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A NEW METHOD FOR THE DETERMINATION OF THE BINDING CAPACITY OF TESTOSTERONE–ESTRADIOL-BINDING-GLOBULIN IN HUMAN PLASMA

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SUMMARY

A new method for the quantitative determination of the testosterone–estradiol-binding-globulin, TeBG, in human plasma is described. The concentration of the globulin is measured in terms of the specific binding capacity in plasma for 5α-dihydrotestosterone, DHT. The method is based on equilibrium partition in an aqueous two-phase system containing 10% (w/w) dextran ($M_w = 4 \times 10^5$), 7% (w/w) poly-(ethylene glycol) ($M_n = 6 \times 10^3$), 0·1 M KSCN in 0·005 M phosphate buffer. In this two-phase system more than 99% of the total plasma proteins partition into the lower phase and the partition coefficients for DHT and testosterone are 1·66 and 1·73 respectively. The concentration of the bound and unbound DHT or testosterone in equilibrium with the plasma proteins was determined from the concentration of the steroid in the upper phase. The method is rapid and simple and requires only small quantities of plasma. In contrast to those for testosterone, the results for the binding of DHT are not affected by the presence of transcortin and endogenous steroids in plasma. The intrinsic association constant for the binding of DHT and testosterone to TeBG and the apparent association constant for the binding to albumin were determined from Scatchard-type binding plots. The constants for DHT were $2{\cdot}2 \times 10^9$ L mol^{-1} for TeBG and $8{\cdot}6 \times 10^4$ L mol^{-1} for albumin. The affinity of TeBG for DHT was found to be about 2·4 times that for testosterone. The specific binding capacity values obtained, expressed as μg DHT bound/100 ml plasma, were: men, 1·76, women: 3·00; pregnant women, 12·7.

INTRODUCTION

THE EXISTENCE in human plasma of proteins that specifically bind certain steroids is well established [1, 2]. A number of methods have been used for the determination of steroid protein interactions as well as the specific steroid-binding capacity of a given plasma sample. The methods used have been those that are generally accepted for the determination of macromolecule-ligand binding, namely equilibrium dialysis [3], gel filtration [4], ultrafiltration [5], gel electrophoresis [6], precipitation [7] and adsorption [8].

These methods are either time consuming (dialysis), or require a large amount of plasma sample (steady state gel filtration). The use of precipitation or adsorption involves a risk since the binding characteristics may not be the same as in solution. Ultrafiltration methods yield much higher binding capacity values than dialysis [5]. In general, accurate binding data will be obtained only when the protein–steroid complex is in equilibrium with unbound steroid as in the dialysis method. The specific steroid-binding activity in plasma has been shown to vary under certain physiological and pathological conditions and this is probably due to a corresponding variation in the level of specific binding proteins in plasma [9]. A rapid, simple and accurate method is desirable when a quantitative assay of the

specific steroid-binding capacity is to be carried out in a large number of samples. The present work concerns the development of a method based on equilibrium partition between two aqueous phases in the type of two-phase systems developed by Albertsson *et al.*[10]. These aqueous two-phase systems are obtained by mixing concentrated aqueous solutions of two polymers, mostly dextran and poly(ethylene glycol). Since the water content of each of the phases is high, generally 85–90% (w/w), the solubility of the proteins is sufficiently high to offset any deleterious effects on the stability of the proteins, in contrast to the situation when protein–steroid interaction has been studied by partition between aqueous and organic phases[11]. Furthermore, partition equilibrium in such aqueous two-phase systems has been used successfully for investigating the binding of cytidine triphosphate to aspartate transcarbamylase[12]. The principle of the equilibrium partition method is similar to equilibrium dialysis provided the binding protein (or proteins) is almost quantitatively partitioned into one of the phases. In the type of phase systems used in this investigation, the partition of proteins between the phases is a function of several factors[10]. We were able to compose a system where the concentration of the plasma proteins in the lower phase was more than 99% of their total concentration in the plasma. In this system the steroids partitioned slightly in favour of the upper phase.

We have investigated the binding of 5α-dihydrotestosterone, DHT, and testosterone to testosterone–estradiol-binding globulin (TeBG). The affinity of TeBG is higher for DHT than for either testosterone or estradiol[12]. Therefore a method for the determination of TeBG, based on the binding of DHT to TeBG, should yield more accurate results on the basis of binding capacity.

EXPERIMENTAL

Equilibrium partition method

The theory of this method is thoroughly discussed in the literature[12] and will be presented here only briefly. In principle, the method involves the determination of the partition coefficient, K_s, of the steroid in the required two-phase system in the absence of the protein

$$K_s = \frac{[S]_u}{[S]_l} \tag{1}$$

where $[S]_u$ and $[S]_l$ are the concentrations of the steroid in the upper and lower phases, respectively. When the steroid is partitioned in the presence of proteins having affinity for the particular steroid and if, as in the present work, the proteins are almost quantitatively partitioned into the lower phase, then

$$[S_t]_l = [S_b]_l + [S_f]_l \tag{2}$$

where $[S_t]_l$ is the total concentration of the steroid in the lower phase, while $[S_b]_l$ and $[S_f]_l$ are the respective concentrations of the steroid bound to the proteins and that which is unbound (free). If the partition of free steroid is independent of the presence of the proteins in the system, then from equation (1):

$$[S_f]_l = [S_f]_u / K_s \tag{3}$$

where $[S_f]_u$ is the concentration of the steroid in the upper phase which, for all practical purposes, can be considered to be free from binding proteins. Thus by determining concentrations of the steroid in the upper and lower phases, and knowing the value K_s, it is possible to determine the concentration of the bound, $[S_b]_l$, and unbound, $[S_f]_l$, steroid in equilibrium with a certain concentration of binding proteins in the lower phase.

Determination of specific binding capacity

The specific binding capacity is a measure of the concentration of the binding protein and can be calculated according to the method of Guériguian and Crépy[13]. Thus, in the present work, where DHT is bound specifically to TeBG and non-specifically to albumin, $[S_b]_l$ in equation (2) can be written as

$$[S_b]_l = [S_{b1}]_l + [S_{b2}]_l \tag{4}$$

where $[S_{b1}]_l$ and $[S_{b2}]_l$ are the respective concentrations of steroid bound to TeBG and albumin. If the set of binding sites on each of the proteins is a set of independent but equivalent sites, then[14],

$$[S_b]_l = \frac{n_1 k_1 [P_T]_l [S_f]_l}{1 + k_1 [S_f]_l} + \frac{n_2 k_2 [P_A]_l [S_f]_l}{1 + k_2 [S_f]_l} \tag{5}$$

where n_1 and n_2 are the number of binding sites on TeBG and albumin, respectively; k_1 and k_2 are the respective intrinsic association constants; and $[P_T]_l$ and $[P_A]_l$ are the concentrations of TeBG and albumin, respectively. Since $[S_f]_l$ (usually 10^{-9} mol L^{-1}) and k_2 (less than 10^5 L mol^{-1}) are small quantities, $1 + k_2[S_f]_l \simeq 1$. Substituting this in equation (5) and simplifying, one finally obtains the following expression[13] for the specific binding capacity, $[P_T]_l n_1$, of TeBG

$$[P_T]_l n_1 = \left\{ \frac{[S_b]_l}{[S_f]_l} - n_2 k_2 [P_A]_l \right\} \left([S_f]_l + \frac{1}{k_1} \right). \tag{6}$$

One can therefore determine $[P_T]_l n_1$ from a single partition equilibrium experiment, provided the concentration of albumin, $[P_A]_l$, in the lower phase is known and if the values of $n_2 k_2$ and k_1, previously determined from binding experiments, are assumed to be constant for albumin and TeBG in all plasma samples.

Materials

Reagents: [1,2-^{3}H]-DHT (SA 44 Ci/mmol) and [1,2-^{3}H]-testosterone (SA 51 Ci/mmol) purchased from New England Nuclear were purified by paper chromatography using the Bush B 3 system[15]. Unlabelled DHT, testosterone, cortisol and human serum albumin were purchased from Sigma Chemicals. Dextran T40 (DT40) ($M_w = 4 \times 10^5$) was purchased from Pharmacia Fine Chemicals, Sweden, and poly(ethylene glycol) (PEG) grade Carbowax 6000 (Mn = 6000) was purchased from Union Carbide, U.S.A. The salts used were of analytical grade and the water was double distilled in quartz apparatus.

Plasma. Blood samples were obtained from seven subjects, two normal men, two normal young women on the eighteenth day of the menstrual cycle and three pregnant women in the eighth month of pregnancy. Plasma samples were obtained

by venepuncture and collected in heparinized plastic centrifuge tubes. These were centrifuged at 11,500 rev./min for 20 min at 4°C in a Sorvall RC 2-B centrifuge within 30 min of the collection. The plasma samples were stored at −20°C or colder until used.

Experimental procedures

Partition coefficient of plasma proteins: Preliminary experiments on the partition of plasma proteins in the aqueous two-phase systems showed that in systems containing 10% (w/w) DT40, 7% (w/w) PEG and rather low concentration of salt, as described below, in a Na_2HPO_4–KH_2PO_4 buffer at pH 7·3, the proteins partitioned into the lower phase and also the solubility of the proteins in these systems was sufficiently high. The partition coefficient, K_P, of the plasma proteins is given by

$$K_P = [P]_u/[P]_l$$

where $[P]_u$ and $[P]_l$ are the concentrations of total protein in the upper and lower phases, respectively. $[P]_u$ and $[P]_l$ were determined using a modification [16] of the Lowry method [17]. The modification is necessary since dextran and PEG interfere with the determination of proteins by the original method.

The value of K_P was determined in several two-phase systems containing 10% (w/w) DT40 and 7% (w/w) PEG but with different concentrations (0·01–0·2 M) of sodium or potassium halides or KSCN and 0·01 or 0·005 M phosphate buffer at pH 7·3. The concentration of plasma was 0·025 g per 5 g of the system. The value of K_P was lowest in the system containing 10% (w/w) DT40, 7% (w/w) PEG, 0·1 M KSCN and 0·005 M phosphate buffer (pH 7·3). K_P in this phase system was 0·0025 at 4° and 0·014 at 23°C. Gel electrophoresis also showed the absence of proteins in the upper phase. The binding studies were carried out in this system at 4°C, because in addition to the almost complete partition of proteins in the lower phase, the stability of the proteins can be expected to be higher at this temperature. Partition of the plasma proteins in this phase system at 4°C was determined as a function of the concentration of plasma in the system and the results are shown in Fig. 1. In the concentration range investigated, K_P was independent of the concentration of the plasma in the system. In phase systems where 0·1 M KCl and KI were used instead of KSCN, the values of K_P at 23°C were almost of the same order, being 0·017 and 0·012 respectively, but the solubility of the proteins was slightly lower.

Partitioning of steroids. Samples containing known activity, between 20,000 and 40,000 d.p.m. of the labelled steroid and varying amounts of between 1·0 and

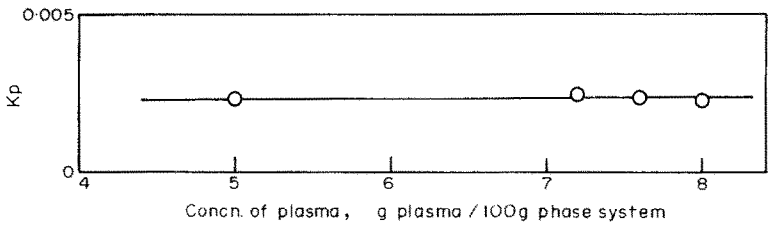

Fig. 1. Overall partition coefficient of plasma proteins as a function of the protein concentration in the two-phase system.

100 ng unlabelled steroid in ethanol, were added to a series of glass tubes and the solvent was evaporated on a water bath at 40°C under a stream of nitrogen. A phase system was prepared by mixing required amounts by weight of 20% (w/w) solution of DT40, 40% (w/w) solution of PEG, 2·0 M KSCN, 0·1 M phosphate buffer and water, so that the final composition was 10% (w/w) DT40, 7% (w/w) PEG, 0·1 M KSCN and 0·005 M phosphate. This mixture was cooled to 4°C, shaken well, and 5 g samples were delivered with a calibrated Cornwall automatic syringe into the tubes containing DHT. The tubes were closed with neoprene stoppers. The contents of tubes were mixed by inverting them about 50 times. The tubes were centrifuged at 2000 *g* for 15 min to speed up the separation of the phases. A 1·0 ml sample was withdrawn from the upper phase, with a disposable tuberculin syringe to which a glass capillary had been fused, and delivered into a polyethylene scintillation vial containing 15 ml of the scintillation mixture. The rest of the upper phase plus the interface was sucked off with a Pasteur pipette attached to a water pump, and 0·6 ml of the lower phase was then transferred in the same way as for the upper phase into a scintillation vial containing 15 ml scintillation mixture. The composition of the scintillation mixture was 5% PPO plus 0·3% dimethyl-POPOP in toluene. The vials were vigorously shaken and left at 4–8°C for 10 h before being transferred to the counting apparatus. Radioactivity was determined by counting each sample for 10 min in a Packard Tricarb model 3310 spectrometer. The counting efficiency determined from the external standard was about 33%. The ratio between the values of d.p.m./ml in the upper and lower phases is equal to the partition coefficient, K_s, of the steroid partitioned (equation 1). The values of K_s obtained for DHT and testosterone were $1{\cdot}66 \pm 0{\cdot}05$ and $1{\cdot}73 \pm 0{\cdot}04$ respectively. The values of K_s were independent of the total concentration of steroids in the system (see Table 1). For the calculation of bound and unbound DHT and testosterone, from the data obtained in binding experiments, the respective mean values of K_s have been used.

Experimental procedure for the determination of binding

The procedure was similar in most respects to that used for the determination of partition coefficients of DHT and testosterone. In binding studies, the steroid sample included, in addition to labelled and unlabelled DHT or testosterone, 0·6 μg of cortisol to block the binding sites on transcortin. After evaporation of the solvent under N_2, 300 μl vol of plasma were added to each tube containing the steroid mixture and incubated for 30 min at 4°C. A phase mixture was prepared by mixing the DT40, PEG, KSCN and phosphate buffer solutions in the same proportions as before but in this instance the weight of water added was 0·3 g less than that required to obtain 5 g of the two-phase system. Since 300 μl of plasma were added to each tube in the binding experiment, the final composition of the system with respect to DT40, PEG, KSCN and phosphate buffer would be the same as before. This phase mixture was cooled and mixed as before and 4·7 g added to each tube containing the incubated mixture of plasma and steroids. The rest of the procedure was identical except that 1·0 ml instead of 0·6 ml samples were withdrawn from the lower phase for the determination of radioactivity and also 0·5 ml H_2O_2 (30%) was added to the scintillation mixture to improve the counting efficiency. This was necessary since the presence of plasma in the samples from the lower phase decreased counting efficiency. The addition of H_2O_2 gave about 95% recovery of the total radioactivity added to the system, when

Table 1. The values of the partition coefficient, K_s, of DHT and testosterone at different concentrations of DHT in the two-phase system*

Steroid	Amount of labelled steroid in the system d.p.m./g system	Amount unlabelled steroid in the system ng	Partition coefficient K_s	Per cent recovery of radioactivity†
DHT	3954	0	1·64	98
	8023	0·1	1·63	99
	8023	1·0	1·67	102
	8023	10·0	1·66	101
	7329	100	1·71	104
	8023	100	1·69	101
	4015	300	1·61	102
Testo-	7329	0	1·73	96
sterone	7898	1	1·71	102
	7228	10	1·77	107
	7329	100	1·71	104
	7329	1000	1·73	104

*The total weight of the system in all experiments was 5 g. See text for experimental details.

†The experimentally determined total radioactivity in the system expressed as per cent of that initially added to the system.

calculated from experimentally determined values for the upper and lower phases. We have, however, used the experimentally determined radioactivity in the upper phase for calculating the concentration of the bound and unbound DHT (or testosterone) in the lower phase.

Thus from equations (2) and (3):

$$[S_b]_l/[S_f]_l = ([S_t]_l/[S_f]_l) - 1$$

or

$$[S_b]_l/[S_f]_l = (K_s[S_t]_l/[S_f]_u) - 1 \tag{7}$$

Since, $[S_t]_l/[S_t]_u = C_l/C_u$, where C_l and C_u are d.p.m./ml in the lower and upper phases respectively,

$$[S_b]_l/[S_f]_l = (K_sC_l/C_u) - 1 \tag{8}$$

C_l is related to the total number of d.p.m., C_t, added to the system and C_u by the expression

$$C_l = (C_t - C_uV_u)/V_l \tag{9}$$

where V_u and V_l are the volumes of the upper and the lower phases respectively. Hence,

$$[S_b]_l/[S_f]_l = \{K_s(C_t - C_uV_u)/V_lC_u\} - 1 \tag{10}$$

Similarly, $[S_b]_1$ can be calculated using the expression

$$[S_b]_1 = (S/C_tV_1)\{[(C_t - C_uV_u)/V_1] - C_u/K_s\} \times 10^3 \quad (11)$$

where S is the moles unlabelled steroid added to the system.

The values of $[S_b]_1/[S_f]_1$ and $[S_b]_1$ plotted in the binding curves have been calculated using equations (10) and (11). The possible effect of transcortin on the binding of DHT or testosterone to TeBG was investigated by performing the binding experiments in the absence of added cortisol.

To ascertain whether the upper phase contained any binding proteins, the upper phase from a system containing only plasma but no steroids was transferred to a tube containing the lower phase of a system containing DHT but prepared by substituting water for plasma. The partition of [^{3}H]-DHT in the phase system so obtained was determined as usual. The plasmas used in these experiments were male and heat-treated pregnancy plasma.

The effect of endogenous steroids on the binding of DHT to TeBG was also investigated by using plasma from which these steroids had been removed by treatment with activated charcoal[18]. Endogenous steroids levels were determined using competitive protein binding methods after paper chromatography[19].

The results reported here for the binding of testosterone are from experiments performed with plasma subjected to charcoal treatment.

The binding of DHT and testosterone to albumin was studied using plasma heated at 60°C for 20 min. This treatment inactivates the specific binding proteins such as TeBG and transcortin but not albumin[8]. The concentration of albumin was determined by the method of Rodkey[20]. The standard curve was obtained using human serum albumin. The measurements on duplicate samples from the same plasma agreed to within ±1·5% of the mean.

Determination of the specific binding capacity of TeBG. In these experiments the total weight of the phase system was 2·5 g instead of 5 g as in the experiments for determination of binding constants, since it was realised that reduction in the amount of the system used did not affect the accuracy of the results. It is an advantage to be able to use smaller samples of plasma and steroids. In this case, therefore, the steroid mixture consisted of 24,000 d.p.m. [1,2-^{3}H]-DHT, 2 ng of unlabelled DHT and 0·2 μg of cortisol. The amount of plasma used was 100 μl and the weight of phase mixture added was 2·4 g. The samples withdrawn for determination of radioactivity were 1·0 ml from the upper phase and 0·5 from the lower phase. The rest of the procedure was identical to that used in the experiments for the determination of the binding curve. From the values $[S_b]_1/[S_f]_1$ and the values of k_1 for TeBG, k_2n_2 for albumin and the concentration $[P_A]_1$ for albumin the specific binding capacity of TeBG in plasma can be determined from equation (6).

The effect of concentration of plasma on the binding capacity of TeBG was studied using plasma samples diluted up to 15 times with water.

RESULTS

The partition coefficient, K_s, for DHT and testosterone (Table 1) and the partition coefficient, K_P, of plasma proteins (Fig. 1) are independent of their respective concentrations in the phase systems within a sufficiently wide range.

Upper phase from systems containing male plasma or heat-treated pregnancy plasma had no effect on the partition coefficient of [^{3}H]-DHT when added to the lower phase of a system containing only steroids but no plasma (Table 2). Therefore no complications are involved in the analysis of the data obtained from partition equilibrium binding experiments.

The concentrations of bound and unbound DHT or testosterone in equilibrium with the binding proteins are $[S_b]_1$ and $[S_f]_1$, respectively.

A Scatchard-type plot [21], where the ratio of the concentrations of bound and unbound steroid is plotted as a function of bound steroid, is useful in analysing the binding data, when the concentration of binding protein is not known. The plots of $[S_b]_1/[S_f]_1$ vs $[S_b]_1$ are shown in Figs. 2–5. These plots would be linear if a single set of equivalent and independent sites were available for the binding of the steroids, as was the case when binding was determined using heat-treated plasma (line 2, Fig. 5). The plots for the binding of these steroids to untreated plasma indicate the presence of at least two sets of binding sites.

The results presented in Fig. 2 show that for DHT, the binding curve (curve 1) obtained in the presence of cortisol in the system was almost identical to the binding curve (curve 2) obtained in the absence of this steroid, indicating that transcortin apparently does not compete with TeBG in the binding of DHT. In

Table 2. Mean partition coefficient of [^{3}H]-DHT in the two-phase system obtained by combining the upper phase from a system containing plasma with the lower phase from a system containing DHT

	Mean	S.D.	n	P *v* Control
Control	1·60	0·098	4	
Male plasma	1·54	0·067	4	$>0·3$
Heat-treated pregnancy plasma	1·54	0·035	4	$>0·2$

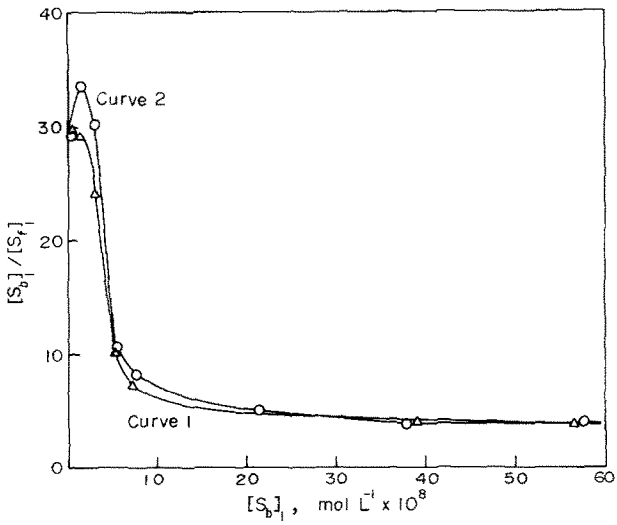

Fig. 2. Scatchard plots for the binding of DHT to plasma proteins in presence and absence of added cortisol. Curve 1, —△— binding in presence of cortisol; curve 2, —○—, binding in absence of cortisol. Plasma from pregnant woman.

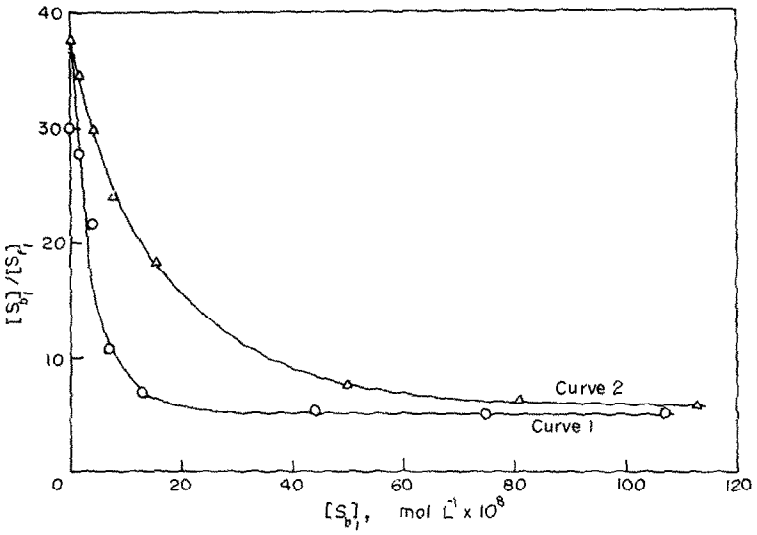

Fig. 3. Scatchard plot for the binding of testosterone to plasma proteins in presence and absence of added cortisol. Curve 1, —○—, cortisol added; curve 2, —△—, no cortisol added. Plasma from pregnant woman.

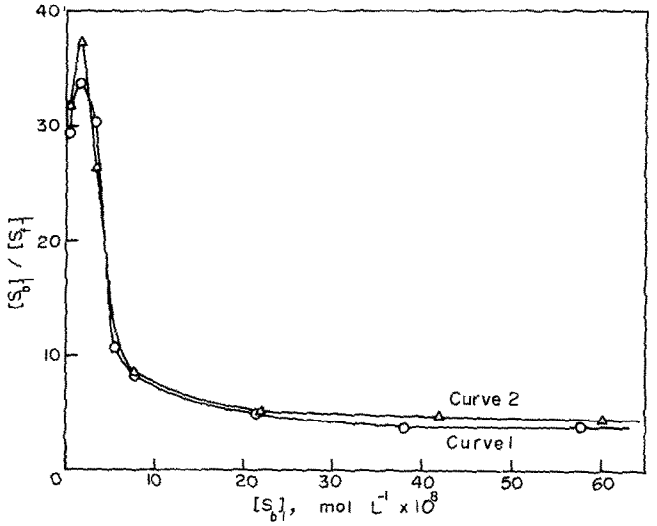

Fig. 4. Scatchard plots for the binding of DHT to plasma proteins in presence and absence of endogenous steroids. Curve 1, —○—, binding data in absence of endogenous steroids; curve 2, —△—, binding data in presence of endogenous steroid. Plasma from pregnant woman.

other words the affinity of TeBG for DHT is much higher than that of transcortin for DHT. It appears, therefore, that it is not necessary to block the binding sites on transcortin with cortisol in the determination of binding of DHT to TeBG. This was in contrast to the experiments in which testosterone was used as ligand (Fig. 3). We have, however, used cortisol in all binding experiments in order to eliminate any doubts about the accuracy of binding parameters.

In Fig. 4, we have compared the binding data obtained for DHT in the presence

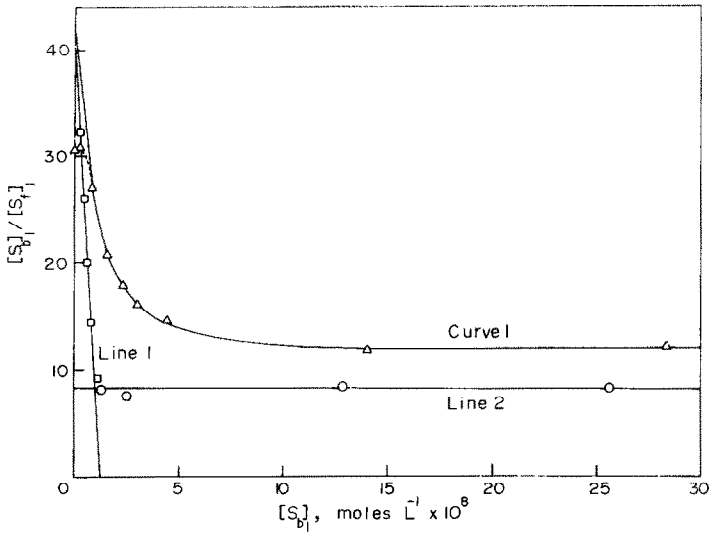

Fig. 5. Scatchard plots for the binding of DHT to: (a) Untreated plasma, Curve 1, —△—; (b) heat-treated plasma, line 2, —○—; (c) to TeBG, line 1, —□—. This binding isotherm was calculated by subtracting co-ordinates of line 2, from the co-ordinates of curve 1 by the method of Rosenthal. Plasma from normal woman.

and absence of endogenous steroids. The curve obtained with plasma stripped of endogenous steroids (curve 1) was similar to that obtained with untreated plasma (curve 2), showing that endogenous steroids do not compete with binding of DHT. This also confirms the high affinity of DHT towards TeBG. The association constants for the binding of DHT to TeBG and albumin were obtained by the graphical method suggested by Rosenthal[22] as illustrated in Fig. 5. The total binding curve in Fig. 5 can be described by equation (12) below, derived from equation (5)

$$\frac{[S_b]_1}{[S_f]_1} = \frac{n_1 k_1 [P_T]_1}{1 + k_1 [S_f]_1} + \frac{n_2 k_2 [P_A]_1}{1 + k_1 [S_f]_1} \tag{12}$$

The first and the second term on the right represent the binding to TeBG and albumin, respectively. The straight line (2) in Fig. 5 represents the binding data obtained using heat-treated plasma, in which only albumin has binding activity. The straight line (1) in Fig. 5 is obtained by subtraction of the co-ordinates of line (2) from those of total curve (curve 1) by the method of Rosenthal. Lines (1) and (2) are described by equations (13) and (14) given below

$$\frac{[S_b]_1}{[S_f]_1} = k_1 n_1 [P_T]_1 - k_1 [S_f]_1 \tag{13}$$

$$\frac{[S_b]_1}{[S_f]_1} = k_2 n_2 [P_A]_1 - k_2 [S_f]_1 \tag{14}$$

Thus the intercept on the ordinate is equal to kn and that on the abscissa is equal to $n[P]$ and the ratio of the intercepts gives k. The intrinsic association constant, k_1, for the binding of DHT to TeBG was calculated from the intercepts of line 1 on

the ordinate and on the abscissa. The values of k_1 obtained in the present work are given in Table 3 which also includes the values of this constant reported by other workers.

The slope of the line (line 2, Fig. 5) representing binding of DHT to albumin is very small, indicating that the association constant, k_2, in this case is very low and cannot be determined with accuracy. Furthermore, since the non-specific binding capacity $n_2[P_A]_1$ of albumin is large, it is not possible to obtain an accurate value for the intercept on the abscissa. However, from a knowledge of the concentration of albumin, $[P_A]_1$, in the lower phase, we can calculate only the effective association constant, n_2k_2, for albumin from the intercept $n_2k_2[P_A]_1$ on the ordinate. This is the usual way of expressing the constant for the binding to albumin. The values of n_2k_2 are also included in Table 2.

The same graphical procedure was used to determine association constants for the binding of testosterone to TeBG and albumin. These results are also included in Table 3 and compared with those reported in the literature.

The binding capacity of TeBG in plasma at various dilutions is shown in Fig. 6 in which the binding capacity in moles L^{-1} is plotted as a function of the amount of plasma added to the system. The binding capacity was calculated using equation (6), with $k_1 = 2{\cdot}2 \times 10^9$ mol L^{-1} and $n_2k_2 = 8{\cdot}6 \times 10^4$ mol L^{-1}, i.e. the mean of the experimentally determined values for these constants. The binding capacity is linear with respect to dilution until the concentration of plasma becomes greater than 40 μl plasma/g system. The factors responsible for the deviation from linearity at higher concentrations of plasma are discussed later. In Table 4 we have compared the values of TeBG binding capacity obtained in the present work with those reported in the literature.

Table 3. The association constants for the binding of DHT and testosterone to TeBG and albumin. The values obtained in this work and those reported in the literature

Steroid	Temperature (°C)	TeBG $k_1 \times 10^{-9}$ (L mol^{-1})	Albumin $k_2n_2 \times 10^{-4}$ (L mol^{-1})	Method†	Source
DHT	4	1·6	9·2	PE	This work
	4	2·8	8·0	PE	This work
	25	2·4*	—	ED	Vermeulen and Verdonck[4]
	25	15	—	PPT	Heyns and De Moor[25]
Testosterone	4	0·93	5·5	PE	This work
	4	1·7	—	ED	Mercier-Bodard and Baulieu[26]
	4	—	3·0	ED	Sandberg *et al.*[27]

*Vermeulen and Verdonck have reported that the affinity of TeBG for DHT is higher by a factor of 3 as compared to its affinity for testosterone. The value given here for DHT has therefore been calculated by multiplying by the above factor the mean value of 8×10^{-8} L mol^{-1} reported by them for the binding of testosterone.

†Abbreviations are: PE, partition equilibrium; ED, equilibrium dialysis; and PPT, precipitation with ammonium sulphate.

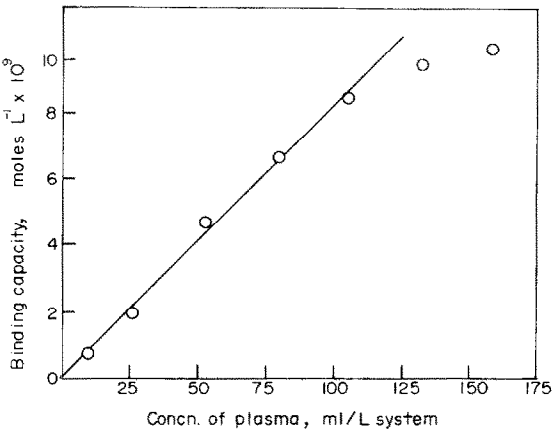

Fig. 6. Specific binding capacity of TeBG for DHT as a function of the concentration of plasma in the two-phase system. Plasma from normal man.

DISCUSSION

The factors which will determine the accuracy of the relative concentration of TeBG in terms of DHT-binding capacity by the present method, are: 1. the accuracy of the determination of bound and unbound DHT; 2. the accuracy of the determination of the binding constant for albumin; 3. the accuracy of the determination of the concentration of albumin. In the present work the concentration of bound and unbound DHT in equilibrium with the proteins has been determined from the concentration of DHT in the upper phase.

Since the proteins were almost quantitatively (more than 99·5%) partitioned into the lower phase, the concentration of DHT in the upper phase should give an accurate measure of the concentration of unbound DHT in the lower phase. Further, since the partition coefficient of DHT is independent of the concentration of DHT in the system (Table 1) it can be used with confidence for the calculation of unbound DHT in the lower phase. The percentage recovery values given in Table 1 show that no errors are likely to be introduced in the values of concentration calculated on the basis of radioactivity measurements.

The apparent association constant values, n_2k_2, for the binding of DHT and testosterone to albumin determined in the present work using heat-treated plasma show that the affinity of albumin for DHT is higher than that for testosterone by a factor of 1·55 (Table 3). The association constant for the binding of DHT to TeBG

Table 4. TeBG binding capacity expressed as μg DHT bound/100 ml plasma. Values obtained in this work and those reported in the literature

Source	Men	Women	Pregnant
This work	1·76	3·00	12·7
Vermeulen *et al.*[28]	1·36	2·13	13·0
Rosner[7]	0·93	1·85	11·9
Corvol *et al.*[6]	0·49	1·42	10·9
Heyns and De Moor[25]	1·07	1·76	12·5

is about 2·4 times that for the binding of testosterone. The difference in the affinity of TeBG for these steroids is almost of the same order as that observed by Vermeulen and Verdonck[4].

The most important evidence that this method gives accurate results for relative concentration of TeBG is that the binding capacity varies linearly with respect to the dilution of plasma (Fig. 6), provided that the concentration of plasma or, in other words, that the binding capacity in the system is not too high. This linear dependence of binding capacity on the concentration of plasma shows the absence of any spurious effects on the equilibrium between the binding proteins and DHT and also that the equilibrium partitioning of the unbound DHT in the system is independent of the concentration of the plasma in the system. The deviation from linear dependence on dilution observed at high concentrations of plasma is also reflected in the corresponding deviation observed in the binding curves at high values of the ratio of bound to unbound DHT. This effect manifests itself when the concentration of plasma in the system is high in proportion to the steroid, so that most of the steroid is bound. Under these conditions the calculations of the concentrations of bound and unbound DHT would be extremely sensitive to small errors in the determination of the unbound DHT and to the presence of trace impurities which contribute to the assay of the DHT, but have themselves a low affinity for the binding protein. Errors in determination of binding data under conditions where the proportion of bound ligand is too high or too low are thoroughly discussed by Steinhardt[23]. Hence to obtain accurate values for the binding capacity, the proportion of DHT to plasma must be such that the ratio of bound to unbound is not too high. From Fig. 6 it is seen that in plasma from a man, the binding capacity determined was accurate up to a concentration of about 40 μl plasma/g phase system when the concentration of DHT was 1 ng/g phase system. The concentration of DHT should be increased with increasing DHT binding capacity, as is the case with pregnancy plasma. Very large concentration, of DHT should be avoided since in this case DHT bound to albumin constitutes a large fraction of the bound DHT. The values for binding capacity obtained in the present work were found to be of the same order as those reported earlier from equilibrium dialysis. They were slightly higher than those based on methods of precipitation with $(NH_4)_2SO_4$, gel filtration or gel electrophoresis. The precipitation method is expected to give fairly low values if there is incomplete precipitation of the bound moiety and also if the precipitation is accompanied by any dissociation of the protein–steroid complex. In gel filtration and gel electrophoresis also, there is a possibility of dissociation of the complex[24], and consequently the binding capacity determined would be too low. In the present work, the binding is studied in solution under conditions of equilibrium between the proteins and DHT. The presence of neutral polymers such as DT40 and PEG is unlikely to affect the binding characteristics of the proteins. In any case the observed linearity of the binding capacity with respect to the concentration of plasma in the system shows that this method would yield accurate results in the determination of the relative concentration of TeBG in different plasma samples and should be useful as a routine method for the determination of changes in the TeBG level in plasma under different physiological and pathological conditions. The method of partition equilibrium is simple and rapid once a suitable two-phase system is developed. The two-phase system used in the present work could also be suitable for binding studies involving plasma proteins in general.

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REFERENCES

1. Sandberg A. A., Rosenthal H., Schneider S. L. and Slaunwhite W. R., Jr.: *Steroid Dynamics* (Edited by G. Pincus, T. Nakao and J. F. Tait). Academic Press, New York (1966) pp. 1–62.
2. Westphal U.: *Steroid–Protein Interactions.* Springer Verlag, Berlin, Heidelberg and New York (1971).
3. Schellman J. A., Lumry R. and Samuels L. T.: *J. Am. chem. Soc.* **76** (1954) 2808.
4. Vermeulen A. L. and Verdonck M.: *Steroids* **11** (1968) 609.
5. Ryan M. T. and Hanna N. S.: *Analyt. Biochem.* **40** (1971) 364.
6. Corvol P. C., Chrambach A., Rodbard D. and Bardin C. W.: *J. biol. Chem.* **246** (1971) 3435.
7. Rosner W.: *J. clin. Endocr. Metab.* **34** (1972) 983.
8. Pearlman W. H. and Crépy O.: *J. biol. Chem.* **242** (1967) 182.
9. Crépy O, and Guériguian J. L.: *Res. Steroids* **IV** (1970) 61.
10. Albertsson P-Å.: *Partition of Cell Particles and Macromolecules*, 2nd Ed. Almqvist & Wiksell, Stockholm and Wiley, New York (1971).
11. Alfsen A.: *Cr. Trav. Lab. Carlsberg* **33** (1963) 415.
12. Gray W. C. and Chamberlain M. J.: *Analyt. Biochem.* **41** (1971) 83.
13. Guériguian J. L. and Crépy O.: *C.R. Acad. Sci. Paris*, Serie D **209** (1969) 1457.
14. Tanford C.: *Adv. Protein Chem.* **17** (1962) 69.
15. Bush I. E.: *Biochem. J.* **50** (1952) 370.
16. Ref. 10. p. 320.
17. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265.
18. Heyns W., Van Baelen H. and De Moor P.: *Clinica chem. Acta.* **18** (1967) 361.
19. Carstensen H., Amér B., Amér I. and Wide L.: *J. steroid Biochem.* **4** (1973) 45.
20. Rodkey F. L.: *Clinica Chem. Acta* **11** (1965) 478.
21. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660.
22. Rosenthal H. E.: *Analyt. Biochem.* **20** (1967) 525.
23. Steinhardt J. and Reynholds J. A.: *Multiple Equilibria in Proteins.* Academic Press, New York (1969) pp. 35–38.
24. Burke C. W.: *Biochim. biophys. Acta* **176** (1969) 403.
25. Heyns W. and De Moor P.: *Steroids* **18** (1971) 709.
26. Mercier-Bodard C. and Baulieu E. E.: *C.R. Acad. Sci. (Paris)* **267** (1968) 804.
27. Sandberg A. A., Slaunwhite W. R., Jr. and Antoniades H. N.: *Recent Prog. Horm. Res.* **13** (1957) 209.
28. Vermeulen A., Verdonck L., Van der Straeten M. and Orie N.: *J. clin. Endocr. Metab.* **29** (1969) 1470.