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# THE BINDING OF TESTOSTERONE TO DIFFERENT SERUM PROTEINS: A COMPARATIVE STUDY

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## SUMMARY

The binding of testosterone to various serum protein fractions has been investigated by fractionation on DEAE-cellulose. In male human sera the mean ratio of testosterone bound to the 17β-hydroxy-steroid binding protein (SBP) to transcortin (CBG)-bound testosterone was found to be 0·50. The corresponding mean ratio in female sera was 5·4. These values correspond to the actual SBP concentrations of $1{\cdot}44 \times 10^{-8}$ M and $8{\cdot}1 \times 10^{-8}$ M, respectively. In contrast to man only easily-dissociable binding sites were found in rat sera. In sera from guinea pigs, at least approximately 50 per cent of the added testosterone was found to be associated with easily dissociable binding sites. The presence of saturable binding sites with comparatively high affinity was, however, demonstrated in both sexes. In the pregnant guinea pig, testosterone was found to be strongly bound to proteins with a very low dissociation rate. These proteins have been further characterized.

## INTRODUCTION

In man steroid hormones are strongly, specifically and reversibly bound to certain plasma globulins. The principal plasma protein which binds the sex hormones 17β-oestradiol and testosterone, the 17β-hydroxysteroid-binding protein (SBP), has recently been purified, and some of its properties are known[1–3]. Testosterone also binds reversibly with corticosteroid-binding globulin (CBG)[4, 5], with α-acid glycoprotein (AAG)[6] and with serum albumin[7], but its associations with them are much weaker than with SBP.

Most of the studies on the testosterone-binding protein fractions have been conducted on human plasma or serum, while the information on such proteins in other mammalian species is rather scant. The purpose of this investigation has been to show to what extent high-affinity binding proteins are involved in the binding of testosterone in sera from humans and some animal species. To characterize and quantify the steroid binding, equilibrium dialysis and fractionation of the binding proteins by DEAE-cellulose chromatography have been used.

## METHODS

### *Serum collection*

Whole blood was collected by venipuncture from male and female blood donors, as well as from women in the last trimester of pregnancy. From rats and guinea pigs the blood samples were drawn by cardiac puncture. After the blood was allowed to coagulate for several hours at 4°C, the serum was collected by centrifugation and processed immediately. The individual sera were depleted of endogenous steroids by incubation with powdered charcoal according to a procedure described by Heyns *et al.*[8]. Incubation for 30 min with 50 mg Norit A per ml serum was found adequate. Four control determinations by the competitive protein binding technique[9] showed that 89·3 ± 3·8 per cent of the cortisol was removed from the sera.

## MATERIALS

[1, 2-$^3$H] Testosterone (S.A. 50·0 Ci/mmol) was purchased from New England Nuclear and was tested for purity by paper chromatography in the system hexane: benzene (2:1)/formamide. [4-$^{14}$C]Cortisol (S.A. 57·9 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Dialysis tubing was obtained from Visking Company, Chicago, Illinois U.S.A. The DEAE-cellulose (Whatman DE-52) was converted into its phosphate form by titration with phosphoric acid as recommended by the manufacturer. The prepared exchanger was stored in 0·0125 M phosphate buffer, pH 6·3, containing 0·03 per cent toluene as a preservative. Other chemicals were standard commercial products of analytical grade.

### *Equilibrium dialysis*

After removal of the charcoal by centrifugation at 12·000 *g*, a 5 per cent dilution of the stripped serum was made in 0·05 M sodium phosphate buffer, pH 7·4. For dialysis, 0·5 ml of diluted serum was transferred to a dialysis bag (Visking 8/32″ seamless) and dialyzed against 5 ml of outside solution in a glass vial equipped with polyethylene cap closure. The system was allowed to equilibrate under gentle agitation for 48 h at 4°C. The outside solution consisted of [$^3$H]-testosterone dissolved in phosphate buffer, 0·625 ng/5 ml. After equilibrium had been reached, 0·25 ml samples of the inside and outside solutions were counted in a liquid scintillation counter.

### *Fractionation of serum proteins*

In addition to charcoal absorption, the samples destined for chromatography were dialyzed twice for 24 h against 100 ml of a phosphate buffer, 0·0125 M, pH 6·3. The contents of the dialysis bags were then incubated with [$^3$H]-testosterone in concentrations of 625 and 62·5 ng/100 ml for male and female sera respectively. Samples from pregnant individuals were incubated with the same concentration of labelled testosterone as in male sera. Incubation was carried out at 4°C under occasional shaking for at least 2 h.

Separation of serum proteins into fractions was accomplished by chromatography on DEAE-cellulose using the method of Sober and Peterson[10]. The phosphate buffers used for elution were: 0·0125 M, pH 6·3; 0·04 M, pH 5·9; 0·1 M, pH 5·8; 0·4 M, pH 5·2. A Whatman water-jacketed column (1·0 × 20 cm) was packed to a height of 11·0 ± 0·5 cm with the prepared DEAE-cellulose. Approximately 100 ml of the starting buffer (0·0125 M) was then precycled before application of the sample. The flow rate through the column was kept constant at 46 ± 2 ml per h. From each eluant, 10 fractions, each 4·6 ml, were collected. The column temperature was kept constant at 4·0 ± 0·1°C.

## RESULTS

### 1. *Behaviour of the human serum albumin-testosterone complex*

The elution pattern resulting from chromatography on DEAE-cellulose of 1 ml 3·6 per cent solution of pure human serum albumin (HSA) incubated with 6·25 ng [$^3$H]-testosterone is shown in Fig. 1. Prior to chromatography this solution was found by equilibrium dialysis to bind 95·6 per cent of the added testosterone. Most of the radioactivity was eluted between the free testosterone (broken line) and the albumin peak. By equilibrium dialysis, this radioactivity was shown to be

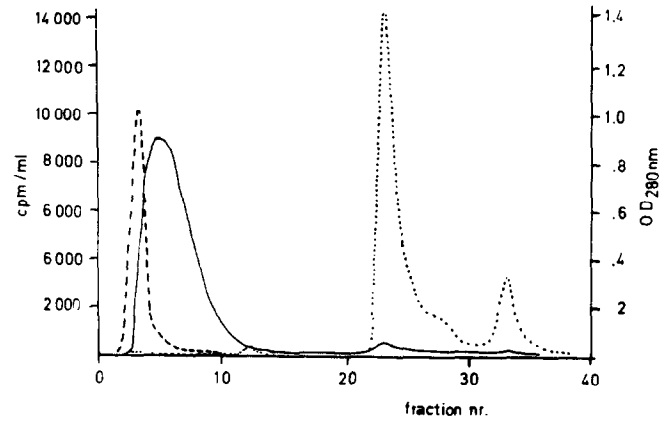


Fig. 1. Chromatography of 1 ml 3·6 per cent HSA solution preincubated with 6·25 ng [1,2-$^3$H]-testosterone. Elution scheme according to Sober and Peterson[10]. Dotted line: optical density (O.D.) at 280 nm. Broken line indicates position of radioactive peak produced by chromatography of a protein-free solution of [1,2-$^3$H]-testosterone.

non-protein bound, showing that any initial HSA-testosterone complex will dissociate almost quantitatively on the column.

2. *Distribution of testosterone among different protein fractions in human sera*

Typical elution patterns of human male and female sera are shown in Figs. 2 and 3. The chromatography system used has been shown by Guerigian and Pearlman[11] to separate the human SBP from CBG. According to the data presented by these authors, the two distinct peaks positioned on the chromatogram at fractions 13–15 and 22–24 are considered to correspond to the elution of SBP and SBG respectively. By equilibrium dialysis, the radioactivity eluted in the fractions preceding the $\beta$-globulin peak proved to be non-protein bound and obviously consisted of testosterone initially bound by albumin together with testosterone dissociated from the other two protein-bound fractions.

The results of 17 fractionation experiments carried out with six normal male sera, 9 normal female sera and two sera from pregnant women are presented in Table 1. The amount of dissociated testosterone, expressed as per cent of the total radioactivity eluted from the column, was found to be significantly higher

Table 1. Binding of testosterone to human serum fractions as revealed by chromatography on DEAE-cellulose following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer, 0·0125 M, pH 6·3. Prior to chromatography, the dialyzed sera were incubated with approximately physiological concentrations of [1,2-$^3$H]-testosterone. B/U: Ratio of bound to unbound testosterone as determined by equilibrium dialysis. SBP/CBG: ratio of radioactivity bound to SBP and CBG fractions

| Sex | % Dissociated | SBP/CBG | B/U | *n* |
|---|---|---|---|---|
| Males | 30·5 ± 7·5 (S.D.) | 0·50 ± 0·30 (S.D.) | 11·3 ± 2·8(S.D) | 6 |
| Females | 19·6 ± 6·9 (S.D.) | 5·41 ± 8·50 (S.D.) | 17·3 ± 5·8(S.D.) | 9 |
| Pregnants | 14·3 ± 3·1 | 1·60 ± 0·44 | 41·0 ± 8·6 | 2 |

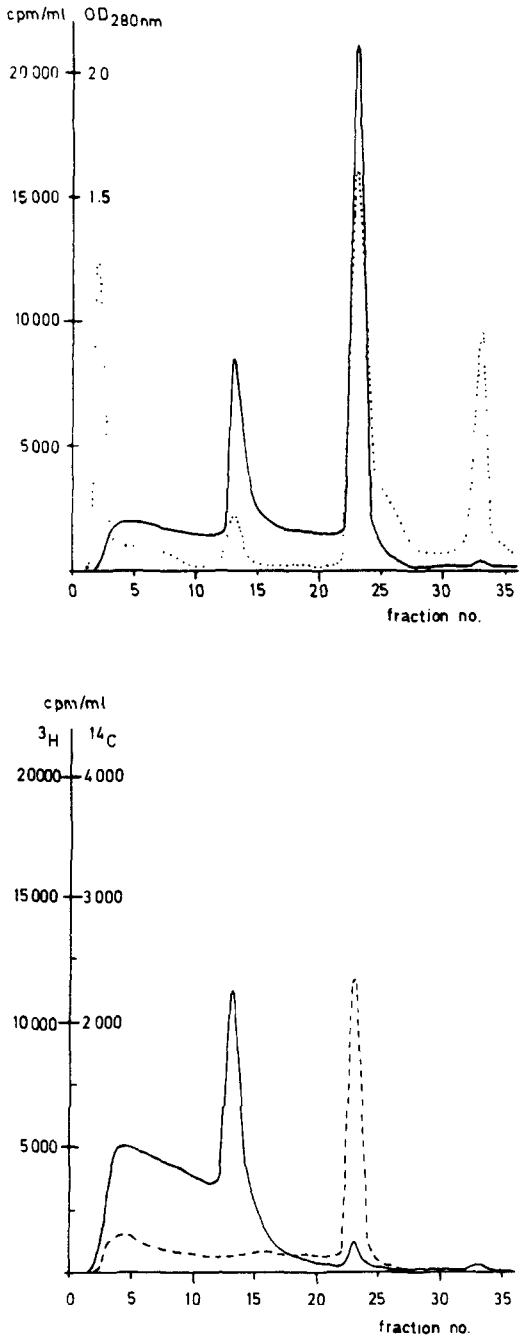


Fig. 2. Upper diagram: DEAE-cellulose chromatography of 1 ml human male serum following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer 0·0125 M, pH 6·3. Prior to chromatography, the stripped, dialyzed serum was incubated with [1,2-$^3$H]-testosterone, 625 ng/100 ml. Dotted line: O.D., 280 nm. Peaks positioned at fractions 13–15 and 22–24 correspond to SBP and CBG respectively[11]. Lower diagram: the serum treated in the same way, but with the addition of [4-$^{14}$C]-cortisol, 25 $\mu$g/100 ml. Broken line: [$^{14}$C] activity.

($P < 0{\cdot}02$) in male than in female sera. However, there was apparently no difference between non-pregnant and pregnant women.

The radioactivity bound in the $\beta$-globulin region (SBP) and in the CBG-albumin region was estimated by subtracting the extrapolated level of dissociated radioactivity from the total radioactivities present in the two respective peaks. Since any albumin-bound testosterone under the applied experimental conditions will be almost quantitatively dissociated (Fig. 1), the radioactivity of the second peak (fractions 22–24) can be referred to as CBG-bound testosterone. The mean ratios of SBP-bound to CBG-bound testosterone were $5{\cdot}41 \pm 8{\cdot}6$ in the female sera and only $0{\cdot}50 \pm 0{\cdot}30$ in the male sera. However, due to the great individual variations in the women, the difference between the two sexes was not significant.

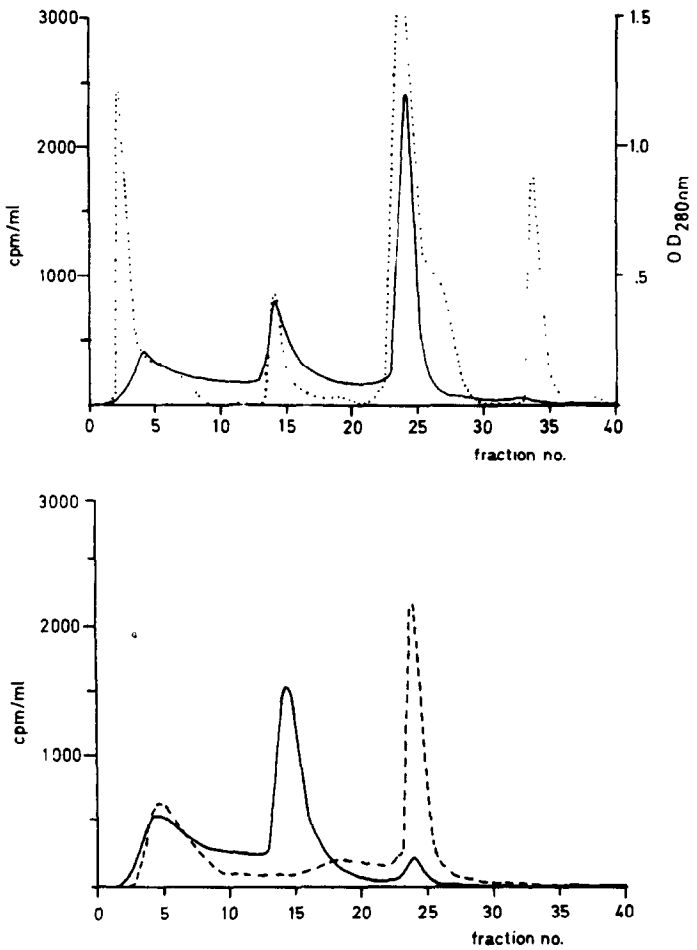


Fig. 3. Upper diagram: DEAE-cellulose chromatography of 1 ml human female serum following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer 0·0125 M, pH 6·3. Prior to chromatography, the stripped, dialyzed serum was incubated with [1,2-$^3$H]-testosterone, 62·5 ng/100 ml. Dotted line: O.D., 280 nm. Peaks positioned at fractions 13–16 and 23–26 are considered to correspond to SBP and CBG respectively [11]. Lower diagram: the serum treated in the same way, but with the addition of [4-$^{14}$C]-cortisol, 25 $\mu$g/100 ml. Broken line: [$^{14}$C]-activity.

The ratio of bound to unbound testosterone obtained from equilibrium dialysis was found to be significantly higher in female than in male sera. The sera from pregnant women showed a still much higher ratio of B/U as compared with the sera from the non-pregnant women.

3. *Effect of cortisol*

Saturation of the CBG binding sites with cortisol produced a striking change in the distribution of the radioactivity on the eluate fractions. In female sera, the addition of [4-$^{14}$C]-cortisol in a concentration of 25 $\mu$g per 100 ml led to a displacement of testosterone from CBG to SBP, as shown in Fig. 3. Also in the male sera, the ratio of testosterone bound by SBP to testosterone bound by CBG was strongly increased. However, while in the female sera, the dissociable fraction increased very little (Fig. 3, lower diagram), the addition of cortisol to the male sera caused mainly a displacement of testosterone from SBG to albumin, with a resultant marked increase of the dissociable fraction (Fig. 2, lower diagram). These effects have also been verified by computer treatment of data from existing literature on concentrations and association constants of the proteins involved. Details of this treatment will be published elsewhere [12].

4. *Rat sera*

The chromatographic patterns of serum samples from rats are presented in Fig. 4. It is evident from the chromatograms that no detectable SBP is present in the sera tested. Easily dissociable binding sites were almost exclusively present in both sexes. As judged from equilibrium dialysis, a sexual difference in the ability of testosterone binding, with the highest values in the females, seemed to exist. However, the limited number of animals do not make any definite conclusions on this point justified (Table 2).

Table 2. Results of chromatography on DEAE-cellulose and equilibrium dialysis of serum samples from rats. Chromatography was carried out following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer, 0·0125 M, pH 6·3. Prior to chromatography, the dialyzed male and female sera were incubated with 625 and 62·5 ng respectively of [1,2-$^{3}$H]-testosterone per 100 ml. B/U: Ratio of bound to unbound testosterone as determined by dialysis

| Sex | Per cent dissociated on the column | B/U | *n* |
|---|---|---|---|
| Males | 95·8 ± 2·3 | 2·44 ± 0·42 | 3 |
| Females | 92·3 ± 3·1 | 4·22 ± 0·32 | 3 |

5. *Sera from guinea pigs*

Typical chromatograms obtained with serum samples from guinea pigs are shown in Fig. 5. The upper left part of the figure presents the elution profile of a male serum showing the presence mainly of dissociated testosterone. The elution pattern of a female serum sample (upper right diagram) shows a considerable amount of dissociated testosterone, but also the elution of testosterone-protein complexes in both the $\beta$-globulin region (fractions 14–16) and the albumin-$\alpha$-

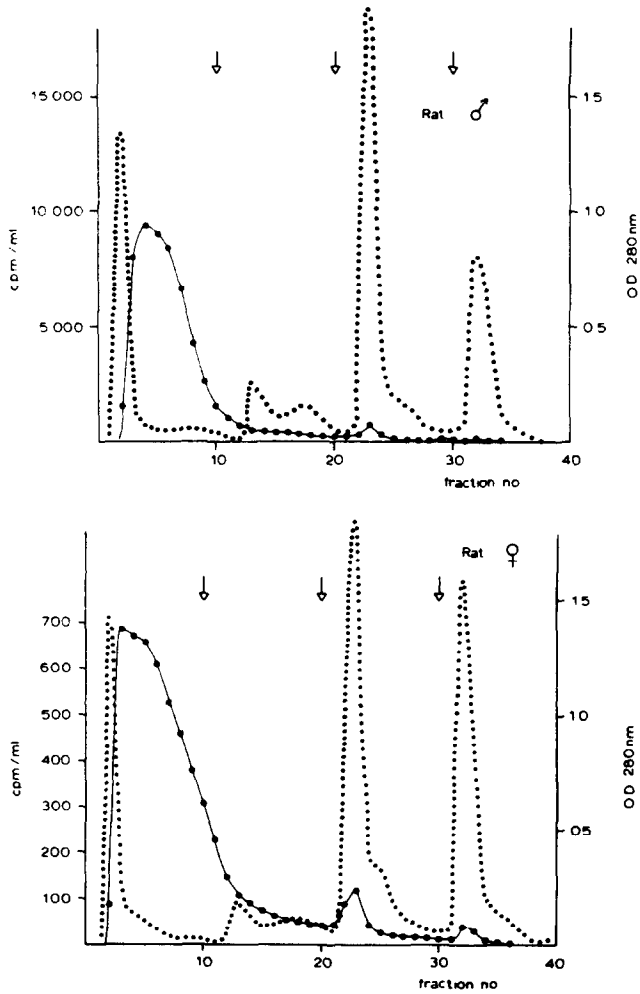


Fig. 4. DEAE-cellulose chromatography of 1 ml samples of rat sera following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer, 0·0125 M, pH 6·3. Male (upper diagram) and female (lower diagram) sera were incubated with 625 and 62·5 ng [1,2-$^3$H]-testosterone per 100 ml respectively. Dotted lines: O.D., 280 nm. Arrows mark changes in phosphate buffer for elution.

globulin region (fractions 22–25). This sexual difference is, however, apparently due to the higher testosterone concentration which was used in the male samples. As can be seen from the lower part of the figure, a lowering of the testosterone concentration to the same level as in the female sera results in an elution pattern comparable to that of the female sera.

The elution pattern of a sample from a pregnant guinea pig is shown in the lower right diagram. This pattern differs fundamentally from that of the non-pregnant animals, the most striking difference being the small dissociable fraction and the elution of two peaks in fractions 23–25 and 33–35 respectively. These two peaks do not necessarily imply the elution of two different proteins, as some overlapping is known to occur between the last two protein fractions [10].

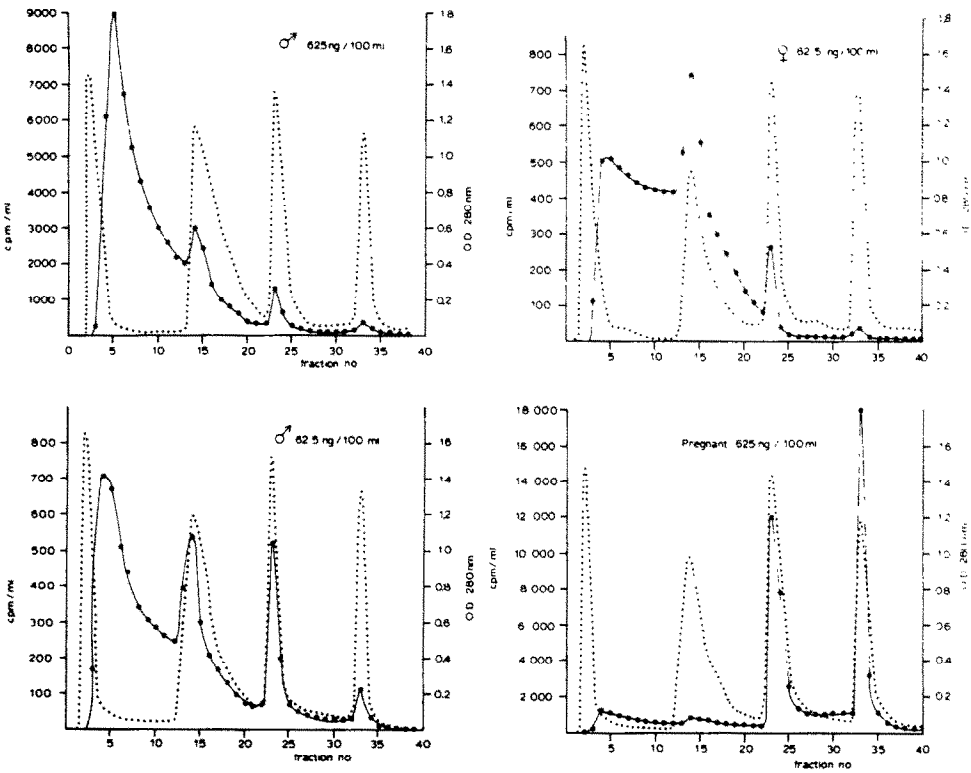


Fig. 5. DEAE-cellulose chromatography of 1 ml samples of guinea pig sera following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer, 0·0125 M, pH 6·3. Prior to chromatography, the sera were incubated with [1,2-$^3$H]-testosterone in concentrations stated at each diagram. Dotted lines: O.D., 280 nm.

A summary of the results obtained with sera from guinea pigs is presented in Table 3. A marked sexual difference was found in the amount of testosterone dissociated prior to the elution of the $\beta$-globulin fraction. As mentioned, this difference may, however, be attributed to the higher testosterone concentrations used for preincubation of the male sera. The binding parameters obtained by equilibrium dialysis did not reveal any sexual difference. In the sera from pregnant animals, a marked increase in the ratio of bound to unbound testosterone was found when compared with the non-pregnant animals. As already mentioned, the dissociable fractions of the sera from pregnant animals were relatively small (mean value: 12·6 per cent as against 45·6 per cent in the non-pregnant females).

6. *Properties of the testosterone-binding protein(s) in the pregnant guinea pig*

A preliminary investigation of the testosterone-binding protein(s) isolated from pregnant guinea pig serum has been carried out. The protein fraction appeared to be heterogenous upon DEAE-cellulose and DEAE-Sephadex chromatography. On isoelectric focusing two poorly resolved main components were found at pH 4·4 and 3·5 respectively. Only one binding component with a mobility corresponding to $\alpha_1$-globulin was observed by paper electrophoresis. The testosterone-protein complex also appeared to be homogenous upon gel filtration on Sephadex G-150 and G-200, giving an elution volume corresponding to a

Table 3. Results of chromatography on DEAE-cellulose and equilibrium dialysis of serum samples from guinea pigs. Chromatography was carried out following charcoal adsorption of endogenous steroids and dialysis against phosphate buffer, 0·0125 M, pH 6·3. Prior to chromatography, the dialyzed sera were incubated with [1,2-$^3$H]-testosterone. B/U: Ratio of bound to unbound testosterone as determined by dialysis

| Sex | [1,2-$^3$H]-testosterone concentration in pre-incubation (ng/100 ml) | Per cent dissociated on column | | | B/U | | |
|---|---|---|---|---|---|---|---|
| | | Mean | S.D. | *n* | Mean | S.D. | *n* |
| Males | 625 | 72·9 | 7·6 | 6 | 2·04 | 0·48 | 13 |
| Females | 62·5 | 45·6 | 8·1 | 4 | 2·78 | 0·81 | 5 |
| Pregnants | 625 | 12·6 | 2·9 | 6 | 58·9 | 21·6 | 5 |

molecular weight of 180,000. A Scatchard plot of data obtained from dialysis at 4·0°C of a 1 per cent serum dilution against increasing amounts of testosterone is shown in Fig. 6. The resulting curve could, by graphical analysis, be resolved into two straight lines, giving rise to two sets of binding sites. The one set gives an association constant of $4{\cdot}6 \times 10^8\ M^{-1}$ and a concentration of binding sites of $2{\cdot}6 \times 10^{-6}$ M, the other set an association constant of $3{\cdot}7 \times 10^7\ M^{-1}$ and a binding site concentration of $1{\cdot}7 \times 10^{-5}$ M. Oestradiol and cortisol did not seem to affect the binding of testosterone to these binding sites.

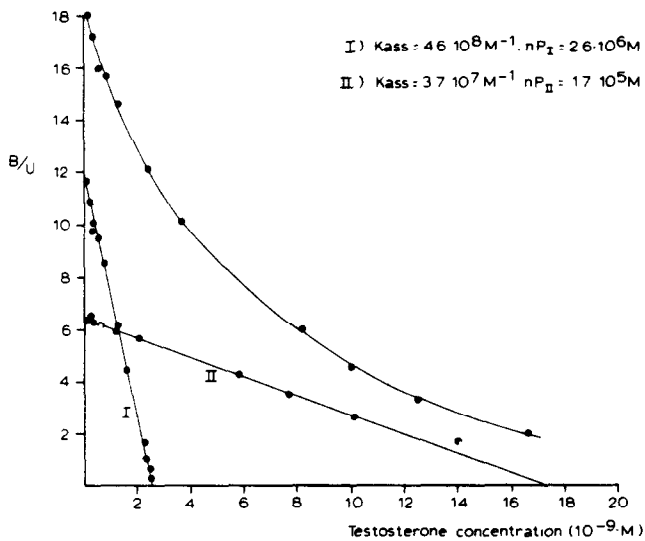


Fig. 6. Scatchard plot of data obtained from dialysis of a 1·0 per cent dilution of pregnant guinea pig serum against increasing amounts of testosterone (upper curve). This curve has been resolved into two straight lines (I and II) by graphical analysis. Temperature: 4·0°C.

## DISCUSSION

In human male sera, the distribution of testosterone on binding proteins of different affinity and dissociability is influenced by the cortisol concentration. Provided testosterone bound to easily-dissociable binding sites is biologically more active than testosterone bound in high affinity, low-dissociability complexes;

then a function of SBP and CBG in male human serum could be to synchronize the biological activity of testosterone with the cortisol concentration. In women, pregnant or non-pregnant, testosterone is mainly bound to high-affinity binding sites, possibly resulting in a deactivation of the testosterone present in circulating plasma.

In the rat, testosterone was found to be bound almost exclusively to easily-dissociable binding sites, in spite of the fact that rat serum is known to contain CBG[13]. In guinea pig sera, too, at least one half of the added testosterone was found to be associated with easily-dissociable binding sites. The presence of saturable binding sites with comparatively low dissociability was demonstrated in both sexes of these animals, but from a quantitative point of view these high-affinity binding proteins apparently are rather unimportant.

The state of protein binding observed in the sera from pregnant guinea pigs seems to be in accordance with the concept of a highly efficient biological deactivation of circulating testosterone.

## REFERENCES

1. W. Pearlman, O. Crepy and J. H. Tou: *J. biol. Chem.* **244** (1969) 1373.
2. H. Van Baelen, W. Heyns and P. De Moor: *Ann. Endocr.* (Paris) **30** (1969) 199.
3. C. Mercier-Bodard, A. Alfsen and E. E. Baulieu: *Acta endocr. (Kbh.)* **64, Suppl. 147** (1970) 204.
4. R. P. Doe and U. S. Seal: *J. clin. Invest.* **42** (1963) 929.
5. M.-C. Lebeau, C. Mercier-Bodard, J. Olds, D. Bourquin, T. Brécy, J.-P. Raynaud and E. E. Baulieu: *Ann. Endocr.* (Paris) **30** (1969) 183.
6. J. Kerkay and U. Westphal: *Biochim. biophys. Acta* **170** (1968) 324.
7. B. E. P. Murphy: *Can. J. Biochem.* **46** (1968) 299.
8. W. Heyns, H. Van Baelen and P. De Moor: *Clin. chim. Acta* **18** (1967) 361.
9. B. E. P. Murphy: *J. clin. Endocr.* **27** (1967) 973.
10. H. A. Sober and E. A. Peterson: *Fed. Proc.* **17** (1958) 1116.
11. J. L. Guerigian and W. H. Pearlman: *J. biol. Chem.* **243** (1968) 5226.
12. O. A. Lea and K. F. Støa: Manuscript in preparation.
13. G. J. Chader and U. Westphal: *Biochemistry* **7** (1968) 4272.

## DISCUSSION

**Munck:** Could you explain a little more what you meant by the physiological effects of these binding proteins being synchronized?

**Lea:** An increase in the cortisol concentration in human male blood displaced testosterone from CBG onto the albumin, and albumin-bound testosterone may be biologically more active than testosterone bound to CBG, so if you look at the diurnal variation, for instance of cortisol, you will perhaps find that when the cortisol concentration is at its highest you also have the highest concentration of testosterone bound to albumin.

**Munck:** But wouldn't you expect that the testosterone that's bound to the albumin, as long as it's bound, is as inactive as any other bound form of testosterone? Are you assuming that there is a bound form which is active? The general assumption is that the only kind that's active is free, and that it is the free form that is regulated by feedback, regardless of how much is bound.

**Lea:** Yes, but the extent to which you can determine the biological activity of free testosterone separately from a protein-bound testosterone depends on whether the dissociation of the protein-testosterone complex is the rate-determining step in your determination of the biological activity, and I think that testosterone bound to albumin is so rapidly dissociated that you cannot distinguish it from free testosterone in most systems.

**Rosner:** A comment and a question: In answer to Dr. Munck's question I think that many people who work in this field feel that the assumptions about the activity of bound versus free steroid are still open to some question. It is still not clear to me that it is only the unbound hormone that is active in all target tissues. I will present some evidence tomorrow that in certain *in vivo* systems, binding doesn't affect hormone action. My question is: Westphal has recently described a progesterone-binding protein in guinea pig plasma, and I wonder if you looked at progesterone competition with the testosterone-binding protein in the pregnant guinea pig?

**Lea:** I am aware of that, but we haven't looked at the competitive effect of progesterone; however, we have observed that progesterone is bound to the same fractions in our DEAE-cellulose chromatography. So it is possible that they are the same.