Steroid-Protein Interaction with Particular Reference to Testosterone Binding by Human Serum

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SUMMARY

The binding affinity of serum protein for testosterone (and also of serum albumin as a reference protein) was determined by a technique based on the principle of equilibrium dialysis but with the use of the cross-linked dextran, Sephadex G-25, in a batchwise fashion on a semimicrom scale. The binding parameters were shown to be dependent on the concentrations of both protein and steroid. The logarithm of the reciprocal of the protein concentration and the logarithm of the reciprocal of the bound steroid concentration (at 50% steroid binding) were in linear relationship. A sensitive and rapid method thus evolved for the detection and quantitative estimation of serum protein, or proteins, exhibiting a specific or high binding affinity for testosterone. This new technique, entailing a novel treatment of the experimental data, may also prove to be useful in the general study of steroid-protein interaction.

Testosterone-binding levels in various human sera were determined in a comparative survey; markedly elevated levels were observed in advanced pregnancy.

METHODS

Principle of Method—The cross-linked dextran, Sephadex G-25 (6), was employed in a technique comparable in principle with that of equilibrium dialysis (7): protein, P, and steroid bound to protein, Sb, were excluded by the dextran gel and remained in the external phase; unbound steroid, Sb, partitioned freely between the external and internal phases of the semipermeable system.

The binding (or combining) affinity of steroid for protein may be conveniently expressed in accordance with the usage of Daughaday (8) and Westphal (9) as Sb/P = 1/P. When Sb/Sb = 1, i.e. at 50% steroid binding, the binding affinity is expressed simply as 1/P. The binding affinity of a steroid for a protein may thus be given either in liters per g or in liters per mole of protein. The binding affinity for serum protein can obviously be given only in liters per g of serum protein. The binding affinity in this instance has the form of an association constant but cannot be calculated in molecular terms as Daughaday (8) has indeed pointed out. Perhaps its designation as a relative binding affinity would be more appropriate when comparing the binding affinity of various sera on an empirical basis.

The binding affinity is related to the association constant according to the following two equations set forth by Klotz (10):

\[
P \frac{S_b}{S_a} = \frac{1}{n k} + \frac{1}{n}
\]

and (the equation utilized in the Scatchard (11) plot),

\[
\frac{S_b}{S_a} P = k \left( n - \frac{S_a}{P} \right)
\]

where k is the intrinsic association constant for each binding site, and n is the number of binding sites in the protein molecule.

This study of steroid-protein interaction was undertaken with a view to understanding the biochemical mechanisms underlying the high uptake in vivo of certain steroid sex hormones by normal and cancerous mammary tissue recently reported (2) from our laboratory. After some acquaintance in the laboratory with available equilibrium dialysis and dextran gel filtration techniques (cited in the "Discussion"), we arrived at a new and relatively simple technique, entailing a novel approach to the determination of the steroid-protein-binding parameters. This technique is described here together with the results obtained on testosterone-binding levels in individual human sera.

Prior studies on the binding of testosterone and related steroid hormones by human plasma and plasma protein fractions have been reviewed by Antoniades, Daughaday, and Slaunwhite (3). A testosterone-binding globulin has been recently reported by Mercier, Alfsen, and Beaulieu (4) and by Mercier (5).

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The symbols, $S_n$, $S_b$, and $P$, have been defined above and are expressed in molar concentration in each instance.

**Experimental Procedure**—A known amount, $a$, of radioactive steroid and a known amount, $P$, of protein were added to Sephadex G-25 in a phosphate buffer at pH 7.4. Equilibration was achieved by shaking at 25° for 1 hour. The radioactivity, $x$, in the external phase was then determined. Bound and unbound steroid, $S_b$ and $S_u$, respectively, in the external phases were calculated from $a$, $x$, and $K'$ (the partition factor for the steroid in the absence of protein). The binding affinity, $1/P$, at 50% steroid binding was thus obtained. A detailed account of the procedure now follows.

To each tube (11 × 45 mm, fitted with a ground glass stopper, penny head 9), containing 200 mg of Sephadex G-25, was added 1 ml of 0.155 M sodium phosphate buffer, pH 7.4. The gel swelled. To facilitate equilibration, two glass beads (diameter, 4 mm) were added; the tubes were gently shaken and allowed to remain overnight at room temperature. The volume, $v_o$, of the internal phase was determined, as described below, to be 0.5 ml. The volume, $v_o$, of the external phase was adjusted from its initial volume of 0.5 ml to a final volume of 1.5 ml on addition of testos-

The tubes (with the glass beads retained) were placed horizontally in a Dubnoff incubator and shaken at 25° for 24 hr in a convenient period, although preliminary experiments indicated that equilibration could be achieved in much less time). The tubes were removed and placed vertically in another water bath at 25° for 1 hour to allow the gel to settle. Duplicate aliquots (0.2 ml each) of the clear supernatant solution were removed for radioassay.

**Factors Influencing $K'$**—It is essential that $K'$ be determined with utmost precision in each experiment since $K'$ varied somewhat from one experiment to the next. A mean value of 1.13 with a relative standard deviation of about 4% was obtained in a total of 33 experiments; quadruplicate determinations of $K'$ in each experiment were in excellent agreement. $K'$ varied only very slightly with the steroid concentration; a more precise value for $K'$ may be selected on plotting $K'$ against $(a-x)$ or against the logarithm of the reciprocal of the molar concentration, $S$, of testosterone in the external phase (see Fig. 1). $K'$ should theoretically be 3 since $v_o/v_i = 3$, an arbitrary ratio which was experimentally convenient. This divergence from theory may be attributed to adsorption of the steroid on the gel matrix in view of similar experiences encountered by Gelotte (12) with a wide variety of compounds (not including steroids, however); in our experience, $K'$ was dependent on the nature of the steroid employed. $K'$ for testosterone was fairly constant over a wide range of steroid concentration according to Fig. 1. The adsorption isotherm for a great many compounds is constant at very low solute concentrations, but other types of adsorption isotherms have also been encountered (13). In our study, $K'$ was determined at very low solute concentrations.

$K'$ may also be influenced by the protein concentration, the presence of other steroids, the ionic strength, $\mu H$, and other factors. However, $K'$ for unbound testosterone in the presence of protein may logically be expected to be the same as that for the steroid in the absence of protein. Direct proof that $K'$ is indeed constant might be difficult to furnish inasmuch as many proteins bind steroid to some extent. The addition of radioinert cortisol to radioactive testosterone did not significantly affect $K'$ for testosterone (see "Results"); the effect of other steroids was not determined.

It should be emphasized that the serum is greatly diluted, about 20- to 200-fold, in 0.155 M phosphate buffer at pH 7.4 when measuring binding affinity. The ionic strength and pH of the system are consequently not significantly altered. The effect of pH on $K'$ was not studied; however, $K'$ is routinely determined in our procedure for measuring binding affinity, and, hence, the determination of binding affinity at a pH other than 7.4 would not be invalidated.

$K'$ Represents Reversible Partitioning of Solute—$K'$ represents a readily reversible partitioning of testosterone between the external phase and the entire gel phase (i.e. the gel matrix and the internal aqueous phase). This was shown, as described below, by chromatography of testosterone on a partition column of Sephadex G-25 under conditions comparable with those employed in the batchwise experiments. It is noteworthy that of the many compounds (all nonsteroidal) similarly tested by Gelotte (12), none showed irreversible adsorption.

A partition column ($\pi, 0.48 cm^2, h, 25.3 cm$), containing 2.5 g of Sephadex G 25 in 0.155 M sodium phosphate buffer at pH 7.4, was employed. Blue dextran, tritiated water, and testosterone-1,2-3H, each in 0.2 ml of the above buffer, were separately applied to the column, and eluted with the same buffer (see Fig. 2). The chromatogram is a composite of individual runs with each solute; two identical columns were prepared which gave the same results when blue dextran and tritiated water were applied. The optical density of the blue dextran solution was measured at 923 mp, an optical density unit is defined as optical density times volume of solution times dilution; 1 optical density unit is equivalent to 289 $\mu g$ of blue dextran. $K_d$ for testosterone, calculated from the basic equation given by Gelotte (12) to describe gel filtration, was found to be identical with the value predicted from $K'$ in the batchwise experiments with Sepha-

![Fig. 1. The partition factor, $K'$, plotted as a function of the logarithm of the reciprocal of the molar concentration, $S$, of testosterone in the external phase. The results of five individual experiments, each at two widely different steroid concentrations, are shown.](http://www.jbc.org/)

*Unpublished data.*
FIG. 2. Chromatography of blue dextran, tritiated water, and testosterone-1,2-3H on a partition column of Sephadex G-25 in 0.155 M sodium phosphate buffer, pH 7.4; for experimental details, see text.

The exchange of tritium with the labile hydrogen of Sephadex, as noted by Marsden (14), was taken into account. The definition of $K_d$ is that given by Gelotte (12) and Flodin (15), and further elucidated by Ackers (16).

To recapitulate, the discrepancy of the partition coefficient, $K'$, from its theoretical value is admittedly a sign of serious interference of the measuring system with the measured quantities, but it appears that Sephadex G-25 can, nevertheless, be validly employed for the purpose described herein.

Other gels, e.g. polyacrylamide, may prove to be similarly useful.

Determination of $v_i$ and $v_e$—Blue dextran, a high molecular weight dextran coupled with a blue chromophore, is completely excluded from Sephadex, a solution of blue dextran is accordingly concentrated upon the addition of Sephadex. $v_i$ and $v_e$ were thus readily determined, employing the equations

$$v_e = v\left(\frac{\text{O.D.}'}{\text{O.D.}''}\right)$$

and

$$v = v_i + v_e$$

where O.D. ́ and O.D. ́ ́ ́ is the optical density (at 263 mμ) before and after the addition of Sephadex; and $v$, the volume of the solution before the addition of Sephadex; $v_i$ and $v_e$ have been defined above. Blue dextran in 0.155 M sodium phosphate buffer, pH 7.4, exhibited a broad maximum of absorption at 263 mμ. Equilibration of blue dextran, in a known volume, $v$, with a known amount of Sephadex G-25 was carried out as in the technique for measuring steroid-protein binding. The volume for $v$, was thus found to be 2.42 ± 0.34 ml per g of Sephadex G-25 (mean ± S.D. of three observations). The concentration of blue dextran was not a factor influencing $v$, when concentrations of 0.2, 0.1, and 0.02 mμ were employed. The ionic strength of the buffer was likewise not of significant influence; e.g. the value for $v_i$ was 2.50 ± 0.18 ml when substitution with 0.00155 M phosphate buffer, pH 7.4, was made. Both values for $v_e$ are in agreement with that stated by the manufacturer as the water regain value, 2.5 ± 0.2 ml per g of Sephadex G-25. A slight loss (3.8%) of weight occurred on drying Sephadex G-25 to constant weight at 105°C for 44 hours. This would introduce a negligible error at most of (0.038/10) 100 or 0.38% in the calculation for ($v_i + v_e$).

CALCULATIONS

$S_a$ and $S_b$ as well as $a$ and $x$, represent radioactivity in the equations given in this section.

Partition Factor, $K'$, in Absence of Protein—Let $a$ equal the radioactivity (counts per min) of total steroid added, and $x$ equal the radioactivity in the external phase. Hence, $a - x$ equals the radioactivity in the internal phase. By definition

$$K' = \frac{x}{a - x} \quad \text{or} \quad x = K'(a - x) \quad (3a)$$

In the absence of protein, $x$ equals $S_a$ in the external phase; and hence

$$S_a = K'(a - x) \quad (3b)$$

The volumes of the external and internal phases, designated respectively $v_e$ and $v_i$, were determined as described above.

$S_a$ and $S_b$ in Presence of Protein—Let $x$ equal $S_a + S_b$ in the external phase after equilibration; hence

$$S_b = x - S_a \quad (4a)$$

Substitution for $S_a$ (Equation 3b) in Equation 4a gives

$$S_b = x - K'(a - x) \quad (4b)$$

Let

$$y = S_a/S_b \quad (4c)$$

Hence from Equations 3b and 4b

$$y = \frac{K'(a - x)}{x - K'(a - x)} \quad (4d)$$

$S_b$ at 50% Steroid Binding—Solving for $x$ in Equation 4d gives

$$x = K'(a - x)\left(\frac{y + 1}{y}\right) \quad (5a)$$

Let

$$c = \frac{y + 1}{y} \quad (5b)$$

Hence

$$x = \left(\frac{K'c}{1 + K'c}\right)a \quad (5c)$$

At 50% steroid binding, $y$ equals 1 and $c$ equals 2.

Hence from Equations 4c, 4e, and 5c,

$$S_b = S_a = \left(\frac{K'}{1 + 2K'}\right)a \quad (5d)$$

Molarity of Steroid—The molarity, $M$, of steroid ($S_a + S_b$) in the external phase is given by the equation,

$$M = \left(\frac{x}{S.A.}\right)\frac{1}{v_e} \quad (6)$$

where $x$ is expressed in millimicrocuries, S.A. (the specific activity) in millimicrocuries per mole of steroid, and $v_e$ in liters.

Regression Line—The equation for the regression line was obtained by the method of least squares as described by Moroney (17).
**DETERMINATIONS AND MATERIALS**

**Tritium Determination**—Radioactive samples were counted in a Packard automatic Tri-Carb liquid scintillation spectrometer, model 314 AX. The voltage was maximal at 1420, with the tap set at 9; the discriminating voltage was 40 to 100; the attenuators were set at 1.

Aqueous samples (each 0.2 ml) were counted after the addition of 5 ml of a phosphor solution, similar in composition to that described by Jacobson et al. (18) but containing 7.5 g of 2,5-diphenyloxazole (PPO), 0.125 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), and 120 g of naphthalene (m.p. 79-80°, Matheson, Coleman and Bell) per liter of dioxane, 0.5 liter of xylene, and 0.3 liter of 95% ethanol. All solvents were analytical grade and were redistilled; the phosphors were obtained from Pilot Chemicals, Inc., Watertown, Massachusetts. The detection efficiency was about 13% and the background count, 55 cpm. The detection efficiency was determined for each run of sample determinations. The small amount of protein present in the aqueous samples did not significantly affect the detection efficiency.

Nonaqueous samples, e.g. methanol-benzene solutions of testosterone-1,2-3H, were evaporated under nitrogen. The residue was dissolved in a toluene-phosphor as previously described (19); the detection efficiency was about 21%.

**Protein Determination**—Serum protein was estimated by the method of Lowry et al. (20) with slight modification, employing bovine serum albumin as a standard.

Sephadex G-25 (fine) and blue dextran were obtained from Pharmacia. Testosterone-1,2-3H (17β-hydroxyandrost-4-en-3-one), specific activity 37.5 Ci per mmole, in 10% methanol-benzene was obtained from New England Nuclear. It was further diluted in the same solvent and stored at 5°.

The radiochemical purity of this material was confirmed just before use by the manufacturer by paper chromatography and isotope dilution analysis. This was confirmed on occasion in our laboratory by reverse phase chromatography (19) on a partition column of siliconized Celite with toluene-70% methanol and by isotope dilution analysis.

The specific activity was reduced, when required, by the addition of a solution of testosterone in the same solvent. Aqueous solutions of testosterone-1,2-3H were prepared just before use by evaporating a suitable aliquot of the methanol-benzene solution under a stream of nitrogen at room temperature. The residue was immediately shaken gently with 0.155 M sodium phosphate buffer, pH 7.4, at room temperature; the solution was centrifuged, or transferred to another vessel, or both, and assayed for tritium. Tritiated water, with a specific activity of 2.07 x 10^6 dpm per ml, was also obtained from New England Nuclear. Bovine albumin (crystalline, A grade) was obtained from Calbiochem. Human serum albumin (at least 96% pure as determined by electrophoresis) was kindly furnished by Dr. Werner Baumgarten, Merck Institute for Therapeutic Research, West Point, Pennsylvania. Human pregnancy serum was procured at the Boston Lying-In Hospital with the kind cooperation of Dr. Kendall Emerson, Jr. Other types of serum or plasma were obtained at the Peter Bent Brigham Hospital with the kind assistance of the staff.

**RESULTS**

**Binding to Bovine Serum Albumin**

The ratio of unbound to bound testosterone, S_u/S_b, plotted against the reciprocal of the bovine serum albumin concentration, 1/P, gave a straight line, see Fig. 3. The equation for the regression line was S_u/S_b equals 2.48 (1/P) + 0.091 in Experiment 3, and, similarly, 2.24 (1/P) + 0.097 in Experiment 5. At 50% steroid binding, 1/P = 0.367 and 0.404 liters per g of protein, and 1/S_b ~ 4.93 x 10^6 and 4.83 x 10^6 liters per g of testosterone in Experiments 3 and 5, respectively.

At 50% steroid binding and when S_u/P is very small (as in Experiments 3 and 5), Equation 2 above may be simplified to an approximate form, 1/P equals nk. To obtain nk, 1/P was multiplied by the molecular weight of bovine serum albumin (taken as 6.9 x 10^6°) since 1/P was expressed above in liters per g. Approximate values for nk were 2.83 x 10^4 and 2.70 x 10^4 liters per g of protein in Experiments 3 and 5, respectively. The ratio, S_u/P, in moles per g of protein, was negligible: 0.513 x 10^-4 at 50% steroid binding in Experiment 3. The reciprocal of the slope, (S_u/S_b)P, of the line (see Fig. 3) gave nk equal to approximately 2.78 x 10^4 liters per g of protein when S_u/S_b equals 1.

![Fig. 3. Binding of testosterone to bovine serum albumin. The ratio of unbound to bound steroid, S_u/S_b, plotted as a function of the reciprocal of the protein concentration, 1/P. The total amount of steroid per tube containing Sephadex was kept constant; the protein concentration was varied. The binding affinity is defined as 1/P in liters per g of protein when S_u/S_b equals 1.](image-url)
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FIG. 4. Binding of testosterone to bovine serum albumin. The logarithm of the ratio of the molar concentration of protein to that of bound steroid, P/Sb, plotted as a function of the logarithm of the reciprocal of the molar concentration of unbound steroid, 1/Su.

10^4 liters per mole. Nonlogarithmic plots of this sort are generally employed (10, 11) to obtain binding constants (n/k).

Binding to Human Serum Albumin

The binding of testosterone to human serum albumin (see Fig. 5) was determined as with bovine serum albumin (see Fig. 3) except that the amount of steroid added per tube containing Sephadex was decreased from one experiment to the next. The binding affinity at 50% steroid binding increased very slightly with increasing values for the reciprocal of the bound steroid concentration. The values for (Sb/Su)/(1/P), calculated from the data shown in Fig. 5, were 3.52 × 10^4, 3.59 × 10^4, 3.86 × 10^4, and 4.28 × 10^4 liters per mole, respectively. A Scatchard (11) plot (not shown) of (Sb/Su)/(1/P) against (Sb/P) afforded a precise value of n/k = 4.19 × 10^4 liters per mole of human serum albumin; estimation of n was not attempted because too extensive an extrapolation would have been required. The binding affinity of testosterone for human serum albumin was thus found to be higher than that for bovine serum albumin (n/k = 2.44 × 10^3. Similar binding values, (Sb/Su)/(1/P), for human serum albumin have been reported by Westphal, Ashley, and Selden (21). Our data for bovine serum albumin are in good agreement with the binding constants (n/k) recently reported by Alfsen (22) and by earlier workers (reviewed by Slaunwhite (7)).

Binding to Pregnancy Serum Protein

The binding affinity of testosterone for pregnancy serum protein of late human pregnancy was determined (see Fig. 6) in the same manner as for human serum albumin (Fig. 5). The binding affinity for pregnancy serum protein increased markedly with increasing values for the reciprocal of the bound steroid concentration (at 50% steroid binding), whereas the binding affinity for human serum albumin increased only slightly.

Logarithm of Binding Affinity Linear Function of Logarithm of Reciprocal of Bound Steroid Concentration

Human Pregnancy Serum and Human Serum Albumin—The values for 1/P and 1/Sb at 50% steroid binding (Figs. 5 and 6) when plotted in log-log fashion (see Fig. 7) gave a linear relationship. The slope of the regression line for human pregnancy serum was steep whereas that for human serum albumin was practically nil. This type of plot proved to be particularly useful, as discussed below, for the quantitative estimation of the testosterone-binding level in serum.

Nonpregnant Female Plasma and Male Serum—Results similar to those described for human pregnancy serum (Fig. 7) were obtained with nonpregnant female plasma and male serum (see Fig. 8); however, the slope of the regression line was not as steep.

Effect of Previous Heat Exposure of Protein on Binding Affinity

The effect of previous exposure of protein to heat is shown in Fig. 7 (human pregnancy serum and human serum albumin) and in Fig. 8 (nonpregnant female plasma and male serum). The binding affinity for plasma or serum was markedly decreased after exposure to heat but the binding affinity for human serum albumin was only slightly lowered.

The binding affinity of testosterone for male serum protein declined sharply as the temperature of exposure was raised from 4–65°C (see Fig. 9). On the other hand, the binding affinity for bovine serum albumin declined only slightly.

The binding affinity, 1/P, for heat-exposed serum protein was consistently low, regardless of the endocrine status of the donor (see below). Thus, a value of 0.31 ± 0.07 (mean ± standard deviation) liters per g of serum protein was obtained on examination of 27 individual sera.

FIG. 6. Binding of testosterone to protein of human pregnancy serum under conditions comparable to those described for human serum albumin (Fig. 5).

FIG. 5. Binding of testosterone to human serum albumin. The ratio of unbound to bound steroid, Sb/Su, plotted as a function of the reciprocal of the protein concentration, 1/P. The reciprocal of the bound steroid concentration, 1/Sb, at 50% steroid binding is indicated. The binding affinity is defined as 1/P in liters per g of protein when Sb/Su equals 1.
FIG. 7. Binding of testosterone to protein of human pregnancy serum and to human serum albumin. A plot on a log-log scale is shown of the reciprocal of the protein concentration, \(1/P\), against the reciprocal of the bound steroid concentration, \(1/S_b\), at 50% steroid binding. The binding affinity is defined as \(1/P\) in liters per g of protein when \(S_b/S_b = 1\). The effect of previous exposure of the protein to heat (for 1 hour at 60°, pH 7.4) on the binding affinity is shown.

Effect of Added Cortisol on Binding Affinity of Pregnancy Serum for Testosterone

Radioinert cortisol was added to a specimen of late pregnancy serum so that the endogenous cortisol concentration (assumed to be 20 μg/100 ml of serum (23)) was increased 5-fold. The binding affinity for testosterone-1,2-3H at \(S_b = 1.2 \times 10^8\) (at 50% steroid binding) was 4.0 and 4.9 before and after addition of cortisol, respectively; \(K'\) was 1.11 and 1.12, respectively, under the same conditions but in the absence of protein. The binding affinity at \(1/S_b = 5 \times 10^8\) was likewise 3.6 and 3.4 before and after addition of cortisol; \(K'\) was 1.06 and 1.13, respectively. The concentration of radioinert cortisol was about 5 times that of radioactive testosterone in these experiments.

It should also be pointed out that influence of other endogenous steroids on testosterone-binding may be minimal in view of the high dilution of serum in this measurement.

Units of Testosterone-binding Activity Defined

Units of testosterone-binding activity may be conveniently

FIG. 8. Binding of testosterone to protein of nonpregnant female plasma and male serum. Compare with the log-log plot similarly described for human pregnancy serum (Fig. 7). The binding affinity is similarly expressed in liters per g of protein.

expressed as the difference in the binding affinity, \(\Delta 1/P\), of testosterone for serum protein before and after heat exposure for 1 hour at 60°, pH 7.4; the binding affinity is measured at 50% steroid binding. Inasmuch as the values for \(\Delta 1/P\) increased markedly with decreasing steroid concentration (i.e. with increasing values for the reciprocal of the bound steroid concentration) (see Figs. 7 and 8), the measurement of testosterone-binding activity was seen to be most sensitive at high dilution of steroid, e.g. when \(1/S_b = 1.2 \times 10^8\) liters per mole at 50% steroid binding; this binding behavior, it should be mentioned, is typical of a system with limited binding capacity, and is therefore in contrast to that of the albumin solutions. The limiting factor in diluting the steroid was of course its specific activity. Measurement of \(\Delta 1/P\) need not always be determined at the same value for \(1/S_b\) although this is desirable; \(\Delta 1/P\) may be

FIG. 9. Binding affinity of testosterone for protein of male serum and for bovine serum albumin, plotted as a function of the temperature to which the protein was previously exposed for 1 hour at pH 7.4. The binding affinity, defined as \(1/P\) in liters per g of protein when \(S_b/S_b = 1\), was conveniently measured when \(1/S_b = 1.2 \times 10^8\) liters per mole.

FIG. 10. Testosterone-binding levels in the serum of men, menstruating women, postmenopausal women, breast cancer patients, and women in late pregnancy. The testosterone-binding levels during the course of a menstrual cycle in one subject are represented by the open circles. The levels are expressed in units of testosterone-binding activity, as defined in the text, in liters per g of serum protein; the measurement was made when \(1/S_b = 1.2 \times 10^8\) liters per mole at 50% steroid binding.
readily calculated for any value for $1/S_b$ from the binding affinities observed at two divergent values for $1/S_b$ in view of the linear relationship between $\log 1/P$ and $\log 1/S_b$.

**Survey of Testosterone-binding Levels in Various Individuals**

Fig. 10 affords a comparison of testosterone-binding levels (in the units defined above) in the serum of various individuals. Testosterone-binding levels in late pregnancy were much higher than those in menstruating women. There appears to be no marked difference in the levels between the sexes although men had somewhat lower levels than women. Widely fluctuating levels were occasionally seen in the groups designated as post-menopausal women and breast cancer patients, suggesting that further study of a larger series of individuals might be instructive.

In view of the physiological variations in serum protein concentration, notably in pregnancy (24), expressing the testosterone-binding level (or activity) per g of serum protein seems appropriate.

**DISCUSSION**

The level of steroid hormone-binding protein, e.g. corticosteroid-binding globulin, is usually expressed either as its binding affinity or binding capacity. For example, Daughaday (8) has employed binding affinity empirically (as discussed under "Methods" above). Recently, however, Daughaday et al. (25) measured corticosteroid-binding globulin as its binding capacity, defined as the micrograms of cortisol bound per 100 ml of plasma at saturation of the corticosteroid-binding globulin-binding sites with added cortisol. The binding capacity would thus appear to be related to $S_b/P$ at maximal steroid binding. Other useful definitions of binding capacity have been given, e.g. "transcortin (or corticosteroid-binding globulin) capacity" (14), which do not, however, always have the same formulation or meaning.

A comparison of steroid hormone-binding levels in serum is valid only if the measurements in dialysis experiments are made at the same concentration of protein or of steroid in view of the complexity of the system: the various serum proteins compete for a given steroid, and, hence, many binding equilibria may exist; moreover, several steroids may compete for the same binding site. Pioneer work has shown that the binding affinity (8) or the percentage of steroid bound to plasma protein (26) was dependent on the steroid concentration and also of course on the nature of the steroid hormone under study. Slaunwhite and Sandberg (26) pointed out, moreover, that the plasma required considerable dilution in order to detect protein with high binding affinity for cortisol in the presence of other proteins; particularly albumin, with low binding affinity. Brunkhorst and Hess (27) recently showed in their study on cortisol-albumin interaction that the binding parameters are dependent on the concentration of both protein and steroid.

In our procedure, the protein concentration is varied until the same degree (50%) of steroid binding is obtained at a given concentration of steroid: a very sensitive and quantitative estimate of testosterone-binding activity was thus obtained. An optimal concentration of steroid was selected following a systematic study, see above, which showed that: (a) a linear relationship exists between the logarithm of the reciprocal of the serum protein concentration, $1/P$, and the logarithm of the reciprocal of the bound testosterone concentration, $1/S_b$, at 50% steroid binding, (b) the slope, $\log P/\log S_b$, of the line in this plot is steep for unheated serum protein (which also has a high affinity for binding testosterone), but it is very low or nil for serum albumin; the term, $S_b/P$, in Equation 2 above becomes negligible at high steroid dilution (i.e. when $1/S_b$ is increased) in the case of serum albumin, whereas $S_b/P$ remains significant with unheated serum protein, especially that of late pregnancy, and (c) the value for $1/P$ at 50% steroid binding is greatly diminished after exposure of serum protein to heat at 60°, whereas serum albumin is only slightly affected in this respect. Corticosteroid-binding globulin has likewise been shown (25) to lose its high affinity for binding cortisol on heat exposure.

Our experimental approach to the calculation of binding parameters with the aid of the above log-log plot is novel. The same degree (50%) of steroid binding is maintained in our procedure as the protein concentration is decreased by decreasing the steroid concentration, whereas in the conventional procedure the steroid binding is increased by increasing the steroid concentration at a given protein concentration, and then plotting the data as in the Scatchard (11) plot. Binding at the 50% level was selected because measurement at this level is precise; the data were plotted in log-log fashion to represent conveniently the wide range in values, particularly of $1/S_b$. The linear relationship which our data seem to indicate is an empirical one since the authors are unable to give, at least at present, a theoretical analysis of the slope. Nonetheless, the usefulness of this log-log plot may be apparent. It permits the ready detection and quantitative estimation (on a relative scale) of a protein (or possibly proteins) of high binding affinity in a complex system of proteins of relatively low binding affinities; it is also proving to be useful as a guide in the purification of serum protein, or proteins, exhibiting a high binding affinity for testosterone and but little for cortisol.

The concentration of both radioactive and endogenous steroid hormone must be known to measure the testosterone-binding level with accuracy. $S_b$ represented, however, virtually only radioactive testosterone inasmuch as the endogenous testosterone, present at levels of only about $10^{-8}$ mg and $10^{-9}$ mg in male and female plasma (28-30), respectively, was diluted at least 100-fold. Underestimation of the testosterone-binding level may be obviated by determining the endogenous testosterone concentration, especially when this is suspected to be very high, and including this value in the calculation for $S_b$. Measurement of both endogenous testosterone and the testosterone-binding level would, in any case, be desirable for a proper assessment of circulating androgenicity; interpretation of the activity of a hormone solely on the basis of its plasma concentration may, as Daughaday (31) has suggested, lead to erroneous conclusions unless the degree of binding of the hormone to serum protein is taken into account.

The Sephadex gel exchange in our technique represents in effect a single dialysis, and hence an *equilibrated* system, whereas in the widely used gel filtration methods (see for example, Reference 32) equilibrium is never reached. The new technique thus affords binding data which are thermodynamically essentially correct, and lend themselves to the calculation of association constants and other parameters. However, it does suffer from a limitation not encountered in conventional dialysis (which also provides an equilibrated system): the internal phase cannot be sampled; the Sephadex gel can be filtered and extracted but this resort may not be feasible. Other limitations have already been mentioned,

3 J. Guériguian and W. H. Pearlman, unpublished data.
e.g. the discrepancy of the partition factor, $K'$, from its theoretical value, but this was readily taken into account. The possible influence of third components, e.g. salts, competing steroids, on $v_i$ and $K'$ was also readily ascertained: a lack of influence of added salts on $K'$ was demonstrated; but since only a very few control experiments were performed, the possibility of such complications should be kept in mind. The new technique, despite these limitations, is precise, simple, and rapid, allowing multiple determinations to be performed simultaneously and conveniently.

Very rapid equilibration is achieved owing to the exceptionally high ratio of the surface area of the Sephadex bead (diameter, 20 to 50 $\mu$m in the dry state) to the volume of the internal phase; conventional dialysis usually requires at least 24 hours for equilibration. This is an important consideration, apart from one of conventional dialysis.


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