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Nonlinear binding of sex steroids to albumin and sex hormone binding globulin

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SUMMARY

We have studied the binding of sex steroids to albumin and sex hormone binding globulin (SHBG) using gel filtration chromatography for the separation of the bound from the free fraction of the steroid. It was found that estradiol binds to the globulin and albumin in a nonlinear manner: a lag period of binding was observed at low concentrations of the proteins, followed by an exponential increase of the bound hormone as the protein concentration increased. The same was observed with dihydrotestosterone (DHT) and albumin but not with globulin. In the presence of a constant concentration of albumin, the increase of SHBG concentrations resulted in a rapid transfer of estradiol from albumin to globulin while the transfer of DHT was moderate. When whole serum was used, the increase of its amount again resulted in the transfer of estradiol from albumin to globulin. Our study showed that a substantial increase of globulin-bound hormone can occur, following small variations of the protein. This offers obvious advantages to the organism, by saving energy, material and time and plays a basic role in estradiol transfer from albumin to the much more biologically active globulin.

INTRODUCTION

It is generally accepted that sex steroids are present in human plasma bound to the proteins albumin and sex hormone binding globulin (SHBG). It is believed that the free hormone circulating in the plasma does not exceed 2% of the total quantity. This small fraction of the hormone was regarded for many years as the biologically active one (1–4). During the past few years, however, an increasing number of reports have appeared, declaring the existence of receptors for SHBG on cell membranes (5–7) and the bioavailability of the albumin-bound and (even more) the SHBG-bound hormone. Various suggestions concerning the new role of SHBG, such as the uptake of steroid hormones by target cells via a receptor-mediated endocytosis (8,9) and the induction of a cellular second messenger cAMP by the complex SHBG-membrane receptor (10) have been made. In any case, the binding and dissociation of the hormone from the protein are essential for their biological action.

Following some indications of nonlinearity in the binding of estradiol to SHBG, we decided to study this phenomenon using molecular sieving chromatography

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for the separation of the bound from the free hormone fraction. At the same time, a preparation of SHBG obtained by a new simple and quick method was used in the experiments in order to avoid the possibility of seriously diminishing or damaging the binding activity of the molecule as a result of the purification procedure. Albumin was also purified in the laboratory because the commercial product was found to contain many other proteins.

MATERIALS AND METHODS

Chemicals

Steroids were products of Sigma, Germany, while radiolabelled steroids were products of Amersham, UK. The specific activity of estradiol (2,4,6,7,16,17-^{3}H) was 140–170 Ci/mmole and that of dihydrotestosterone (DHT) (1,2,4,5,6,7-^{3}H) 100–150 Ci/mmole. Sephadex G-100, G-200 and DEAE-cellulose were products of Sigma. Triton X-100 was a product of Serva, Germany. The scintillation cocktail for the measurement of radioactivity contained 4 g PPO and 0.4 g POPOP (Sigma) per liter of a toluene-Triton X-100 mixture (2:1).

Sex hormone binding globulin was purified from the serum of pregnant women by acetone and ammonium sulfate fractionation and ion-exchange chromatography. The procedure, performed at 4°C was as follows: 100 ml of serum were diluted with 150 ml of Tris-saline buffer (0.05 M Tris-HCl pH 8.0, NaCl 0.9%) and 212.5 ml (0.85 vol) of acetone, precooled at –25°C, was added dropwise, under vigorous stirring. 15 min following the addition of acetone, the heavy precipitate formed was centrifuged for 10 min at 12,500 *g* and discarded. 87.5 ml (0.35 volumes) of acetone was then added to the supernatant under the same conditions. The new precipitate, obtained by centrifugation, was dissolved in Tris-saline buffer to a final volume of 62 ml. To this solution, 38 ml of a cold (4°C) saturated solution of ammonium sulfate in Tris-saline buffer were added dropwise under vigorous stirring, and 20 min later the precipitate was centrifuged for 10 min at 12,500 *g* and discarded. An additional 32 ml of ammonium sulfate solution was added to the supernatant, in order to achieve a 53% final saturation. After stirring for 30 min, the precipitate, obtained by centrifugation as above, was dissolved in 20 ml of 0.05 M Tris-HCl buffer pH 8.0, dialyzed for 3 h against 500 ml of the same buffer and chromatographed on a 3 × 30 cm DEAE-cellulose column, pre-equilibrated with the same buffer. The column was eluted with a linear 0–0.25 M NaCl gradient (400 + 400 ml) in the same buffer (Fig. 1.) The tubes containing the active protein were pooled and 1.2 volumes of acetone were added dropwise under stirring. The pellet, obtained after centrifugation, was dissolved in 5 ml of Tris-HCl buffer (0.05 M, pH 8.0) and used in the experiments. This product exhibited practically only one protein fraction as shown by chromatography with Sephadex G-200. The pattern of this protein coin-

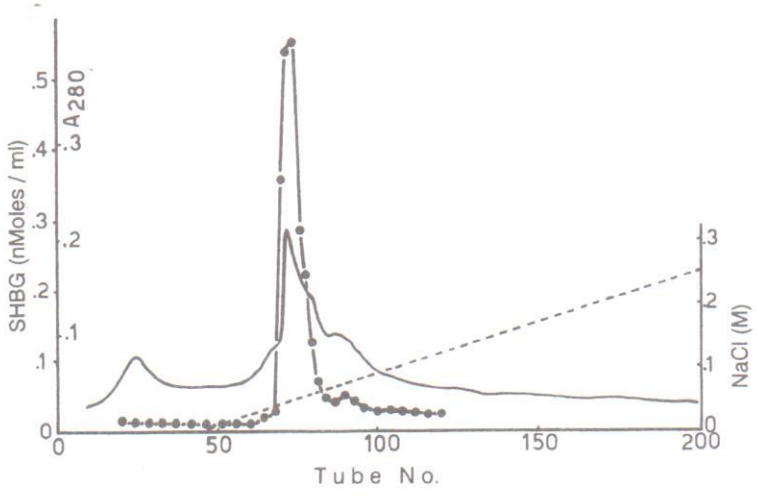

Fig. 1 : Ion-exchange chromatography of the SHBG preparation obtained following ammonium sulfate fractionation. A 3 × 30 cm DEAE-cellulose column was used, pre-equilibrated with 0.05 M Tris HCl buffer, pH 8.0 and eluted with a 0–0.25 M NaCl gradient in the same buffer. Fractions of 5 ml were collected and assayed with labelled estradiol for binding activity by a DCC method. Solid line A_{280}, filled circles SHBG, expressed in nmoles of bound estradiol per ml of solution, and dashed line NaCl concentration.

Table I : Summary of the SHBG purification procedure.

Purification step	*Total protein (mg)*	*SHBG (nmoles)*	*Purification factor*	*Yield (%)*
Serum	7300	17.6	1	100.0
Acetone fractionation	22.3	14.2	267	80.7
Ammonium sulfate fractionation	13.7	12.8	388	72.7
Ion exchange chromatography	2.8	10.6	1579	60.2
Second acetone fractionation	2.4	10.3	1787	58.5

cided exactly with the binding activity. In polyacrylamide gel electrophoresis, this product also gave practically one band, with some traces of albumin (results not shown). A summary of the purification procedure is presented in Table I. The whole procedure was completed in less than 24 h.

Albumin was purified from human serum by DEAE-cellulose chromatography. A 2.3 × 30 cm column, pre-equilibrated with 0.05 M Tris-HCl buffer pH 8.0 was used, eluted with a 0–0.4 M NaCl gradient in Tris buffer. The albumin fraction from the column, detected by a colorimetric method using bromocresol green (diagnostic kit of Sigma), was concentrated by polyethyleneglycol (Serva) and filtered through a Sephadex G-200 column (3.5 × 55 cm) equilibrated with the same Tris buffer. The albumin fraction was chromatographed once again on a DEAE-cellulose column, and the product obtained was used in the experiments. Whole serum used in the experiments was obtained from a healthy 32 year-old woman.

Gel filtration of the protein–steroid complex

A 3.5 × 60 cm column of Sephadex G-100 (Superfine), equilibrated with 0.05 M Tris-HCl buffer pH 8.0, was used throughout the experiments. Steroid (15 pmoles), was incubated with the protein solution for 15 min at 0°C (ice-bath). The final volume was 2 ml in all cases (except for the case of serum), in order to avoid differences in dilution during the chromatography of various samples. The sample was loaded on the column and chromatographed with Tris buffer at 4°C with a flow rate of 0.5 ml/min. Fractions of 5 ml were collected, and 0.4 ml of the tube content was added to 4 ml of scintillation fluid and measured for radioactivity in an LKB 1211 Rackbeta β-counter. Typical gel filtration patterns are presented in Figures 2A and 6A. In competition experiments between albumin and SHBG, as well as in the case of whole serum experiments, the curves were derived based on

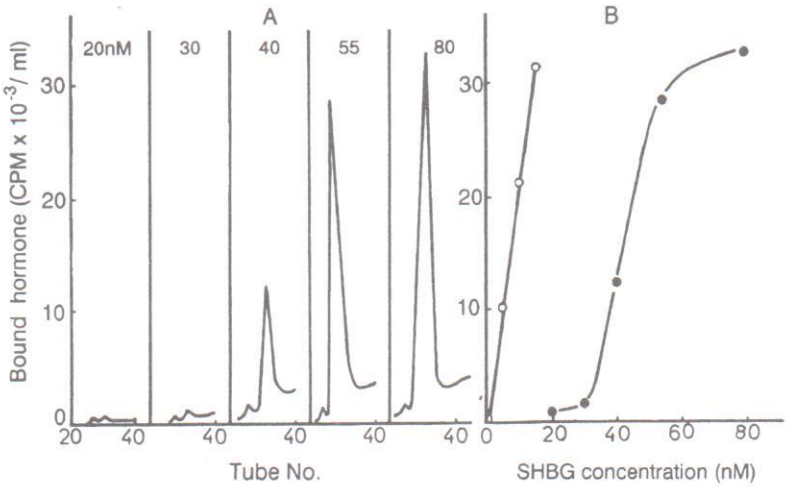

Fig. 2 : Binding of sex-steroids to SHBG. (**A**) Sephadex G-100 gel filtration pattern of the SHBG-estradiol complex. Various concentrations of SHBG are indicated in the figure. (**B**) Curves of hormone binding to SHBG, derived on the base of gel filtration. Filled circles, estradiol; open circles, dihydrotestosterone.

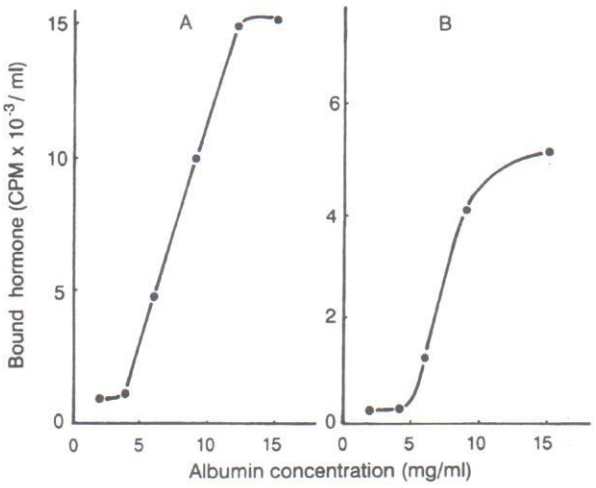

Fig. 3 : Binding of sex-steroid to albumin. The curves were derived on the basis of the elution pattern of the albumin-hormone complex from a Sephadex G-100 column, in a way similar to that presented in Figure 2. (**A**) Estradiol; (**B**) dihydrotestosterone.

the bound hormone appearing at the peaks of the two proteins.

RESULTS

Binding of estradiol and dihydrotestosterone to SHBG

As shown in Figure 2, binding of estradiol to SHBG was not observed until the concentration of the latter exceeded 32 nM. Exceeding that concentration, the binding of the hormone followed an exponential increase, reaching a plateau at concentrations over 70 nM.

The lag period of estradiol binding was observed only as a consequence of SHBG variation. Variations of the hormone concentration did not influence the filtration picture to any degree (results not shown).

When dihydrotestosterone (DHT) was used, the lag period of the binding was not observed but, following the increase of SHBG concentration, the increase of the binding was linear (Fig. 2B).

Binding of estradiol and DHT to albumin

The binding of estradiol to albumin gave a profile which was in some respects similar to that observed with SHBG. There was a lag period in the binding at low concentrations of the albumin, however, the increase of the binding which was observed after 2 mg/ml concentration was less exponential compared to that observed with SHBG (Fig . 3A).

The profile of DHT binding to albumin was similar to that observed in the case of estradiol (Fig. 3B), with a somewhat longer lag period. Yet the maximal DHT bound to high concentrations of albumin was much lower compared to estradiol.

Competition of SHBG with albumin for the binding of estradiol

When a stable concentration of albumin, corresponding to its mean concentration in the human serum, and increasing concentrations of SHBG were used, a lag period in the binding of estradiol to SHBG was observed, without differing substantially from that observed when SHBG was present alone in the incubation mixture. The binding of estradiol to albumin remained practically stable during this period. When SHBG started to bind estradiol, there was a rapid increase of globulin-bound hormone, followed by a

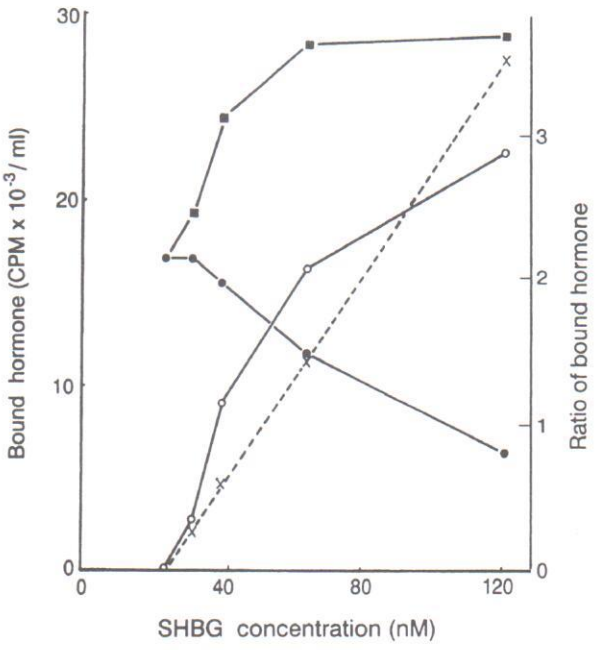

Fig. 4 : Competition of SHBG with albumin for the binding of estradiol. A constant concentration of albumin (40 mg/ml) was used and the curves were derived on the basis of the elution of the complexes from a Sephadex G-100 column as described in Materials and Methods. Filled circles, albumin-bound estradiol; open circles SHBG-bound estradiol; filled squares, total bound estradiol; crosses, ratio of SHBG to albumin bound estradiol.

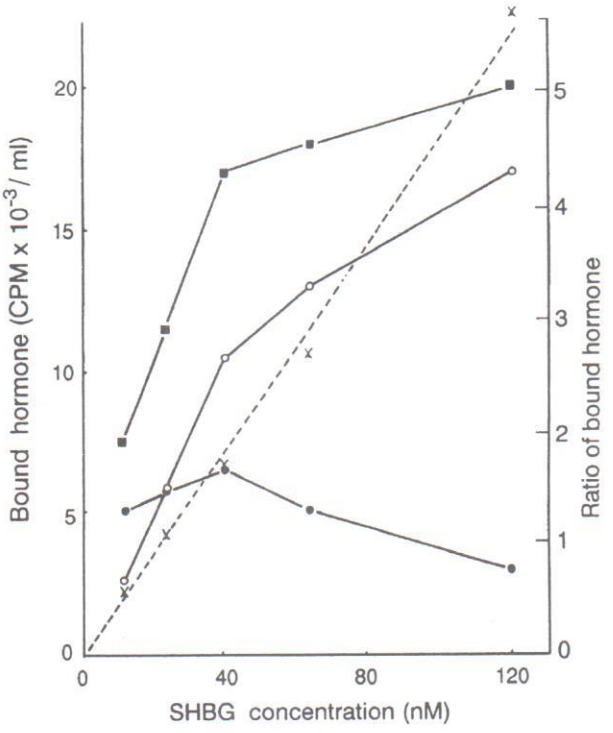

Fig. 5 : Competition of SHBG with albumin for the binding of dihydrotestosterone. A constant concentration of albumin (40 mg/ml) was used and the curves were derived as described in Figure 4. Filled circles, albumin-bound DHT; open circles, SHBG-bound DHT; filled squares, total-bound DHT; crosses, ratio of SHBG to albumin bound DHT.

moderate decrease of albumin-bound hormone. This resulted in a rapid increase of the total bound estradiol which reached a plateau very quickly (Fig. 4). It is notable that the ratio of SHBG to albumin bound estradiol gave a completely linear curve throughout the range of SHBG concentrations.

Competition of SHBG with albumin for the binding of DHT

In the case of DHT, the picture obtained was quite different from that with estradiol. There was practically no lag period in the binding of DHT to SHBG while an increase of albumin-bound DHT was observed as SHBG was increasing up to 40 nM. After that point, it started decreasing slowly. As a consequence of the above, the total bound DHT increased at a moderate rate. However, the ratio of SHBG to albumin-bound DHT followed, again as in the case of estradiol, a completely linear curve which however started from zero concentrations of SHBG (Fig. 5).

Binding of estradiol to serum proteins

The binding of estradiol to serum proteins is shown in Figure 6. Increased volumes of human serum incubated with estradiol were filtered through Sephadex G-100. Two peaks of radioactivity corresponding to SHBG and albumin-bound hormone (according to the order of appearance) were obtained (Fig. 6A). On the basis of this elution profile, the binding curves presented in Figure 6B were prepared.

It is evident from the figure that the lag period for the albumin binding is shorter than that for SHBG binding. This resulted in an exclusive binding of estradiol to albumin when the total amount of serum proteins was low, while a transfer of estradiol from albumin to SHBG was observed as the total protein quantity was increased. The amount of total bound hormone increased exponentially following the albumin binding until the beginning of SHBG binding. Then increased at a slower rate and reached a plateau with high quantities of serum. Interestingly, the SHBG to albumin ratio again increased quite linearly.

DISCUSSION

Plasma proteins binding with sex steroids were first reported about 30 years ago (11,12). Since this time, many studies have been conducted regarding both their structure and function (8,13). The discovery of receptors for steroid hormones, however, a few years later, focused great interest on them. This appears to be the main reason why very important questions concerning the biological function of these proteins and their role in the regulation of hormonal action remain unanswered or at least are still the subject of controversy.

A few years ago, we had some indications (14) that there is a nonlinear relationship between SHBG concentration and bound steroids. We decided to study the binding of sex hormones to SHBG and albumin using molecular sieving for the separation of the complex from the free hormone. The results presented here clearly confirmed the indications.

Thus, the concentration of the hormone does not affect the formation of the complex which is proportional to the protein concentration (although in a non linear manner). It is clear, in fact, that the binding of

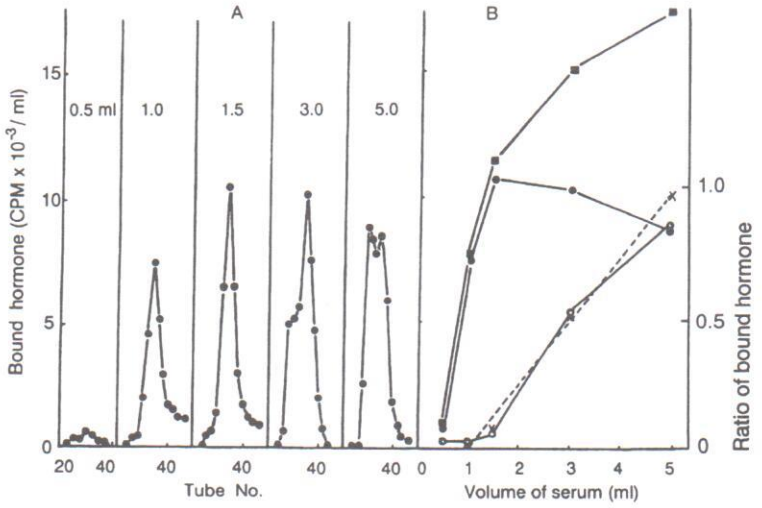

Fig. 6 : Binding of estradiol to whole serum proteins. (**A**) Sephadex G-100 gel filtration pattern of SHBG and albumin-bound estradiol. The volumes (ml) of serum used are indicated in the figure. (**B**) Curves of the hormone bound to SHBG and albumin, derived on the base of gel filtration presented in (A). Filled circles, albumin-bound estradiol; open circles, SHBG-bound estradiol; filled squares, total bound hormone; crosses, ratio of SHBG to albumin bound estradiol.

estradiol to both SHBG and albumin is characterized by a lag period as the concentration of the protein increases. This period is followed by an exponential phase (more profound in the case of SHBG than albumin) and then it reaches a plateau. It is evident that, during the exponential phase, very small increases in the protein concentration result in a disproportionately high binding of the hormone to the protein. When dihydrotestosterone was used, a lag period was observed only with albumin, while the binding to SHBG followed a linear relationship with protein concentration.

It must be noted that the concentration of albumin in vivo is much higher than concentrations where the exponential phase is observed. This means that in vivo the regulatory role, concerning the distribution of the hormone between albumin and SHBG, must belong to the latter, whose exponential phase is observed at concentrations usually present in vivo.

In our experiments with stable concentrations of albumin and increasing concentrations of SHBG, this role was very clear. When the concentration of globulin entered the exponential phase, a quick transfer of estradiol from albumin to SHBG was observed. Interestingly, with DHT which binds to SHBG linearly, the rate of the binding was slower at low concentrations, compared to that observed without the presence of albumin, accompanied by a parallel small increase of albumin-bound hormone. Only at high concentrations of SHBG was a transfer of hormone from albumin to SHBG observed. The concentration of SHBG at the 'turning point', however, was about the same with that observed in the case of estradiol.

It is interesting that in both cases (estradiol and DHT), the ratio of the hormone bound to SHBG to that bound to albumin increased quite linearly. The only difference is that the zero value was at the beginning of the axes in the case of DHT and at the point of the beginning of exponential phase in the case of estradiol.

When whole serum was used in the experiments, the increase of its amount resulted in a transfer of estradiol from albumin (on which it was exclusively bound when the quantity was lower than 1.5 ml) to SHBG. Again the ratio of SHBG to albumin bound hormone exhibited a linear dependency.

Some interesting conclusions could be drawn from these observations. First, it is evident that the non linear binding of estradiol to SHBG offers to the organism an advantage from the point of view of saving biological material, energy and time during the transfer of the hormone from albumin to SHBG. Second, the linear increase of the ratio of SHBG to the albumin-bound hormone and its direct dependence on SHBG concentration, indicates that albumin plays the role of 'reservoir' for sex steroids while SHBG is the active molecule which exclusively determines the time and the velocity of the detaching hormone from albumin to be used in various metabolic pathways.

During our study, a paper appeared (15) presenting evidence of a nonlinear binding of the contraceptive steroids levonorgestrel, 3 keto-desogestrel and gestodene to SHBG. This nonlinear relation was derived from a large number of data points collected in several clinical studies using various contraceptives. On the base of their results, the authors point out that this nonlinearity must be taken into account when the quantities of given contraceptives are calculated. This seems also to be the case with regard to the estimation of the real concentration of sex steroids acting in each case based on our findings.

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