid-to-HSA [3]. Differences in polar interactions between steroid and HSA could

influence the competitive behaviour of the steroids for a binding site on the HSA molecule. However, competition between steroids is not found under physiological conditions (molar ratios steroid-to-HSA <0.01) [2]. Our results do show the existence of competition between fatty acid and steroid (Fig. 3). This phenomenon may be important in steroidogenesis for example. In the case of the Leydig cells for instance, it is known that partial stripping of fatty acids from the HSA molecule during the passage of serum to the interstitial fluid is a necessary condition to give HSA its biological effect on steroidogenesis [10]. Fatty acids display two high affinity sites in domain three ($K = 10^8\,M^{-1}$) and two sites of lower affinity in domain two ($K = 10^6\,M^{-1}$) [23]. Reed [24] showed for bovine serum albumin that Lys-349 and Lys-473 are involved in the binding of most long chain fatty acids. Lys-116 plays a minor role in this binding. Therefore, in a competitive displacement reaction between fatty acid and testosterone, one would expect that the steroid is displaced only at molar fatty acid-to-HSA ratios of 2 and higher, as, the lower affinity fatty acid binding sites will be occupied only at molar fatty acid-to-HSA ratios higher than 2–3. In this study, this has been confirmed by looking at the displacement of testosterone from its primary binding site on the HSA molecule. Apparently HSA and bovine albumin have comparable binding characteristics towards steroids and fatty acids.

The observed binding behaviour further indicates the existence of cooperativity between secondary fatty acid binding sites and the primary testosterone binding site. In case of identical binding site(s) for fatty acid and steroid it was anticipated that the tangent of the displacement curve of testosterone with increasing oleic acid concentrations would be steeper, as the affinity constant for the secondary fatty acid binding sites is at least 100 times larger than the affinity constant for the primary testosterone binding site. Evidently, there is no common binding site for testosterone and oleic acid on the HSA molecule. It is more likely that the apparent allosteric behaviour of HSA [25] is involved in the displacement process between the two ligands. Allosteric effectors such as the long-chain fatty acids can influence the conformational structure of HSA, thus changing the nature of ligand binding sites and binding affinities [25].

One would expect the T45 fragment to display a similar displacement curve as HSA, as both the primary and secondary fatty acid binding sites are, in principle, present in both proteins. As this is not observed, it is obvious that the characteristics of the oleate binding sites are changed in the T45 fragment, probably as a result of a reduced affinity for oleic acid by the binding sites in domain three. This is in agreement with other binding studies performed with drugs like warfarin and diazepam, where it was found that the binding properties are changed, when the binding to HSA and its derived fragments are compared [16, 17]. In these studies, it was stated that the stability of the 3-D structure was largely due to an interaction between domain one and the other two domains. Lack of domain one changes the 3-D structure from a U-shaped form to a more oblate ellipsoid one, thereby changing the hydrophobic and polar characteristics of the remaining domains [7, 26]. As the fatty acid binding sites are located in these regions, a diminished hydrophobic interaction will apparently change the affinity constants for fatty acid binding to the T45 fragment and perhaps also the number of binding sites for fatty acid. Therefore, the displacement of testosterone by oleic acid in the case of the T45 fragment shows other characteristics than the displacement of testosterone by oleic acid in the situation of albumin.

Around physiological pH, HSA is able to adopt two different isomeric forms. The conformational change is the N–B transition [7–9, 16, 19, 21, 27 and others]. The molecular mechanism of this transition has been extensively investigated using the T45 and P46 fragments [7, 16, 17, 26, 28]. Changes in the binding properties of ligands to HSA, due to variation in plasmogenic and cellular conditions (e.g. in the liver [29] or with Leydig cells in the testes [10]) have been linked to changes in HSA conformation (the N–B transition). The results of the pH-dependent CD measurements (Fig. 4), assuming the binding of steroids like testosterone and pregnenolone to HSA and P46 fragment (Fig. 2), can therefore be linked to an N–B or N–B-like transition [8]. The effect of the pH on $[\Theta]$ must be due to a change in the protein structure, because no changes are known to occur in the steroids in the given pH-region. The N–B transition is a proton-induced change in the protein, which can be directly influenced by calcium ions [7, 8] or ligands like fatty acids [25]. pK-shifts of histidine residues located in the domain one are involved in the transition and lack of domain one makes this conformational change almost absent in the T45 fragment [7]. The absence of a pH-dependent change in molar ellipticity of the steroid–T45 fragment complex, and the similarity in steroid binding behaviour and other characteristics of the P46 fragment and intact HSA supports this statement. When comparing Figs 2 and 4, one might therefore conclude that changes in the pH dependency of the binding of the steroids to the proteins and changes in the molar ellipticity of the steroid–protein complexes originate from one and the same phenomenon.

Plasma albumin typically carries 0.5–2.0 mol fatty acid/mol of albumin [24]. However, nutritional habits may increase or decrease the fatty acid-to-HSA ratio, thereby affecting the steroid binding capacity of HSA. An increase in the amount of fatty acids may result in a decrease of the HSA-bound fraction of steroid. This could be a situation which is perhaps unfavourable for the human body. In previous work [10], it has been shown that HSA having a molar ratio of oleic acid-to-HSA of less than 1 is able to activate the stimulation of steroid production in Leydig cells, whereas at oleic acid-to-HSA ratios of more than 2 HSA is unable to do so. This process could be a natural feedback mechanism to overcome a prolonged rise in free steroids, or on the other side to avoid a prolonged shortage of free steroid.

Reed *et al.* [12] and Horie *et al.* [11] found evidence for a surface induced conformational

change in albumin that facilitates release of ligands across the cell-membrane of hepatocytes. As mentioned, in a previous study we found that HSA stimulates the Leydig cell steroid production and that this stimulation is dependent on its fatty acid content and correlates with the potential to undergo conformational changes [10]. Therefore HSA, with its large binding capacity, but low affinity, and its enormous flexibility due to the N–B transition, may be an important and underestimated factor in the control and transport mechanism of, for example, steroids and to their passage across cell membranes [28]. It is very possible that fatty acids are able to modulate this process, although further studies are necessary to verify this phenomenon.

REFERENCES

1. Westphal U, *Steroid Protein Interactions*. Springer, Berlin, 1971.
2. Södergård R, Bäckström T, Shanbhag VP and Carstensen H, Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature. *J Steroid Biochem* **16**: 801–810, 1982.
3. Shanbhag VP and Södergård R, The temperature dependence of the binding of 5α-dihydrotestosterone, testosterone and estradiol to the sex hormone globulin (SHBG) of human plasma. *J Steroid Biochem* **24**: 549–555, 1986.
4. Dunn JF, Nisula BC 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 Endocrinol Metab* **53**: 58–68, 1981.
5. Mooradian AD, Pamplona DM, Viosca SP and Korenman SG, Effect of free fatty acids on the bioavailability of plasma testosterone and dihydrotestosterone. *J Steroid Biochem* **29**: 369–370, 1988.
6. Reed MJ, Cheng RW, Beranek PA, Few JD, Franks S, Ghilchik MW and James VHT, The regulation of the biologically available fractions of oestradiol and testosterone in plasma. *J Steroid Biochem* **24**: 317–320, 1986.
7. Bos OJM, Labro JFA, Fischer MJE, Wilting J and Janssen LHM, The molecular mechanism of the neutral-to-base transition of human serum albumin. *J Biol Chem* **264**: 953–959, 1989.
8. Wilting J, van der Giesen WF, Janssen LHM, Wiedeman MM, Otagiri M and Perrin JH, The effect of albumin conformation on the binding of warfarin to human serum albumin. *J Biol Chem* **255**: 3032–3037, 1980.
9. Wilting J, 't Hart BJ and de Gier JJ, The role of albumin conformation in the binding of diazepam to human serum albumin. *Biochim Biophys Acta* **626**: 291–298, 1980.
10. Meisert R, Bos OJM, van der Linden R, Fischer MJE, Wilting J, Janssen LHM, Hoogerbrugge JW and Rommerts FFG, The stimulatory effect of albumin on LH stimulated Leydig cell steroid production depends on its fatty acid content and correlates with conformational changes. *Mol Cell Endocrinol* **82**: 23–32, 1991.
11. Horie T, Mizuma T, Kasai S and Awazu S, Conformational change in plasma albumin due to interaction with isolated rat hepatocyte. *Am J Physiol* **254**: G465–G470, 1988.
12. Reed RG and Burrington CM, The albumin receptor effect may be due to a surface-induced conformational change in albumin. *J Biol Chem* **264**: 9867–9872, 1989.
13. Sharpe RM and Cooper I, Intratesticular secretion of a factor(s) with major stimulatory effects on Leydig cell testosterone secretion *in vitro*. *Mol Cell Endocrinol* **37**: 159–168, 1984.
14. Kremer JMH, A simple method for isolating large scale quantities of human serum albumin. *Vox Sang* **42**: 223–224, 1982.
15. Bos OJM, Fischer MJE, Wilting J and Janssen LHM, A procedure for isolating a large peptic fragment of human serum albumin. *J Chromatogr* **424**: 13–21, 1988.
16. Bos OJM, Fischer MJE, Wilting J and Janssen LHM, Drug binding and other physico-chemical properties of a large tryptic and a large peptic fragment of human serum albumin. *Biochim Biophys Acta* **953**: 37–47, 1988.
17. Bos OJM, Remijn JPM, Fischer MJE, Wilting J and Janssen LHM, Location and characterization of the warfarin binding site of human serum albumin. *Biochem Pharmacol* **37**: 3905–3909, 1988.
18. Kragh-Hansen U, Minchiotti L, Brennan SO and Sugita O, Hormone binding to natural mutants of human serum albumin. *Eur J Biochem* **193**: 169–174, 1990.
19. Vansterkenburg ELM, Wilting J and Janssen LHM, Influence of pH on the binding of suramin to human serum albumin. *Biochem Pharmacol* **38**: 3029–3035, 1989.
20. Watanabe S, Circular dichroism study of the interaction between conformationally altered human serum albumin and testosterone. *J Steroid Biochem* **23**:177–183, 1985.
21. Peters T Jr, Serum albumin. *Adv Protein Chem* **37**: 161–245, 1985.
22. Rauen HM, *Biochemisches Taschenbuch* Part I, pp. 497 and 516. Springer, Berlin, 1964.
23. Kragh-Hansen, U, Molecular aspects of ligand binding to serum albumin. *Pharmacol Rev* **33**: 17–53, 1981.
24. Reed RG, Location of long chain fatty acid-binding sites of bovine serum albumin by affinity labeling. *J Biol Chem* **261**: 15619–15624, 1986.
25. Dröge JHM, Janssen LHM and Wilting J, The fatty-acid-induced conformational states of human serum albumin investigated by means of multiple co-binding of protons and oleic acid. *Biochem J* **250**: 443–446, 1988.
26. Bos OJM, Fischer MJE, Wilting J and Janssen LHM, Mechanism by which warfarin binds to human serum albumin. *Biochem Pharmacol* **38**: 1979–1984, 1989.
27. Foster JF, Some aspects of the structure and conformational properties of serum albumin. In: *The Albumin Structure, Function and Uses* (Eds. Rosenoer VM, Oratz M and Rothschild MA), pp. 53–84. Pergamon Press, Oxford, 1977.
28. Bos OJM, Albumin, the 'Jack-of-all-trades' in the protein world. *Pharm Weekbl Sci Ed* **11**: 242–243, 1989.
29. Wilting J, Kremer JMH, Ijzerman AP and Schulman SH, The kinetics of the binding of warfarin to human serum albumin as studied by stopped-flow spectrophotometry. *Biochim Biophys Acta* **706**: 96–104, 1982.