

# A Critical Evaluation of Simple Methods for the Estimation of Free Testosterone in Serum

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## ABSTRACT

The free and nonspecifically bound plasma hormone levels generally reflect the clinical situation more accurately than total plasma hormone levels. Hence, it is important to have reliable indexes of these fractions. The apparent free testosterone (T) concentration obtained by equilibrium dialysis (AFTC) as well as the fraction of serum T not precipitated by 50% ammonium sulfate concentration (non-SHBG-T; SHBG, sex hormone-binding globulin), often referred to as bioavailable T, appear to represent reliable indexes of biologically readily available T, but are not well suited for clinical routine, being too time consuming. Several other parameters have been used without complete validation, however: direct immunoassay of free T with a labeled T analog (aFT), calculation of free T (FT) from total T and immunoassayed SHBG concentrations (iSHBG), and the free androgen index (FAI = the ratio 100T/iSHBG). In the view of substantial discrepancies in the literature concerning the free or bioavailable T levels, we compared AFTC, FT, aFT, FAI, and non-SHBG-T levels in a large number of sera with SHBG capacities varying from low, as in hirsute women, to extremely high as in hyperthyroidism. All these indexes of

bioavailable T correlated significantly with the AFTC concentration; AFTC and FT values were almost identical under all conditions studied, except during pregnancy. Values for aFT, however, were only a fraction of either AFTC or FT, the fraction varying as a function of SHBG levels. Also, the FAI/AFTC ratio varied as a function of the SHBG levels, and hence, neither aFT nor FAI is a reliable index of bioavailable T.

The FT value, obtained by calculation from T and SHBG as determined by immunoassay, appears to be a rapid, simple, and reliable index of bioavailable T, comparable to AFTC and suitable for clinical routine, except in pregnancy. During pregnancy, estradiol occupies a substantial part of SHBG-binding sites, so that SHBG as determined by immunoassay overestimates the actual binding capacity, which in pregnancy sera results in calculated FT values that are lower than AFTC. The nonspecifically bound T, calculated from FT, correlated highly significantly with and was almost identical to the values of non-SHBG-T obtained by ammonium sulfate precipitation, testifying to the clinical value of FT calculated from iSHBG. (*J Clin Endocrinol Metab* 84: 3666–3672, 1999)

TESTOSTERONE (T) circulates in plasma nonspecifically bound to albumin, specifically bound to sex hormone-binding globulin (SHBG), and, in a small percentage, unbound. Although the free and nonspecifically bound hormone fraction in plasma (commonly referred to as the bioavailable fraction) reflects only partially the hormone available at the cellular level in specific tissues where partial dissociation of the specific steroid-protein complex may occur and inactive prohormones may be converted intracellularly to active hormones, there is good evidence that this fraction reflects more accurately the clinical situation than the total hormone levels in plasma.

T bound to albumin represents the nonspecifically bound T fraction (1) and is linearly related to free T (FT):  $AT = K_a C_a \times FT$ , where AT is the albumin-bound T,  $K_a$  is the association constant of albumin for T,  $C_a$  is the albumin concentration, and FT is the free T fraction. The sum of FT and AT (*i.e.* the non-SHBG-bound T) is for convenience often referred to as bioavailable fraction in plasma. As the total serum T concentration is subject to variations in the concentration of the binding proteins, it is not a reliable index of bioavailable T. For example, in hyperthyroidism T levels are high, whereas bioavailable T levels are normal (2). Hence, there is a clinical need for a reliable index of bioavailable androgens.

Several methods have been used to estimate free or bioavailable T in plasma. FT can be measured by equilibrium dialysis [apparent FT concentration (AFTC)] (2), the method of choice for measurement of the free fraction of steroids *in vivo* (3). Alternatively, the non-SHBG-bound, biologically readily available fraction may be obtained by precipitation of SHBG-bound T with ammonium sulfate (non-SHBG-T) (4, 5). As both are rather time-consuming procedures, many researchers use an indirect parameter of FT, the free androgen index (FAI), which is obtained as the quotient 100 T/SHBG (6). FT can also be estimated directly by an immunoassay method involving an analog ligand (aFT). The latter technique is often considered the easiest and fastest method for measuring FT (3), but the values obtained with this analog ligand immunoassay are substantially lower than values obtained by dialysis (7, 8).

At equilibrium, binding of T to plasma proteins can be represented by Eq I:  $T = FT + P_1T + P_2T + P_3T + \dots + P_nT$  (Eq I). For each protein, applying the law of mass action, we have:  $[FT] + [P] \rightleftharpoons [PT]$  or  $[FT] = ([PT]/(K \times [P]))$ , and in the presence of several binding proteins:  $[FT] = ([P_1T]/(K_1 \times [P_1])) = [P_2T]/(K_2 \times [P_2]) = \dots = ([P_nT]/(K_n \times [P_n]))$  (Eq II), where  $[P_1T]$ ,  $[P_2T]$ ,  $\dots$ ,  $[P_nT]$  are the concentrations of T bound to proteins 1, 2,  $\dots$ , n, respectively;  $K_1$ ,  $K_2$ ,  $\dots$ ,  $K_n$  are the association constants of proteins 1, 2,  $\dots$ , n, respectively, for T; and  $[P_1]$ ,  $[P_2]$ ,  $\dots$ ,  $[P_n]$  are the free binding sites on protein 1, 2,  $\dots$ , n, respectively. As the binding capacity of albumin is very high with respect to the concentration of T, the following equation holds true for the ratio of albumin-

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bound T (AT) to unbound T:  $(AT/FT) = K_a C_a$  or  $AT = (FT \times K_a C_a)$  (Eq III), where  $K_a$  equals  $3.6 \times 10^4$  L/Mol (9, 10);  $C_a$  is the albumin concentration, *i.e.*  $\pm 43$  g/L or (mol wt, 69,000)  $\pm 6.2 \times 10^{-4}$  mol/L; and  $K_a C_a$  is  $\pm 22$ .

The only other protein that binds T is SHBG; binding of T to transcortin or orosomucoid is negligible (2). As the binding of other steroid hormones normally present in plasma can be omitted from the calculation (9), it follows:  $FT = ([T] - (N \times [FT])) / (K_t \{SHBG - [T] + N[FT]\})$  (Eq IV), where  $K_t$  is the association constant of SHBG for T, and  $N = K_a C_a + 1$ . This yields a second degree equation that can be solved either for FT or SHBG (2, 7, 9).

Several immunoassay methods are available for measurement of serum SHBG levels. If the concentration of immunoassayable SHBG (iSHBG) is a reliable measure of SHBG binding capacity (11), a reliable value of FT can thus easily be calculated. Conversely, when the AFTC is determined by equilibrium dialysis, the SHBG binding capacity (cSHBG) can be calculated.

It is surprising that whereas calculation of free T from total T and iSHBG is a simple and rapid procedure, the reliability of the calculated FT has never been extensively studied by comparing these values to those obtained by dialysis (AFTC). Indeed, Södergaard *et al.* (11) calculated FT from SHBG and albumin concentrations, but did not validate the method, whereas Wilke and Utley (7) compared calculated FT values in women to data obtained by direct analog ligand immunoassay of FT (aFT), but not to AFTC. The manufacturer of a kit for aFT measurement claims that aFT corresponds to  $0.42 \text{ AFTC} + 9.8 \text{ pg/mL}$  ( $r = 0.67$ ) in males and to  $0.79 \text{ AFTC} + 1.07 \text{ pg/mL}$  ( $r = 0.75$ ) in women, but values obtained in clinical routine appear to be substantially lower (7, 8).

It is thus not surprising that there are substantial discrepancies in the literature concerning the free or non-SHBG-bound bioavailable T levels obtained by a variety of only partially validated methods, as has recently been illustrated (8). We decided therefore to compare the AFTC values, generally considered as the index of choice for evaluation of free T levels, to calculated FT and aFT levels as well as to the FAI in sera from subjects with normal, low, and high SHBG binding capacities, respectively. Finally, we also compared the non-SHBG-T obtained by the ammonium sulfate precipitation technique with the FT levels as well as the nonspecifically bound T levels, calculated from T and iSHBG (*i.e.*,  $FT \times N$ ).

## Materials and Methods

### Assay procedures

Total serum T was measured by RIA using a commercial kit (Biosource Technologies, Inc., Fleurus, Belgium). Concentrations are given in nanomoles per L; for conversion to nanograms per dL, multiply by 28.84. The interassay coefficient of variation (CV) is below 10% for the whole concentration range.

Serum concentrations of SHBG were measured by immunoradiometric assay (IRMA), using a commercial kit (Orion Diagnostica, Espoo, Finland) and are referred to as iSHBG; the interassay CV is 8%.

In our reference procedure for estimation of free T, AFTC is obtained from serum total T and the free T fraction determined by equilibrium dialysis on diluted serum at 37 C with use of  $[^3\text{H}]\text{T}$  as described previously (2, 9); the SHBG binding capacity is calculated from AFTC using the above-mentioned second degree equation (see introduction; Eq IV), taking a value of  $1 \times 10^9$  L/mol for the association constant of SHBG for

T at 37 C and a value of  $3.6 \times 10^4$  L/mol for that of albumin for T (10); this value is corrected for serum dilution, and AFTC in undiluted serum is then calculated. The interassay CV is 7.8%. The SHBG binding capacities thus calculated from AFTC are further referred to as cSHBG.

The same second degree equation (introduction; Eq IV) is used for calculation of FT from serum total T and serum iSHBG measured by IRMA. The FAI is calculated from total T and iSHBG:  $\text{FAI} = (100 \times T) / \text{SHBG}$  (Eq V), with both T and SHBG expressed in nanomoles per L (6).

Direct estimation of serum free T by an analog ligand RIA (aFT) was performed using a commercial kit from Diagnostic Products (Los Angeles, CA); the binding capacity of SHBG calculated from aFT using the above-mentioned second degree equation is further referred to as aSHBG.

Non-SHBG-T was obtained from serum total T and determination of the non-SHBG-bound T fraction by ammonium sulfate precipitation, the method involving incubation with a tracer dose of  $[^3\text{H}]\text{T}$  at 37 C, and precipitation of SHBG-bound hormone with ammonium sulfate at a final concentration of 50%, followed by centrifugation and counting of radioactivity in the supernatant (5).

### Serum samples

Sera from men were obtained from a randomly selected subgroup of sera from healthy, ambulant men participating in a population study on the influence of age on plasma T levels.

Sera from postmenopausal women were obtained from women consulting the menopause clinic for check-up. Pregnancy sera were obtained in third trimester pregnancy. Sera were also obtained from hyperthyroid subjects; hyperthyroidism was confirmed on the basis of suppressed TSH with elevated  $\text{FT}_4$  and  $\text{FT}_3$  levels. Finally, sera were also obtained from a small group of women investigated for mild clinical hyperandrogenism.

### Statistics

Correlation of results obtained by different methods was estimated using the method of least square regression.

## Results

### Comparison of FT and AFTC

In a first series of experiments we compared the FT values calculated from serum T using the iSHBG and the actual measured albumin serum concentration to the AFTC determined by equilibrium dialysis in a group of ambulant men ( $n = 28$ ), aged 25–80 yr, with serum T concentrations varying between 1.63–31.0 nmol/L.

As can be seen in Fig. 1a, AFTC and FT values differed very little; the mean values ( $\pm \text{SEM}$ ) were  $330 \pm 36.4$  and  $332 \pm 37.1$  pmol/L for AFTC and FT ( $\text{FT} = 1.002 \text{ AFTC} + 0.877 \text{ pmol/L}$ ), respectively, with a correlation coefficient of 0.987.

As calculation of FT from T and iSHBG requires an additional measurement of the serum albumin concentration, we studied the influence of variations of the albumin concentration on the FT values. In a series of 30 ambulant subjects, the mean albumin concentration was  $6.29 \pm 0.4$  (SD)  $10^{-4}$  mol/L ( $43.4 \pm 0.26$  g/L), with as extremes 5.62 and  $7.01 \times 10^{-4}$  mol/L (38.8 and 48.4 g/L). Hence, we decided to study the influence on FT of a variation in albumin concentration ranging from  $5.8$ – $7.2 \times 10^{-4}$  mol/L (*i.e.*, 40, 45, and 50 g/L), respectively. In subjects ( $n = 30$ ) with an iSHBG concentration varying between 13.3–91.6 nmol/L, the mean ( $\pm \text{SE}$ ) FT was  $322 \pm 37.1$  pmol/L at the actual albumin concentration,  $340 \pm 40.9$  pmol/L, assuming an albumin concentration of 40 g/L,  $320 \pm 37.8$  pmol/L assuming an albumin concentration of 45 g/L, and  $303 \pm 35.4$  pmol/L

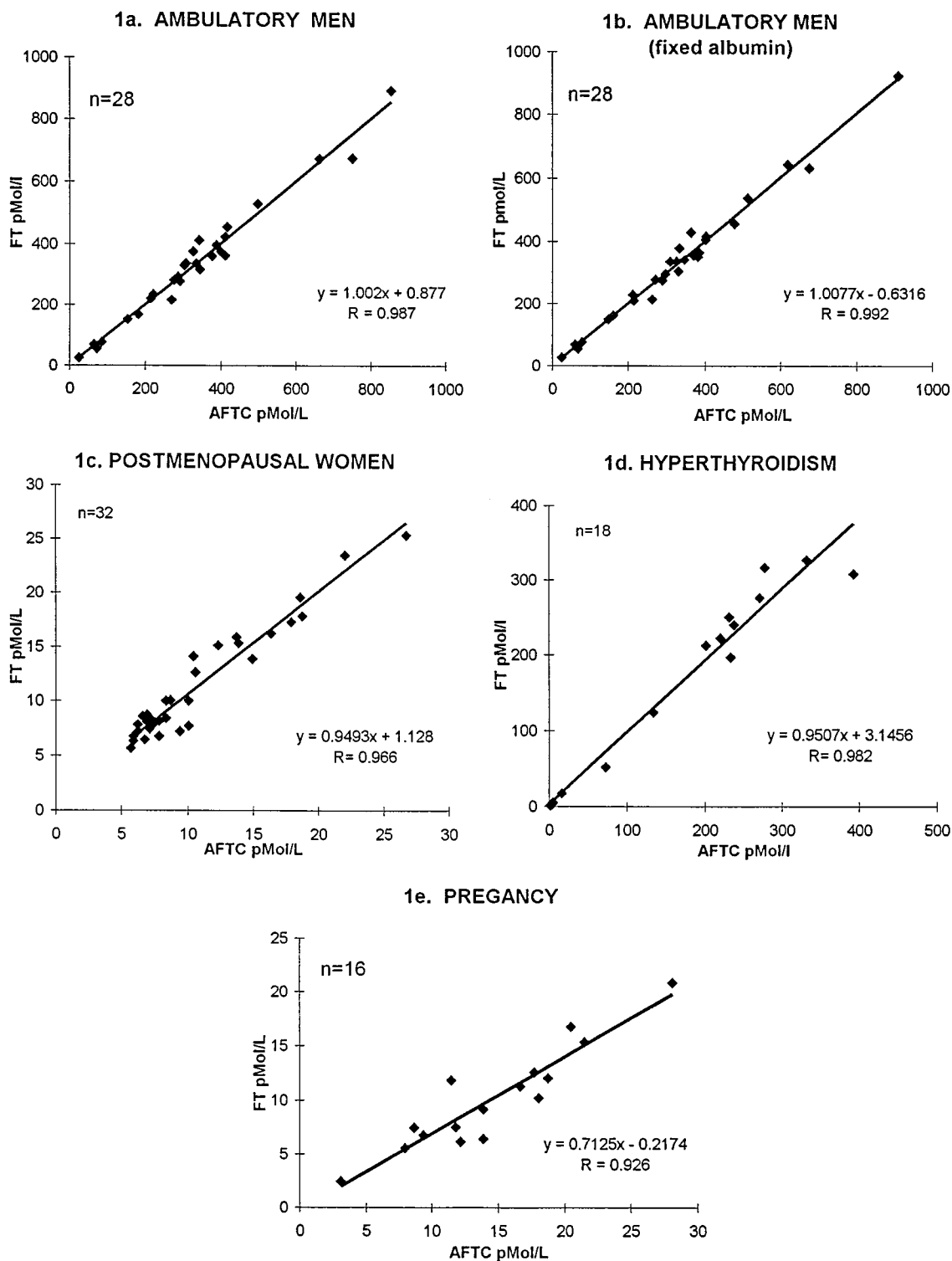


FIG. 1. Comparison of AFTC determined by equilibrium dialysis with FT calculated from T and SHBG by immunoassay (iSHBG) in a) 28 ambulant men, aged 25–80 yr, using the actual albumin concentration in the calculation of FT and AFTC; b) the same 28 men assuming a fixed albumin concentration of 43 g/L ( $6.2 \times 10^{-4}$  mol/L) in the FT calculation; c) 32 postmenopausal women (assuming a fixed albumin concentration of 43 g/L); d) 18 patients of either sex with hyperthyroidism (assuming a fixed albumin concentration of 43 g/L); and e) 16 serum samples obtained during third trimester pregnancy, using the actual albumin concentration in the FT calculation.

assuming a concentration of 50 g/L albumin. Using a value of  $3.6 \times 10^4$  L/mol for the association constant of albumin for T, the calculated albumin-bound T varied from 7.14 nmol/L

(40 g/L albumin) to 7.80 nmol/L (50 g/L albumin). In view of the relatively unimportant changes in FT, when the albumin concentration varies by as much as 25%, we concluded

that for routine purposes FT could be calculated assuming an albumin concentration of 43 g/L ( $6.2 \times 10^{-4}$  mol/L) if one is not dealing with sera from patients with marked abnormalities in plasma protein composition, such as in nephrotic syndrome or cirrhosis of the liver, or with sera obtained during pregnancy, in which cases the actual albumin concentration should be taken into account. As expected, the use of a fixed albumin concentration in the calculations did not affect the observed correlation between FT and AFTC concentrations; FT values were almost identical to AFTC levels ( $FT = 1.008 \text{ AFTC} - 0.632 \text{ pmol/L}$ ) with a correlation coefficient of 0.992 (Fig. 1b).

A similar study was performed with sera from 32 postmenopausal women with T concentrations ranging from 461–1553 pmol/L. The FT concentrations (mean  $\pm$  SEM,  $11.4 \pm 1.04$  pmol/L) were almost identical to AFTC concentrations ( $11.1 \pm 1.04$  pmol/L;  $FT = 0.949 \text{ AFTC} + 1.14$  pmol/L;  $r = 0.966$ ; Fig. 1c). To assess whether the close correlation between FT and AFTC persisted in subjects with high SHBG, we performed a similar study using sera ( $n = 18$ ) from patients of either sex with hyperthyroidism (SHBG, 41–204 nmol/L). Again, similar values were obtained by both methods; mean ( $\pm$ SEM) FT and AFTC were  $142 \pm 30.5$  and  $146 \pm 31.6$  pmol/L, respectively ( $FT = 0.951 \text{ AFTC} + 3.16$  pmol/L;  $r = 0.982$ ; Fig. 1d). A similar study was performed on sera from a small group of women with mild clinical hyperandrogenism ( $n = 12$ ); the mean ( $\pm$ SE) AFTC and FT were  $25.0 \pm 3.12$  and  $22.9 \pm 2.77$  pmol/L, respectively ( $FT = 0.850 \text{ AFTC} + 1.56$  pmol/L), with a correlation coefficient of 0.979.

Finally, we compared FT levels to AFTC levels obtained for third trimester (weeks 24–37) pregnancy sera ( $n = 16$ ). As the mean albumin concentration was only  $32 \pm 1$  g/L ( $4.6 \pm 0.14 \times 10^{-4}$  mol/L), we used the actual albumin concentration in the calculation. The mean ( $\pm$ SEM) value for AFTC ( $14.6 \pm 1.73$  pmol/L) was significantly higher than that for FT ( $10.06 \pm 1.39$  pmol/L;  $FT = 0.713 \text{ AFTC} - 0.217$  pmol/L;  $r = 0.926$ ; Fig. 1e).

#### Comparison of aFT and AFTC

In the next series of experiments we compared the aFT levels as measured directly by analog ligand RIA to AFTC levels. In a group of 28 men, mean ( $\pm$ SEM) aFT values ( $65.5 \pm 7.28$  pmol/L) were only a fraction (one fifth) of AFTC values ( $303 \pm 34.0$  pmol/L;  $aFT = 0.186 \text{ AFTC} + 4.38$  pmol/L), albeit there was a significant correlation between these values ( $r = 0.937$ ; Fig. 2a). In sera from women ( $n = 8$ ), aFT (mean  $\pm$  SEM,  $4.85 \pm 0.832$  pmol/L) was around 30% of AFTC values ( $15.95 \pm 4.27$  pmol/L). Our data furthermore suggest that there exists a positive correlation ( $P < 0.05$ ) between the specific binding capacity for T (*i.e.* cSHBG) and the aFT/AFTC ratio (Fig. 2b).

Using the aFT values to calculate the SHBG capacity (aSHBG) in a normal population ( $n = 34$ ; T ranging from 0.312–46.7 nmol/L), we obtained a mean ( $\pm$ SEM) value of  $2.75 \pm 0.16 \times 10^{-7}$  mol/L for aSHBG compared to  $5.5 \pm 0.43 \times 10^{-8}$  mol/L cSHBG by the dialysis method ( $aSHBG = 2.1331 \times \text{cSHBG} + 156$  nmol/L;  $r = 0.638$ ).

#### Comparison of FAI and AFTC

We next evaluated the reliability of the FAI ( $FAI = 100 \text{ T/iSHBG}$ ) as a parameter of bioavailable T. This parameter, obtained in 28 healthy subjects, was highly significantly correlated with AFTC ( $r = 0.848$ ;  $FAI = 0.132 \text{ AFTC} + 7.273$ ; Fig. 3a). However, the ratio FAI/AFTC varied from 0.12–0.26, indicating that in the individual case FAI is a rather unreliable index of bioavailable T. In 18 hyperthyroid subjects, the correlation coefficient was 0.946 (Fig. 3b), whereas the FAI/AFTC ratio varied between 0.17–0.39. The FAI/AFTC ratio is negatively correlated with the number of free binding sites on SHBG ( $\text{cSHBG} - \text{cSHBG-T}$ ; Fig. 3c).

#### Comparison of iSHBG and cSHBG

As iSHBG concentrations are not necessarily identical to the concentrations of binding sites (cSHBG), we compared iSHBG to cSHBG values calculated assuming an albumin concentration of 43g/L. In 28 normal men, the mean ( $\pm$ SEM)

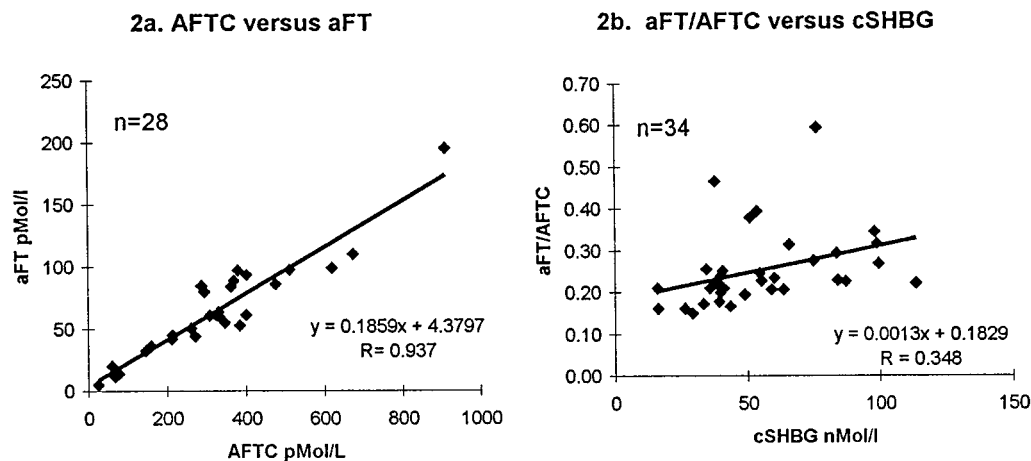


FIG. 2. a, Comparison of AFTC determined by equilibrium dialysis (fixed albumin concentration) and aFT determined by analog-tracer immunoassay in 28 ambulatory men. B, The ratio of aFT/AFTC as a function of the SHBG binding capacity calculated from AFTC (cSHBG) in 34 normal subjects of both sexes.

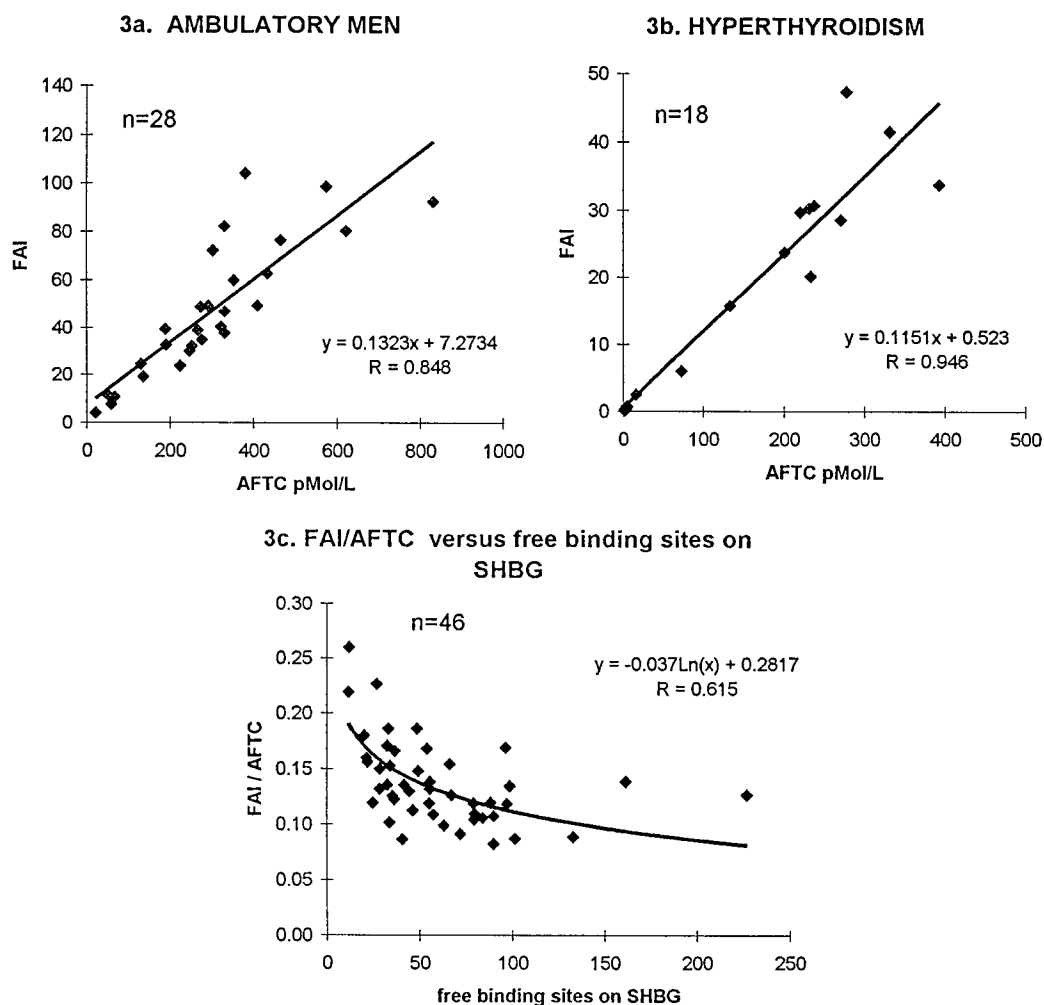


FIG. 3. Comparison of FAI and AFTC (fixed albumin concentration) in 28 ambulant men (a) and 18 hyperthyroid subjects of either sex (b) and the correlation between the FAI/AFTC ratio (AFTC in pmol/L) and the free binding sites on SHBG (mmol/L) (c). The free binding sites are calculated as the difference between the molar concentration of cSHBG and the molar concentration of SHBG-bound T: cSHBG–cSHBG-T.

cSHBG was  $56.1 \pm 5.06$  nmol/L compared to  $48.04 \pm 4.48$  nmol/L for iSHBG (SHBG =  $1.09$  iSHBG +  $3.64$  nmol/L;  $r = 0.963$ ). In our series of hyperthyroid subjects ( $n = 18$ ), the mean iSHBG concentration was  $96.6 \pm 11.58$  nmol/L in comparison with a cSHBG of  $93.9 \pm 10.98$  nmol/L ( $0.962$  cSHBG +  $6.238$  nmol/L;  $r = 0.912$ ). A close correlation between iSHBG (mean  $\pm$  SEM,  $54.25 \pm 5.30$  nmol/L) and cSHBG ( $49.96 \pm 6.32$  nmol/L) was also observed in sera from women with clinical hyperandrogenism ( $r = 0.977$ ; iSHBG =  $0.820$  cSHBG +  $13.273$  nmol/L), whereas in a group of 32 postmenopausal women, at low T levels the correlation coefficient was only 0.806. In the third trimester pregnancy sera, for which AFTC values were higher than FT values, cSHBG values calculated from AFTC were correspondingly significantly lower (mean  $\pm$  SEM,  $161.7 \pm 7.09$  nmol/L) than iSHBG ( $198.0 \pm 2.6$  nmol/L).

#### Comparison of non-SHBG-T and FT

As at least part of the albumin-bound T is bioavailable, we compared non-SHBG-T determined by the ammonium sulfate precipitation technique to FT and to the calculated non-

specifically bound T (*i.e.* non-SHBG-bound T) in 24 subjects of either sex with T concentrations varying between 0.485–25.2 nmol/L. As shown in Fig. 4, the correlation coefficient for non-SHBG-T *vs.* FT was 0.974, with non-SHBG-T corresponding to approximately 20 times FT, whereas the non-specifically bound T calculated from T and iSHBG, assuming a fixed albumin concentration of  $6.12 \times 10^{-4}$  mol/L, was  $\pm 23$  times FT, with a similar correlation coefficient as for FT, nonspecifically bound T being a multiple of FT.

#### Discussion

Measurement of AFTC by equilibrium dialysis at 37 C is probably the physiologically most representative method and the most exact for estimating free T (3) if care is taken that the labeled tracer T used for measurement of the FT fraction is highly purified. Indeed, impurities that do not bind to SHBG might significantly increase the apparent FT fraction. An alternative to the use of labeled T is direct measurement of T in the dialysate, provided the necessary very high sensitivity of the RIA for T can be achieved (12). As the association constant ( $K_d$ ) of SHBG for T (and hence the FT con-

## 4. nonSHBG-T versus FT

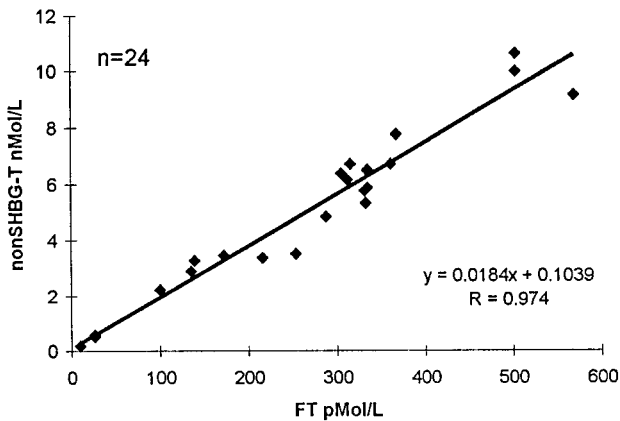


FIG. 4. Correlation between non-SHBG-T as determined by the ammonium sulfate precipitation method and FT calculated from T and iSHBG in subjects of either sex covering a broad range of T levels.

centration) varies with temperature, strict control of the temperature is required when applying these dialysis techniques, even though according to Södergard and co-workers (11), a variation in  $K_t$  by as much as 30% would influence FT to only a moderate degree. In any case, equilibrium dialysis is a rather laborious and time-consuming procedure, hence the search for alternative methods.

Direct measurement of the FT concentration by an analog ligand immunoassay procedure is an attractive and simple alternative. Our study indicates, however, that the direct measurement of aFT by analog ligand RIA, although showing a generally good correlation with AFTC, is not a reliable index of FT; the aFT represents a variable fraction (20–60%) only of AFTC. This is confirmed by the fact that calculation of the SHBG binding capacity from aFT (aSHBG) yields values that are multiples of all values previously reported in the literature. Similar findings were reported by Wilke and Utley (7) as well as by Rosner (8). Our data show, moreover, that the aFT/AFTC ratio is SHBG dependent. Winters *et al.* (13) arrived at a similar conclusion.

The FAI also appears not to be a reliable index of FT. Indeed, the ratio FAI/AFTC, which should be constant if FAI reflects AFTC, varies in fact by as much as a factor of 2.5. Combining Eq III, IV, and V (see introduction and *Materials and Methods*), one obtains:  $FAI/FT = (100[(SHBG - SHBG-T) - K + N])/SHBG$ . From the latter equation it follows that the FAI/AFTC ratio is correlated to the number of free binding sites on SHBG (Fig. 3c). The FAI/AFTC ratio will be high when the number of occupied binding sites is small related to the SHBG binding capacity (*e.g.* in women), whereas the ratio will, conversely, be low when a substantial proportion of the binding sites is occupied (*e.g.* in adult men). Based on a similar calculation, Kapoor *et al.* (14) came to the conclusion that the FAI is not valid for adult males.

The overall excellent correspondence between FT and AFTC levels indicates that FT is a reliable index of unbound T. Calculation of FT from total T and immunoassayable SHBG represents a simple and rapid method that under all conditions studied, except for pregnancy serum, yielded val-

ues very close, if not identical, to those obtained by equilibrium dialysis (AFTC).

Whereas our data show that within the physiological range of 40–50 g/L ( $5.8\text{--}7.2 \times 10^{-4}$  mol/L), the albumin concentration does not significantly affect FT values, it should be realized that this is only valid for these physiological concentrations. Moreover, there is good evidence that at least part of the albumin-bound T might be bioavailable (1). Hence, when the albumin concentration is expected to deviate significantly from normalcy, the actual albumin concentration should be determined, and FT and albumin-bound T calculated accordingly.

Such a situation exists during pregnancy. In our third trimester sera, the mean albumin concentration was  $32 \pm 1$  g/L. Hence, the actual albumin concentration had to be used to calculate FT and cSHBG, and although the AFTC is higher than that in nonpregnant women, the nonspecifically bound T concentration is in the normal range. Bammann *et al.* (15) as well as Wilke and Utley (7) observed increased AFTC as well as FT levels in pregnancy serum, although, as they pointed out, increased androgenicity is not a normal clinical feature of pregnancy. The normal concentrations for the bioavailable, albumin-bound T may explain the absence of virilization in the presence of increased FT and AFTC.

As to the observed differences between AFTC and FT concentrations, and thus between cSHBG and iSHBG concentrations in pregnancy serum, this is the consequence of the occupation of a substantial fraction of the SHBG-binding sites by estradiol. Indeed, AFTC as determined by dialysis is dependent upon the number of binding sites available for T. In the presence of competing steroids in concentrations corresponding to a substantial fraction of the T concentration, cSHBG will be significantly lower than iSHBG, as the latter measures all SHBG molecules regardless of whether they are available for T binding. Knowing the estradiol concentration as well as the association constants of estradiol for albumin and SHBG, the concentration of estradiol bound to SHBG can be calculated. For term pregnancy, with an estradiol concentration around 20 ng/ml (73.4 nmol/L), it can be calculated that about 50 nmol/L SHBG is occupied by estradiol and not available for T binding; this explains why the calculated cSHBG is significantly lower than the iSHBG value measured by IRMA, whereas FT is falsely lower than AFTC as a consequence of inclusion of the binding sites actually occupied by estradiol in the calculation of FT.

The excellent correlation of non-SHBG-T with FT and with the calculated nonspecifically bound T, which is a multiple of FT, respectively, is a strong argument in support of the validity of the calculated nonspecifically bound T as a parameter of the bioavailable fraction of T. Non-SHBG-T measured by ammonium sulfate precipitation was around 20 times the FT, whereas the calculated nonspecifically bound T, using an association constant of albumin for T of  $3.6 \times 10^4$  L/mol, was around 23 times the FT. The value for the association constant, which is obtained using pure human albumin, might be slightly lower in serum due to the presence of lipids, in which case  $N(K_a C_a + FT)$  might be closer to 20. In any case, the calculated nonspecifically bound T reliably reflects the non-SHBG-T.

In conclusion, this study shows that neither aFT nor FAI

is a reliable parameter of FT. The similar values of FT and AFTC as well as iSHBG and cSHBG obtained under various physiopathological conditions, provided no competing steroids are present in a high concentration, show that the calculated FT is a reliable index of FT, that calculated non-specifically bound T reflects reliably non-SHBG-T (bioavailable T), and that immunoassayable SHBG is a reliable measure of SHBG-binding sites.

### References

1. Manni A, Partridge WM, Cefalu W, et al. 1985 Bioavailability of albumin bound testosterone. *J Clin Endocrinol Metab.* 61:705-710.
2. Vermeulen A, Stoica T, Verdonck L. 1971 The apparent free testosterone concentration, an index of androgenicity. *J Clin Endocrinol.* 33:759-767.
3. McCann DS, Kirkish LS. 1985 Evaluation of free testosterone in serum. *J Clin Immunoassay.* 8:234-236.
4. O'Connor S, Baker HWG, Dulmanis A, Hudson B. 1973 The measurement of sex steroid binding globulin by differential ammoniumsulfate precipitation. *J Steroid Biochem.* 4:331-339.
5. Cumming DC, Wall SR. 1985 Non sex hormone binding globulin bound testosterone as a marker of hyperandrogenism. *J Clin Endocrinol Metab.* 61:873-876.
6. Mathur RS, Moody LO, Landgrebbe S, Williamson HO. 1981 Plasma androgens and sex hormone binding globulin in the evaluation of hirsute patients. *Fertil Steril.* 35:29-37.
7. Wilke TJ, Utley DJ. 1987 Total testosterone, free androgen index, calculated free testosterone and free testosterone by analog RIA compared in hirsute women and in otherwise normal women with altered binding of sex hormone binding globulin. *Clin Chem.* 33:1372-1375.
8. Rosner W. 1997 Errors in the measurement of plasma free testosterone. *J Clin Endocrinol Metab.* 82:2014-2015.
9. Vermeulen A. 1973 Testosterone in plasma: a physiopathological study. *Verh. Koninkl Acad Geneesk Belg.* 35:95-180.
10. Moll Jr GW, Rosenfield RL, Hecke JH. 1981 Estradiol-testosterone binding interaction and free plasma estradiol under physiological conditions. *J Clin Endocrinol Metab.* 52:868-874.
11. Södergard R, Bäckström T, Shanbhag V, Carstensen H. 1982 Calculation of free and bound fractions of testosterone and estradiol 17 $\beta$  to human plasma protein at body temperature. *J Steroid Biochem.* 26:801-810.
12. Sinha-Hikim I, Arver S, Beall G, et al. 1998 The use of a sensitive equilibrium dialysis method for the measurement of free testosterone levels in healthy cycling women and in human immunodeficiency virus-infected women. *J Clin Endocrinol Metab.* 83:1312-1318.
13. Winters SJ, Kelly DE, Goodpaster B. 1998 The analog free testosterone assay: are the results in men clinically useful? *Clin Chem.* 44:2178-2182.
14. Kapoor P, Luttzell BM, Williams D. 1993 The free androgen index is not valid for adult males. *J Steroid Biochem Mol Biol.* 45:325-326.
15. Bammann BL, Coulam CB, Jiang N. 1980 Total and free testosterone during pregnancy. *Am J Obstet Gynecol.* 137:293-298.