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A THEORETICALLY SOUND AND PRACTICABLE EQUILIBRIUM DIALYSIS METHOD FOR MEASURING PERCENTAGE OF FREE TESTOSTERONE

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Summary—Free testosterone measured in serum equilibrated *in vitro* is considered a good index of biologically available testosterone even though a large part of free testosterone *in vivo* is derived locally from rapid dissociation of testosterone bound to albumin. The most accurate method for measuring free testosterone, however, is unsettled. The classical method—equilibrium dialysis—has been questioned because of the dilution of serum that it entails and the previous inability to achieve identical results with diulted and undiluted serum. Essentially identical measurements of free testosterone were achieved in diluted and undiluted charcoal-stripped serum by using the dialysis method and calculation reported here. The measured free testosterone in undiluted whole serum from women was only 4–6% lower than the estimated physiological values. These results were obtained using a validated calculation, controlling pH, using physiological bicarbonate buffer at 37°C, maintaining a constant free ligand concentration for dilutions, measuring the water gain by the dialysis bag, and using highly purified labeled testosterone. The mean free testosterone for normal women was 0.17 ng/dl (0.11–0.23) and for hirsute women was 0.49 ng/dl (0.27–0.71). The testosterone not bound to testosterone-estradiol binding globulin, calculated from free testosterone and albumin concentrations, was close to the production rate/min of testosterone. The method should be adaptable to other ligands.

INTRODUCTION

The free (non-protein bound, dialyzable) testosterone (FT) generally has been considered to be a biologically active fraction of the total testosterone present in plasma [1–3]. The major portion of testosterone in women is bound to testosterone-estradiol binding globulin (TeBG), a smaller portion to serum albumin. Since testosterone bound to albumin dissociates rapidly, it also has been considered biologically active [4–7]. FT and non-TeBG bound testosterone (NTBT) are equally well correlated to the MCR and blood conversion ratios of testosterone [7], and there is parellelism of FT and NTBT with respect to the unidirectional extraction of testosterone by brain [6]. Therefore, both FT and NTBT are valid measurements for determining the androgenic status of women with acne, hirsutism, oligomenorrhea and infertility [5, 8–10].

Equilibrium dialysis has been the classical method for determining FT [5, 8, 11–13] and remains the reference method for other methodologies, although there have been some objections to it [3, 14–17] and there has been controversy over whether diluted or undiluted plasma should be employed [8, 12, 18].

Our purpose is to report both a novel calculation of percentage of free ligand from equilibrium dialysis data and a method of dialysis under physiological conditions. It is also to establish that equilibrium dialysis, when performed as described, is an accurate method for determining FT and is practical for clinical use. In addition, by the simple expedient of measuring the albumin concentration in addition to determining the FT, the NTBT can be calculated, yielding a value that is close to the biologically available testosterone.

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EXPERIMENTAL

Serum samples

Blood from volunteer normal, premenopausal women without endocrinopathy, hirsute women, or normal men was allowed to clot and was centrifuged within 30 min. The serum was stored at −20°C. All experimental work used serum pools. Each pool contained samples from 3–10 hirsute or normal women.

Materials

Essentially globulin-free human serum albumin and diatomaceous earth, grade III, were purchased from Sigma Chemical Company (St Louis, MO). Activated charcoal was Norit A from Matheson, Coleman and Bell (Norwood, OH). Sodium chloride was Fisher Scientific Company's Certified Biological grade (Fairlawn, NJ). Unlabeled testosterone was from Steraloids, Inc. (Wilton, NH). [1,2,6,7-^{3}H(N)]testosterone (T) (98.8 Ci/mmol) and [7-^{3}H(N)]T (25.0 Ci/mmol) were from New England

Nuclear Corp. (Boston, MA). Absolute ethanol and ethyl acetate were redistilled. All solvents were HPLC grade. Bromcresol green albumin reagent and albumin standards were obtained from American Monitor (Indianopolis, IN). Diethylmalonic acid was from Calbiochem (Lajolla, CA). All other buffer salts were from Sigma Chemical Company. All other chemicals were reagent grade quality. Regenerated cellulose dialysis tubing was Visking tubing, size 8, from Union Carbide (Chicago, IL). A wire rack to hold 24 glass-stoppered, 15-ml centrifuge tubes at 13° from horizontal was made by E.S. Umstot. The wire rack and sample tubes were incubated in a Dubnoff metabolic shaking incubator. Sterilization filters were Millex-GS, 0.22 m, from Millipore Corporation (Bedford, MA). DEAE-Sephadex was from Pharmacia Fine Chemicals (Piscataway, NJ). The MPS-1 ultrafiltration apparatus and YMT filters were from Amicon Corporation (Danvers, MA).

Dialysis buffer

The 20–25 mM bicarbonate buffer was 145 mM Na^+, 5.0 mM K^+, 1.2 mM Ca^{+2}, 1.0 mM Mg^{+2}, 1.2 mM $(HPO_4)^{-2}$, 0.2 mM $(SO_4)^{-2}$, 129 mM Cl^-, and 1.5 mM N_3^-. It was made from 1.848 g (22 mM) $NaHCO_3$, 7.02 g NaCl, 0.375 g KCl, 0.133 g $CaCl_2$ 0.203 g $MgCl_2 \cdot 6\ H_2O$, 0.166 g $NaH_2PO_4 \cdot H_2O$, 0.028 g Na_2SO_4, and 0.100 g NaN_3 per l. The amount of bicarbonate was determined by the amount needed to produce a final pH of 7.40 ± 0.02 in the exterior solution at 37°C with a particular tank of 5% CO_2:95% N_2, and thereafter was a constant. The buffer was stable for at least 4 weeks if kept stoppered and stored at 2–6°C.

Purification of labeled testosterone

Using well mixed diatomaceous earth–ethylene glycol (2g:ml), 0.5 cm i.d. × 8.0 cm chromatography columns were packed and washed with 3.0 ml of isooctane. Nitrogen gas pressure was used to produce solvent flow. Labeled testosterone (labeled T) [0.1 mCi] was added to the column in isooctane. To elute the testosterone, 5 ml of isooctane were followed by 10 × 1.0 ml of 9% ethyl acetate in isooctane. The major peak fractions were pooled, the solvent was evaporated under N_2, and the purified testosterone was redissolved in absolute ethanol and stored at −20°C. It was repurified at least every 2 months.

Charcoal stripping of serum

Six-hundred mg of activated charcoal were added to 4 ml of serum, and the mixture was incubated at room temperature for 30 min. After centrifugation to remove most of the charcoal, the remaining charcoal was removed by passing the serum through Millex-GS sterilizing filters. A clear, charcoal-free serum was obtained and stored at 2–6°C.

Serum not treated with charcoal has been designated "whole serum".

Equilibrium dialysis

Dialysis tubing (1.0 cm wide, flat) was cut into 17 cm strips and soaked in two washes of distilled water for 20 min each. A knot was tied in one end of each tube, the knot was tightened with the aid of a paper towel, and the resulting bag was placed horizontally in buffer.

Serum was incubated for 15 min or longer with labeled T so that 0.2 ng of steroid was associated with 0.5 ml of undiluted serum in a 13 × 100 mm test tube. Typically, this was about 96,000 cpm of [1,2,6,7-^{3}H]T (0.27 ng) which was dried under N_2 and to which 0.6 ml of undiluted serum was added. When diluted serum was used, less radioactive steroid was added. (See section on Dilution.)

The buffer remaining in a bag was almost completely removed with a photographic strip film squeegee, 0.500 ml of sample was pipetted into the bag, and a knot was tied in the open end and tightened. Excess tubing beyond the knot was cut off. The bag was rinsed to remove any serum from the outside, blotted dry with tissue, weighed immediately to the nearest mg, folded in the middle, and gently wedged into the bottom of a 15 ml conical glass centrifuge tube that already contained 1.5 ml of the bicarbonate buffer. The air in the tube was displaced by a stream of 5% CO_2:95% N_2 (or O_2), a glass stopper was inserted, a drop of water was applied to seal it, and the stopper was wrapped with a small piece of parafilm.

The tubes were incubated for 4 h at 37°C while held in a wire rack at 13° from the horizontal with gentle shaking at 90 oscillations per min.

After dialysis, the pH of the exterior solution of experimental tubes was measured. The dialysis bag was then removed from the tube, rinsed briefly with water, blotted as before, weighed, one end cut off, and the contents squeezed out with forceps into a 12 × 75 mm borosilicate tube.

A 1.0 ml aliquot of the exterior solution and a 0.2 ml aliquot from the dialysis bag (0.3 ml if diluted serum was used) were pipetted into counting vials. To equalize counting efficiency, 0.8 (0.7) ml of buffer was added to the 0.2 (0.3) ml aliquots. Ten ml of 0.4% 2,5-diphenyloxazole (PPO) in toluene were added to each counting vial, shaken for 10 min on an Eberbach shaker, and counted in a Searle liquid scintillation counter for 20 min or 80,000 gross cpm for experimental work, 10 min or 40,000 cpm for clinical work. The counting efficiency was 49.7%.

The percentage of free testosterone in the original undiluted serum (pFT) was calculated using the following formula which contains an integral correction for dilution. A mathematical derivation is given in the appendix.

$$\% \text{ Free steroid in undiluted serum} = \frac{E \times V_o}{(E \times V_o) + (B \times V_t) - (E \times V_w)} \times 100 \quad (I)$$

where,

E = exterior solution, steroid cpm/ml

V_o = original serum water volume $= \frac{\text{ml sample in bag} \times 0.95}{\text{sample dilution factor}}$

B = bag interior solution, steroid cpm/ml

V_t = total volume of the bag at equilibrium, ml

V_w = water volume of the bag at equilibrium, ml

The original serum water volume, V_o, is the ml of undiluted serum in the dialysis bag × 0.95, which corrects for the volume occupied by serum proteins. For 0.496 ml (0.500 ml pipetted minus 0.004 ml lost above the knot) of undiluted serum, V_o is 0.471 ml. For 0.496 ml of a 1/5 dilution, $V_o = 0.0942$.

The total volume of the dialysis bag at equilibrium, V_t, is the sum of the following components: the volume of the sample added to the bag (+0.500 ml), the sample lost when the tubing above the final knot is cut off (−0.004 ml), the residual buffer in the bag after using the film strip squeegee (+0.009 ml), the water evaporated from the knots during dialysis (+0.009 ml), and the weight (1 g = 1 ml) change due to water gain during dialysis (variable). For a 0.500 ml serum sample, $V_t = 0.514 +$ water gain. For clinical use, 0.500 ml and water gain may be adequate for V_t since only a +4% error is introduced if the small volume gains and loses are ignored. These volume changes were determined gravimetrically.

The final dialysis bag water volume, V_w, is equal to: V_t − (ml of serum sample × 0.05). for 0.500 ml of an undiluted serum sample, this simplifies to V_t − 0.025 ml.

Unless stated otherwise, the experimental work was performed in quadruplicate. Intrassay precision between the groups of four was ±1.0% coefficient of variation (CV), $n = 16$. The pFT ranges of normal women, hirsute women, and normal men were determined in duplicate. Interassay precision was ±2.8% CV at 1.35 pFT, $n = 12$.

Total testosterone

Total testosterone was determined by RIA after sample extraction and chromatography on diatomaceous earth–ethylene glycol, 2:1 w/v [19].

TeBG and albumin

TeBG binding capacity was measured by an ammonium sulfate precipitation method [20]. Albumin was assayed using bromcresol green [21].

FT and NTBT, ng/dl

Once the pFT and concentrations of total testosterone and albumin have been determined, the ng/dl or molar concentration of FT and NTBT can be calculated as follows:

Free testosterone (FT) concentration in serum

$$= \frac{\text{pFT(T)}}{100}$$

Non-TeBG-bound T (NTBT) concentration in serum

$$= \left[(K_a)(\text{M})\left(\frac{\text{pFT}}{100}\right)(\text{T})\right] + \left[\left(\frac{\text{pFT}}{100}\right)(\text{T})\right]$$

where,

T = total testosterone concentration

M = molar concentration of albumin (68,500 mol. wt)

K_a = association constant for albumin and testosterone ($3.28 \times 10^4\ M^{-1}$)

Ultrafiltration

Whole serum ultrafiltrate was produced using Amicon's MPS-1 apparatus at 37°C and pH 7.50 [22].

RESULTS

Calculation of per cent free ligand

Table 1 compares pFT results obtained from one set of dialysis data using the formula reported here and seven other formulas reported in the literature. The raw data came from quadruplicate dialyses of a charcoal-stripped pooled serum, a 1/19.6 dilution of that pool, and a 1/20 dilution of a 4.2 g/dl solution of human serum albumin. The suitability of these formulations for use in their reported applications is not being questioned. Rather, the results obtained by these methods are given to explore the breadth of their application. Similar values of pFT for diluted

Table 1. Comparison of methods of calculating percentage of free ligand directly from dialysis data

Method of calculation	pFT in serum, undiluted	pFT in same serum, 1:19.6 dilution	pFT in albumin, 1:20 dilution of 4.2 g/dl
1. Sandberg [23]	1.89	23.48	53.37
2. Florini [24]	1.81	(23.44)	(53.26)
3. Oppenheimer [25]	(5.29)	40.95	61.46
4. Rivarola [11]	1.89	1.20	2.67
5. Westphal [26]	1.89	1.54	5.41
6. Chopra [27]	1.86	a	a
7. Kley [12]	1.58	(23.48)	(53.37)
8. Umstot (this work)	1.48	1.45	5.13

Methods 1, 2, 3, and 7 had no dilution factor. Numbers in parentheses indicate author either did not test or recommended against this dilution state, but values are included for comparison.

[a]Author made dilution values equivalent to undiluted serum values by definition, therefore an independent dilution value cannot be obtained.

and undiluted samples were obtained only with Westphal's formulas and that of this paper. Westphal's formulas contain no provision for the partial specific volume of proteins or water gain of the dialysis bag.

Proof of equilibrium and stability of the system

The dialysis system using undiluted serum equilibrated in about 3 h as shown in Fig. 1. No significant changes were detected in the pFT of redialyzed sera after an initial dialysis of 18 h.

Purity of Tritium-labeled T

After 2 months of storage at −20°C, the purified [1,2,6,7-^{3}H]T showed 0.4% radioactivity impurity when it was repurified. A 1% nonbinding impurity could theoretically cause up to a 200% increase in apparent pFT. However, no significant change in the serum control pFT values was detected. Whatever the nature of the impurity in the purified radiolabeled T, it had no effect on pFT.

When labeled T was exposed to air for 20 min after being dried under N_2, the pFT was 1.02 ± 0.01 SD, compared with a pFT of 1.03 ± 0.02 SD when serum was added immediately. Thus, no oxidation of labeled T occurred in the time tested.

The balance of the 5% CO_2 used to adjust the final pH of the system can be either N_2 or O_2. In a test of five different sera, the mean pFT using 5% CO_2:95% N_2 differed from that using 5% CO_2:95% O_2 by only $0.2\% \pm 0.8$ SD.

Effect of the addition of labeled T

The mass associated with radiolabeled T added during the experiment can be calculated from its specific activity and the counting efficiency. The effect of this added mass on dialysis equilibrium, hence on the pFT, obeys the law of mass action. Figure 2, generated from a computer model of an equation by Vermeulen [8], shows this effect to be nearly linear over the range of concentrations used in this study. The effect of added testosterone with and without a correction based on this approximate linearity is illustrated in Fig. 3. Corrections are not needed for undiluted clinical specimens since the effect of added labeled T is so small; the standard addition of 80,000 cpm of [1,2,6,7-^{3}H]T causes only a 1.7% elevation of FT in normal females and 0.4% elevation in normal males.

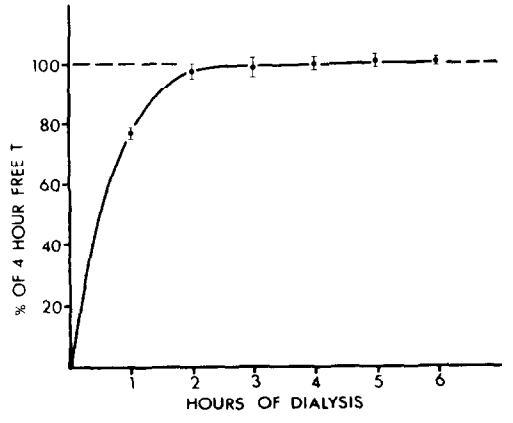

Fig. 1. Time for equilibrium of undiluted serum pFT to be established. Limits are ±1 SD.

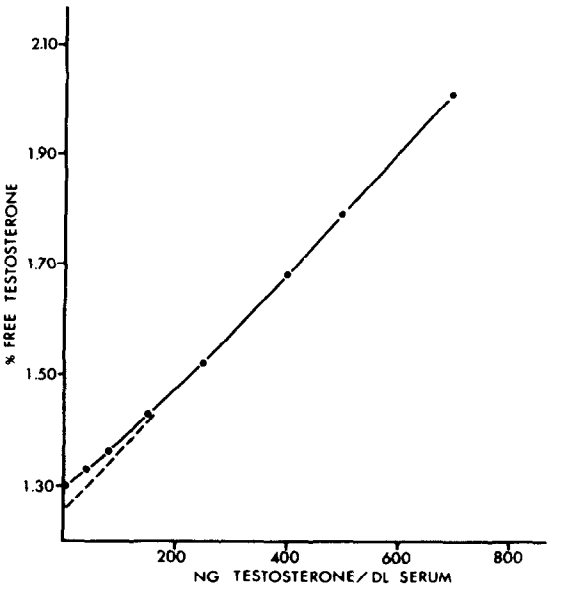

Fig. 2. Computer-generated pFT with increasing testosterone mass. Assumed 0.8 μg T/dl TeBG and 4.2 g/dl albumin. The computer program solves a quadratic rearrangement of the statement by Vermeulen [8] of the law of mass action for FT [10]. The dotted line is a linear extrapolation of the upper portion of the graph.

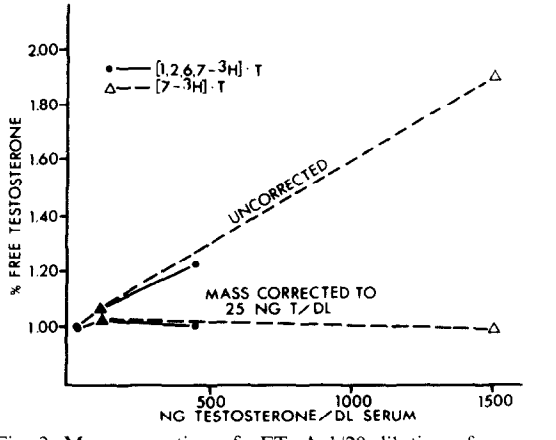

Fig. 3. Mass correction of pFT. A 1/20 dilution of serum was used. For [1,2,6,7-^{3}H]T there were 450 ng T/100,000 cpm, for [7-^{3}H]T there were 1500 ng T/100,000 cpm. Mass correction:

$$\frac{\text{Theoretical pFT at initial ng T/dl}}{\text{Theoretical pFT at dialysis ng T/dl}} \times \text{Uncorrected pFT} = \text{Mass corrected pFT at initial ngT/dl}$$

TeBG of this serum = 1.2 μgT/dl binding capacity. Mean SD at each point = ±0.03. The theoretical pFT was obtained by the computer program used for the data in Fig. 2. For this figure, 25 ng T/dl was the initial T concentration.

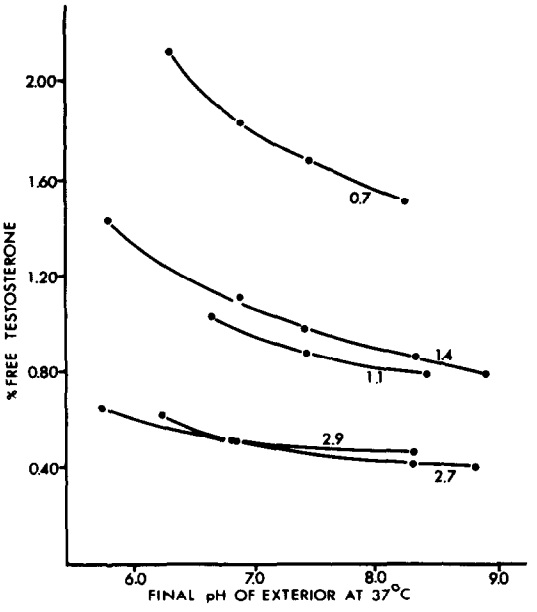

Fig. 4. Effect of pH on pFT in serum. Each line is a different serum, 1/20 dilution, and the numbers beside the lines are the TeBG binding capacities of the undiluted serum (μg T/dl). Average CV = $\pm$2.6%.

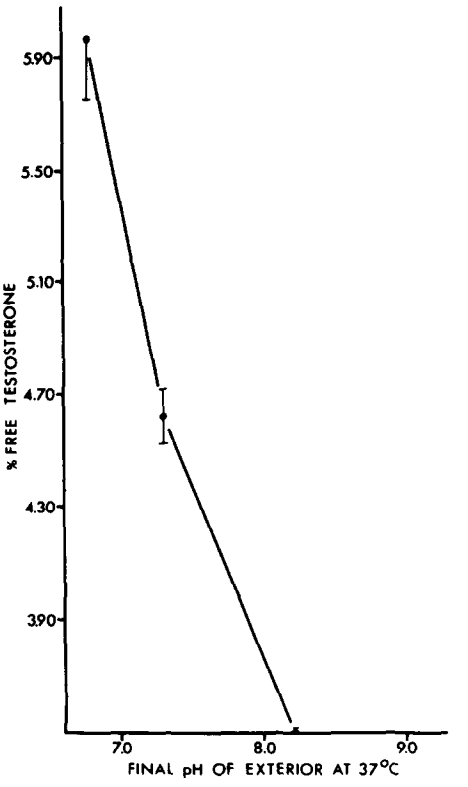

Fig. 5. Effect of pH on pFT in a human serum albumin (HSA) solution. A 1/20 dilution of 4.2 g/dl HSA was used. pFT values are for 4.2 g/dl. Each point, $n = 3$. Limits on points are $\pm$1 SD. No measurable TeBG was present.

Effect of pH on pFT

Figure 4 shows that decreasing pH causes a curvilinear increase in pFT. The pH was lowered by reducing the $NaHCO_3$ concentration and was raised by not using 5% CO_2. When the $NaHCO_3$ concentration was reduced, the NaCl concentration was increased to maintain 145 mM Na^+. For a serum with an initial pH of 8.8, it took 40 min to reach the final pH of 7.40 in the dialysis system. Over 3 h (the time required to reach equilibrium) of dialysis time remained. The bicarbonate ion (5–30 mM) itself caused only a 1.5% $\pm$ 3.2 SD elevation in FT. Therefore, the changes in Fig. 4 were due to pH, not to time or bicarbonate ion concentration. Note that sera with lower TeBG values have higher slopes of pFT versus pH. Using purified human serum albumin (Fig. 5), an even higher slope of pFT vs pH was observed.

Effect of Buffers on pFT

After establishing that the pH of the dialysis system must be controlled, several buffers were tested to see if they would be suitable. PIPES [piperazine-*N*-*N*-*bis*(2-ethanesulfonic acid)], TRIS–HCl, diethylmalonic acid, and imidazole all elevated pFT to some extent (9–33% at 50 mM concentration). HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 50 mM, elevated pFT 2% but was statistically significant at only an $\alpha = 0.10$ level, using a one-tailed *t*-test, and is acceptable for clinical use. Phosphate was not tested because it precipitates calcium from 1.2 mM calcium chloride at pH 7.4. The bicarbonate buffer system of the blood was therefore used for dialysis. Major inorganic ions were included in the buffer to give it a physiological composition. Sodium azide was included as a preservative and had no effect on pFT. A system with 1.5 mM NaN_3 yielded a pFT of 2.75 $\pm$ 0.05 SD, while the same serum in a system without NaN_3 yielded a value of 2.73 $\pm$ 0.08 SD.

Dilution

The most comprehensive test for validating a free ligand method is dilution since practically all uncontrolled variables will appear as a dilution effect. If the known individual variables are controlled, any subsequent dilution effect must be caused by some other variable. For studies on the effect of dilution, the amount of radiolabeled T added was such that the FT concentration was calculated to remain constant if testosterone were the only variable affecting FT. For a 1/5 sample dilution this was typically 20,000 cpm/0.5 ml and for a 1/20 sample dilution it was about 9,000 cpm/0.5 ml.

When serum was diluted with buffer at constant pH and the theoretical effect of testosterone mass was corrected as described above, the pFT decreased (Fig. 6, WHOLE serum lines). This is the predicted effect if substances competing with testosterone for binding sites were reduced in concentration as occurs with dilution. When these substances were removed from another aliquot of the same serum pool by charcoal

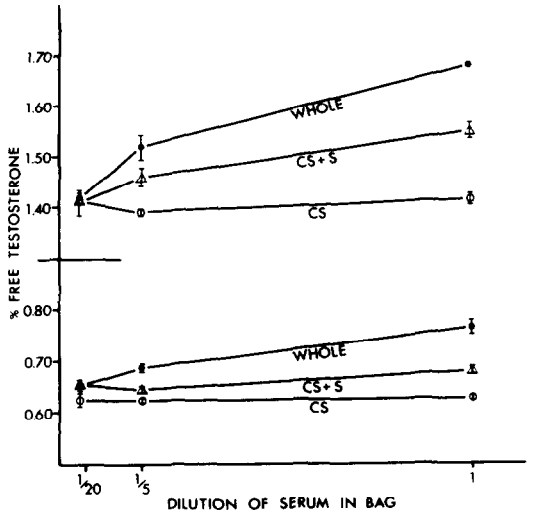

Fig. 6. Dilution. WHOLE = whole serum, i.e. not charcoal-stripped. CS = charcoal-stripped serum. CS + S = charcoal-stripped serum plus added steroids, which were 50 ng/dl of 5-androstenediol, 200 ng/dl of 4-androstenedione, 20 ng/dl of dihydrotestosterone, 15 μg/dl of cortisol, and 700 ng/dl of dehydroepiandrosterone. Limits are ± 1 SD. Upper and lower panels are two different serum pools. Reminder: pFT results are already corrected for sample dilution by the formula used (I).

stripping (95% of cortisol and 98% of testosterone were removed) and the pFT was plotted as a function of dilution, a horizontal line resulted (Fig. 6, CS lines). That is, dilution had no effect on the result. Diluting the charcoal-stripped serum after adding steroids, which compete with testosterone, produced a result similar to diluting whole serum (Fig. 6, CS + S lines), but not as pronounced. Adding these selected steroids elevated the pFT about 10%, whereas endogenous substances competing with testosterone elevated the pFT 21% $\pm$ 9 SD.

In whole, unstripped serum, such as would be used for clinical testing, the effect of dilution of endogenous substances competing with testosterone is a possible problem, since the exterior solution will dilute any substance which can pass through the membrane. This exterior dilution together with any sample dilution may be referred to as system dilution. Figure 7 shows the effect of system dilution on pFT and the extrapolated pFT values in the absence of all dilution, i.e. the physiologic state. Thus, pFT as measured by equilibrium dialysis of undiluted serum was 6.3 $\pm$ 2.6 SD lower than the estimated physiological equilibrium pFT. A more direct test was performed using the ultrafiltrate of a serum as the exterior solution rather than the buffer. Dialyzing against buffer yielded 4% $\pm$ 2 SD lower pFT values than dialyzing against the ultrafiltrate. By definition, the ultrafiltrate caused no system dilution, so that by this test, -4% is the error in the method due to the unavoidable dilution inherent to dialysis.

Association constants

The association constant (K_a) for albumin and testosterone binding was 3.28 $\pm$ 16 $\times$ $10^4 M^{-1}$ at 37°C and pH 7.4, assuming the molecular weight of albumin is 68,500. This average was derived from experiments with three serum albumin preparations obtained by DEAE-Sephadex chromatography in which no specific binding was found, and from Sigma's human serum albumin.

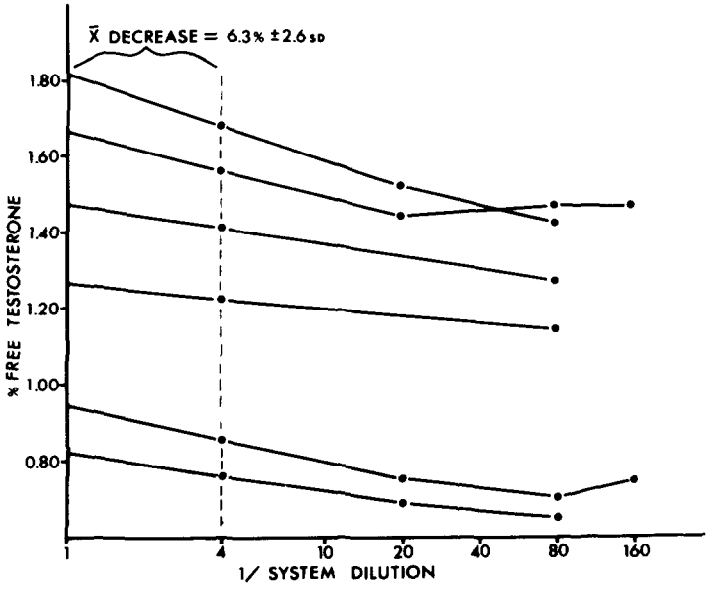

Fig. 7. System dilution and extrapolated physiological values. All samples were whole sera. A 1:1 system dilution is the extrapolated physiological condition. A 1:4 system dilution is 0.5 ml of undiluted serum in a bag in 1.5 ml of exterior solution, which is the standard dialysis arrangement. Average CV = 1.8% $\pm$ 0.7

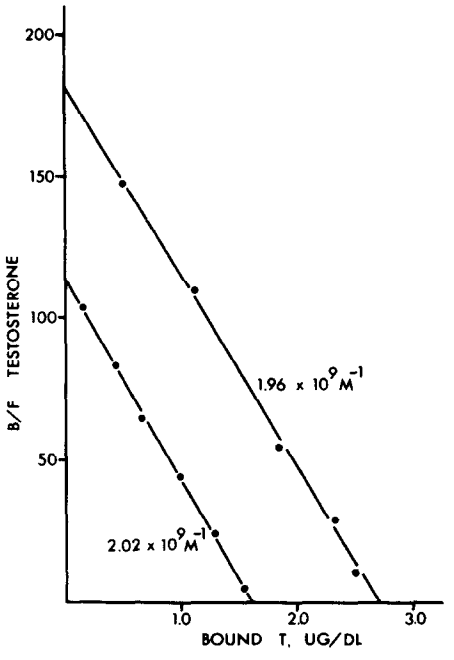

Fig. 8. Association constant (K_a) for TeBG-testosterone. A 1:5 dilution of charcoal-stripped serum was used. Each Scatchard line is a different serum pool. The number beside each line is the K_a (TeBG-T) for that pool. B/F is bound over free. Each point, $n = 3$. Average CV of pFT was $\pm 1.5\%$.

The K_a for TeBG and testosterone binding was 1.98 ± 0.03 SD $\times 10^9 M^{-1}$ at 37°C in three charcoal-stripped pools when determined by Scatchard analysis [28]. Nonspecific binding was subtracted from total binding to yield specific binding. Figure 8 shows specific binding from two of the pools, each with only one class of binding sites.

Clinical values for FT

Table 2 shows the ranges found for normal women and men and for hirsute women.

Table 2. Testosterone fractions: Values for normal and hirsute women and normal men

	Mean	±2 SD range	Data range
Normal women, $n = 9$			
FT, ng/dl	0.17	0.07–0.26	0.11–0.23
NTBT, ng/dl	3.7[b]	—	—
FT, %	0.75	0.45 1.05	0.63–1.01
NTBT, %	16.2[b]	—	—
Total T, ng/dl	23	10–35	12–31
Hirsute women, $n = 13$			
FT, ng/dl	0.49	0.22–0.97[a]	0.27–0.71
NTBT, ng/dl	11.4[b]	—	—
FT, %	1.22	0.58–2.29[a]	0.72–2.27
NTBT, %	26.4[b]	—	—
Total T, ng/dl	43	22–76[a]	30–99
Normal men, $n = 18$			
FT, ng/dl	7.54	3.83–13.5[a]	4.76–12.5
NTBT, ng/dl	175[b]	—	—
FT, %	1.50	0.89–2.12	0.99–2.05
NTBT, %	32.4[b]	—	—
Total T, ng/dl	541	290–935[a]	333–884

[a]Log transformed data.
[b]Assumes 4.3 g/dl albumin concentration.

NTBT and testosterone production rate

Since blood makes a single pass through the body in about 1 min (resting body blood transit time), the amount of testosterone cleared from the plasma in that minute divided by the amount of total plasma testosterone that passed through the body in that minute gives the fraction of testosterone that was cleared. The fraction cleared *in vivo* should be close to the fraction of NTBT determined *in vitro*, if the theory of the biological availability of albumin-bound testosterone is correct. Unlike Pardridge's single-pass studies in animals [6], the fraction cleared using (ligand cleared/min)/(total ligand passed/min) reflects homeostatic conditions in humans. Over a sufficiently long time interval, the amount cleared equals the amount produced, assuming metabolic homeostasis. The production rates (PR) for certain populations can be obtained from the literature (MCR × plasma concentration = PR).

As seen in Table 3, the percentage NTBT, as determined by the method presented in this work, is close to the percentage testosterone cleared and produced by the body. Percentage was used rather than mass to eliminate the error due to differences in total testosterone measurements

DISCUSSION

A great variety of methods have been used for free ligand determinations. Equilibrium dialysis is probably the oldest and most widely used method. However, some authors have raised objections to it [3, 14–17], particularly with regard to problems associated with dilution, and a wide range of normal values of FT and the binding constants of serum proteins and testosterone have been obtained [5, 8, 11–13]. Although ultrafiltration of undiluted serum has been recommended to overcome these variables [17], ultrafiltration has some potential problems that do not exist for dialysis: ligand adsorption, seiving effects, low count levels for the free ligand, and concentrated protein interactions [12, 29]. Consequently, this work was undertaken to develop a theoretically and technically sound equilibrium dialysis method and calculation.

The data in Table 1 indicate the importance of the formula used to calculate pFT from equilibrium dialysis data. Since the same dialysis data were used for all of the values obtained, the differences in results are due solely to the different formulas employed. Only the results of the two-step calculation of Westphal [26] and those of this paper are essentially independent of dilution. Westphal routinely used a 1/10 dilution, and for his formula to be used for undiluted serum the final protein concentration in the dialysis bag must be measured in addition to that of the original serum. When this is done, the value for the undiluted serum sample in Table 1 becomes 1.56. The results from the calculations of Vermeulen *et al.* [8] using an expression of the law of mass action to

Table 3. Production rate of testosterone and NTBT

Production rate determination	Normal women		Hirsute women		Normal men
Literature values					
MCR, liters plasma cleared 24 h	590[a]	650[b]	1160[a]	918[c]	1490[c]
T plasma concentration, ng/dl	38[a]	40[b]	80[a]	78[c]	800[c]
Body surface area, m^2	1.61[d]	1.61[d]	1.89[c]	1.89[c]	2.00[c]
Calculated values[e]					
T production rate (PR), ng/min	156	180	644	497	8280
Plasma flow, ml/min	3190	3190	3750	3750	3650
Total T in volume of plasma flow, ng/min	1210	1276	3000	2925	29200
$\frac{\text{PR, ng/min}}{\text{total T,ng/min}} \times 100 =$ % produced and cleared in one pass of total T in plasma	12.9	14.1	21.5	17.0	28.4
Testosterone produced and cleared per blood pass, mean %	13.5		19.3		28.4
NTBT, mean % at 37°C (this work)	16.2		26.4		32.4

[a]Kirschner and Bardin, [30].
[b]Vermeulen *et al.* [4], mean of 3 normal women.
[c]Vermeulen and Ando, [7], males 31 to 42 years only, ngT/dl estimated for males.
[d]Based on 165 cm height, 56 kg weight.
[e]Assuming that the body blood transit time = 1 min, that the cardiac output (blood flow) = 3.2 l/min/m^2, that the average whole-body hematocrit for women = 42% packed cell volume × 0.91 = 38%, and that the average whole-body hematocrit for men = 47% packed cell volume × 0.91 = 43%, Ganong [31].

calculate FT, and from that the pFT, also are not affected by dilution. The formula presented in this paper has been verified mathematically (Appendix).

To compare this formula (I) with that commonly used by other investigators for calculating pFT from dialysis of diluted serum, both formulas have been written in the dilution forms below (also see Appendix).

This paper:

$$\frac{(\text{free/ml})/\text{dilution}}{(\text{free/ml})/\text{dilution} + \text{bound/ml}} \times 100 = \text{pFT in undiluted serum} \quad \text{(II)}$$

Ref. [11]:

$$\frac{(\text{free/ml})}{(\text{free/ml}) + \text{bound/ml}} \times \frac{100}{\text{dilution}} = \text{pFT in undiluted serum}$$

[11] rearranged:

$$\frac{(\text{free/ml})/\text{dilution}}{(\text{free/ml}) + \text{bound/ml}} \times 100 = \text{pFT in undiluted serum} \quad \text{(III)}$$

The difference between the formulas is the placement of the dilution term. Both are percentage calculations where the part is divided by the whole and multiplied by 100. In this application, the part is the "free/ml" and the whole is "the free/ml plus the bound/ml."

In (III) the part "free/ml" is divided by the dilution factor in the numerator but not in the denominator, so the whole is greater than the sum of its parts. This arithmetic error causes an underestimate of pFT in diluted serum, the underestimate being greater with increasing dilution. Table 1, method 4, illustrates this.

Several investigators have noted that values of FT for undiluted serum obtained by applying (III) to data from dialysis of diluted serum are dilution dependent [3, 11, 18]. The arithmetic error in (III) is one cause of this dilution dependency. In addition, changes in the free ligand concentration with dilution are nonlinear due to the nature of the law of mass action [3], and if no attempt is made either to keep the free ligand concentration approximately constant or to correct for the changes in concentration, an error will be introduced. Using saline (uncontrolled pH) or impure labeled T can produce dilution-dependent results as well. The data in Fig. 6 (CS lines) show that the dialysis method and formula of this paper correct for dilution *per se*; the pFT values are the same within experimental error, with and without sample dilution. While this formula corrects for pure dilution and the dialysis method holds other variables constant, this formula cannot correct for the effects of diluting nontestosterone substances present in serum that bind to TeBG ("WHOLE" lines in Fig. 6). Therefore, undiluted serum should be used in equilibrium dialysis rather than diluted serum, measuring the water gain of the bag by weight differences.

Opposing views have been reported in the literature as to whether FT is affected by pH [32–34]. From a study of testosterone binding to purified TeBG, Lata *et al.* [34] concluded that binding was unaffected by pH. Studies with albumin have shown that steroid binding is affected by pH [33]. Figures 4 and 5 show that pH affects pFT and the effect is greater in sera with low TeBG levels than in those with high TeBG, and greater still in the albumin solution. These data are compatible with the conclusion of Lata *et al.* [34] that testosterone binding to TeBG is pH-independent and that to albumin is pH-dependent. It is clear that pH must be controlled. The bicarbonate buffer is physiological, but HEPES is satisfactory and more convenient for clinical assays.

Use of the described dialysis technique and formula for calculating pFT obviates the following

criticisms of equilibrium dialysis in the literature: The pFT obtained is dilution-dependent due to changes in testosterone concentration that are not taken into account [3, 16]; the association constant of testosterone binding to albumin changes with dilution [15]; and any correction for dilution is prone to error [8, 12, 15, 17].

This dialysis technique is convenient and practical. It utilizes a small volume of plasma or serum (0.5 ml) that makes it applicable to clinical work. Because of the small sample and exterior (1.5 ml) volumes, the system reaches equilibrium in 3 h. By continuing dialysis to 4 h, it is unnecessary to time the length of dialysis precisely. Twenty-four tubes can easily be run by a technician in 8 h.

Although NTBT is quantitatively closer to the amount of testosterone that is produced and cleared than is FT, the calculated values of biologically available testosterone for normal females, hirsute females, and normal males (Table 3) are still lower than the NTBT and much lower than the single-pass, unidirectional extraction of testosterone by rat brain for the same groups [6, 35]. However, if NTBT is preferred over FT as an *in vitro* measure, the method reported here will yield reliable results.

An equilibrium dialysis technique and a formula for calculating percentage FT from dialysis data that are theoretically sound and practicable for routine use have been presented. Accurate pFT and NTBT results, which are only 4–6% lower than the estimated physiological values, are obtained. The problem of dilution inherent to dialysis has been minimized by using undiluted serum and a small exterior volume. The importance of using an amount of labeled T that will not affect the measured FT and the need to control pH have been demonstrated. A physiological bicarbonate buffer was used. The formula for calculating pFT has been validated and is applicable to diluted as well as undiluted specimens, although undiluted specimens, which require measuring the water gain of the dialysis bag, should be used for the clinical measurement of pFT. The fact that constant results were obtained with dilution of charcoal-stripped sera is an overall verification of the dialysis system and calculation. This method should be applicable to all steroid and other nonionic ligands that are stable for the period required to reach equilibrium.

APPENDIX

The equation relating free and bound steroid (or other ligand) is as follows:

$$[SP] = [S] + [P]$$

where [S] is the free steroid concentration, [SP] is the bound steroid concentration, and [P] is the concentration of unbound protein sites. The dissociation constant for this reaction is by definition:

$$K_d = \frac{[S][P]}{[SP]} = \frac{[S_o][P_o]}{[SP_o]} \quad (A1)$$

where the first ratio indicates concentrations one would find at the conclusion of dialysis while the subscript "o" indicates original quantities (before dilution or dialysis).

Recognizing that the protein components are constrained to the same volume (that of the dialysis bag) so that the ratio of original to diluted protein concentration is equivalent to the mole per bag ratio for the protein, and assuming that the case is one of moderate dilution of a strongly binding species that is not close to saturation, then the mole per bag ratio of unbound sites is approximately equal to one (i.e. $P/P_o \simeq 1$). Equation (A1) can be rearranged to yield

$$[S_o] = [S]\frac{SP_o}{SP} \quad (A2)$$

The definition of percentage of free steroid in the original sample is defined

$$\%\,S_o = \frac{S_o}{S_o + SP_o} \times 100 = \frac{[S_o]V_o}{[S_o]V_o + SP_o} \times 100 \quad (A3)$$

Where V_o is the volume of the original sample.
Substitution of equation (A2) for $[S_o]$ in (A3) yields

$$\%\,S_o = \frac{[S]V_o}{[S]V_o + SP} \times 100 \quad (A4)$$

Assuming that at the conclusion of dialysis free steroid is distributed equally throughout the water in the system, then the free steroid inside the dialysis bag is measured by the free steroid outside the dialysis bag, and the protein-bound steroid, SP, is given by the difference between the total steroid and the free steroid inside the bag. Since these concentrations are measured as cpm/ml in the exterior (E) and as cpm/ml total in the bag (B), equation (A4) may be rewritten

$$\%\,S_o = \frac{EV_o}{EV_o + (BV_t - EV_w)} \times 100 \quad (A5)$$

where V_t is the total bag volume and V_w is the free water volume of the bag. Since E is measured in the absence of protein volume, V_o is now the free water volume of the original sample. (A5) is (I) in the text.

If a serum sample were diluted prior to dialysis, equation (A4) could be written in terms of the dilution factor (D) and of the volume of diluted serum used in the dialysis (V_d) as follows:

$$\%\,S_o = \frac{[S]V_d/D}{[S]V_d/D + SP} \times 100 = \frac{[S]}{[S] + [SP]\,D} \times 100 \quad (A6)$$

The equation commonly used [11] for correcting for dilution is

$$\%\,S_o = \frac{[S]}{[S] + [SP]} \times \frac{100}{D} = \frac{[S]}{[S]D + [SP]D} \times 100 \quad (A7)$$

Equations (A6) and (A7) differ in the first term of their denominators. In the limit that $[S] \ll [SP]$ these differences would be negligible with the two equations yielding comparable results. As the degree of dilution increases, however, this assumption becomes invalid, and equation (A7) increasingly gives % S values that are too low. For dialysis of average undiluted normal female serum as described in this paper for percentage of free testosterone, this error is 2.4%, and with the serum diluted 1/5 it is 13.3%; for normal men the error is 5.8 and 23.7%, respectively.

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