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SOME STUDIES ON THE BIOLOGICAL SIGNIFICANCE OF FREE TESTOSTERONE

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SUMMARY

It is generally admitted that the free, non-protein-bound, hormone fraction represents the biologically active fraction in plasma. As far as testosterone is concerned, there exists some indirect clinical evidence and a few experimental data supporting this hypothesis, but a more direct approach to this problem is hampered by difficulties in estimating the free fraction.

Using equilibrium dialysis of diluted plasma and extrapolating the results to undiluted plasma, using a mathematical model, the authors determined the apparent free testosterone concentration (AFTC), which corresponds in first approximation to the free testosterone concentration *in vivo*.

This AFTC was determined in normal adult, (pre)adolescent and hypogonadal males, in male diabetics, as well as in hirsute and pregnant women, and in hyperthyroid patients of both sexes; the values were compared with total plasma testosterone levels. The AFTC in normal males aged 20–50 yr varied between 5·5 and 20 ng/100 ml and decreased with age; this decrease was already significant in the age group 50–70 yr, whereas total testosterone concentration in these subjects was not different from younger adults. In females, the AFTC varied between 0·2 and 0·75 ng/100 ml. In (pre)adolescent males plasma testosterone levels and AFTC increased as a function of sexual maturity, but whereas sometimes adult plasma testosterone levels were already observed before full sexual development was achieved, adult AFTC levels were only obtained in subjects with full sexual maturity. In male hypogonadism, the decrease in AFTC was more pronounced than the decrease in total testosterone.

In female hirsutism, plasma testosterone levels and AFTC were variable, but the AFTC was increased more frequently than total plasma testosterone.

In pregnancy, whereas plasma testosterone levels were increased, the AFTC was normal or moderately decreased. In hyperthyroidism, finally, plasma testosterone levels were often increased, but the AFTC was normal.

The above-mentioned results support the hypothesis that only the free fraction is biologically active.

In order to test this hypothesis more directly, the authors investigated whether there exists a parallel between the free fraction and the metabolic clearance rate of testosterone. This was confirmed within the same subject and within a group of subjects of the same sex. Finally a significant correlation was observed between the AFTC and the formation of 5α-androstanediol.

As the latter is formed essentially in peripheral target tissues, this suggests that target tissues metabolize only the free non-protein-bound fraction.

IT IS generally considered that only the free non-protein-bound hormone fraction is biologically active. For steroid hormones this was first suggested by Westphal in 1955[1]. Slaunwhite *et al.*[2] in 1962, as well as Matsui and Plager[3] in 1966, advanced some experimental evidence that in both *in vivo* and *in vitro* systems, that only the non-transcortin-bound cortisol is active.

As far as testosterone is concerned, there is some indirect evidence that only the free fraction is biologically active: indeed, in hyperthyroidism[4] and in pregnancy[5] high total testosterone levels due to increased testosterone binding globulin (TeBG) levels are not accompanied by signs of virilisation, whereas in

some cases of hirsutism the total plasma testosterone may be normal, while the binding index is decreased[6]. The determination of the free testosterone concentration, however, presents considerable difficulties.

Indeed, the *in vivo* determination of the free testosterone fraction is practically impossible; hence, an index of free testosterone concentration has to be derived from *in vitro* studies under non-physiological conditions.

Equilibrium dialysis and ultrafiltration techniques[7], however, probably approach physiological conditions; unfortunately, due to the elevated binding affinity of plasma proteins for testosterone, resulting in a free testosterone fraction of the order of 1 per cent of total concentration, these techniques yield results subject to important errors when applied to undiluted plasma. Therefore diluted plasma has to be used, but the mathematical extrapolation of the results, obtained with diluted plasma, to undiluted plasma has often been highly simplified[8, 9].

Indeed, since testosterone is bound to plasma proteins with a limited binding capacity, of the order of the physiological testosterone concentration, binding is not a linear function of dilution. Moreover, in equilibrium dialysis the testosterone concentration in the plasma at equilibrium is significantly different from the original testosterone concentration, a fact which has often not been taken into consideration. Nevertheless, using data obtained by equilibrium dialysis of diluted plasma, it is possible to calculate an "apparent free testosterone concentration" [10], which should be a close approximation of the situation *in vivo*.

Indeed, as albumin, transcortin and the TeBG all bind testosterone, we have the following equation at equilibrium:

$$\Sigma_1 = (S) + (SA) + (SP_1) + (SP_2). \tag{1}$$

We could show[10] that at physiological testosterone concentration, the binding of testosterone to transcortin is negligible. Moreover, as the ratio of albumin-bound (SA) to free testosterone (S) is a constant, defined by the albumin content of plasma, equation (1) becomes:

$$\Sigma_1 = N(S) + (SP) \tag{2}$$

where Σ_1 is the total plasma testosterone concentration and (SP) is the TeBG-bound testosterone.
As at equilibrium

$$\begin{aligned} &(SP) = K(S)(P) \\ &\text{and } (P) = \Sigma\rho_T - (SP) \end{aligned} \tag{3}$$

where $\Sigma\rho_T$ equals the total concentration of binding sites on TeBG, (P) the free binding sites at equilibrium, and K the association constant of TeBG and testosterone, we have:

$$\Sigma_1 = N(S) + \frac{S \times K\Sigma\rho_T}{1 + (S)K}. \tag{4}$$

If it is accepted that between individuals there exist only quantitative and no qualitative differences in TeBG and hence that K is constant, this equation (4)

can be easily solved for $\Sigma\rho_T$. Indeed, as K and N are known and as (S) and Σ_1 (concentration of T at equilibrium) in diluted plasma can be determined by dialysis, it follows that $\Sigma\rho_T$ be calculated. From this value, and knowing the T concentration in undiluted plasma (Σ_1), (S) in undiluted plasma can be calculated.

Using this technique we have determined the "apparent free testosterone concentration" in several groups of subjects.

RESULTS

We found that in adult males, there exists a linear decrease of the free plasma testosterone fraction with age: in young adults (20–50 yr) the free fraction corresponded to $2{\cdot}05 \pm 0{\cdot}08$ per cent of total plasma testosterone; it decreased to a mean value of $1{\cdot}72 \pm 0{\cdot}12$ per cent in the age group 50–70 yr and to $1{\cdot}2 \pm 0{\cdot}14$ per cent in the age group 70–85 yr. This corresponds to a mean free testosterone concentration of $11{\cdot}6 \pm 0{\cdot}7$ ng/100 ml in the age group 20–50 yr, $8{\cdot}6 \pm 1$ ng/100 ml in the age group 50–70 yr and $4{\cdot}5 \pm 0{\cdot}8$ ng/100 ml in the age group 70–85 yr.

In *prepubertal boys* the free fraction was very low and only when full sexual maturity is achieved did we find a normal adult free T fraction.

In *normal females* the free testosterone fraction corresponded to $0{\cdot}98 \pm 0{\cdot}07$ per cent with a free T concentration varying between 0·2 and 0·75 ng/100 ml.

The free testosterone fraction was significantly increased in *hirsutism* but decreased in *pregnancy*, in *hyperthyroidism* and in *male hypogonadism*.

Our results are compatible with the view that the apparent free testosterone concentration reflects androgenicity better than total testosterone, and support the view that only free testosterone is biologically active.

If, as it is assumed, only the free fraction is biologically active at the target level, it can be expected that the metabolism of testosterone will be more rapid when the free fraction is increased and as metabolism at the target organs is different from splanchnic metabolism it can be expected that the metabolic pattern will also be different.

In order to minimize the influence of other factors that might affect testosterone metabolism, the effect of variations in free fraction on the metabolic clearance rate of testosterone was studied within the same subject at different levels of plasma testosterone. As can be seen in Table 1, the M.C.R. expressed in Liters/m^2, increased significantly with increasing free fraction.

Moreover, we observed a significant correlation ($r = 0{\cdot}64$, $n = 22$) between the free testosterone fraction and the M.C.R. This is suggestive evidence for a more rapid metabolism of the free non-specifically bound testosterone fraction.

Similarly, the decreased testosterone production rate, notwithstanding the high plasma T levels in hyperthyroidism observed by Dray *et al.*[6] and the decreased M.C.R. in hyperthyroidism observed by Gordon *et al.*[11] is compatible with this view. It is evident, however, that other factors also influence testosterone metabolism[12]: for example, differences in M.C.R. between males and females[13] or changes after the administration of certain drugs[14] cannot be explained exclusively by differences in testosterone binding but are also attributable to differences in enzymatic activity.

In order to determine whether differences in binding also have an effect on the metabolic pattern of testosterone, we determined the metabolites formed from testosterone in a group of young males and compared them with the metabolic pattern observed in a group of senescent males ($n = 14$).

Table 1. Free T fraction and Metabolic clearance rate (M.C.R.).

Females	TeBG mol/l	F.T. %	T ng/100 ml	M.C.R. (1/m²)
	1×10^{-7}	1	28	420
		1·8	22·50	680
	8×10^{-8}	1·2	42	400
		1·6	1000	670
	$8{\cdot}3 \times 10^{-8}$	1·2	36	400
		1·3	207	470
		1·5	605	480
		2·6	3100	580
Males				
	6×10^{-8}	1·6	232	580
		2·5	1950	1000
		3·1	2950	1100
	4×10^{-8}	2·2	650	950
		3·0	1500	1200
	8×10^{-8}	1·6	900	600
		2·2	2100	980

In the group of young males the free testosterone fraction was $2{\cdot}08 \pm 0{\cdot}12$ per cent whereas in the elderly males the free testosterone fraction was $1{\cdot}42 \pm 0{\cdot}12$ per cent old. The results are shown in Table 2.

It can be seen that male senescence is characterized by a relative increase of 5β over 5α metabolites, but more importantly by a decrease of the relative importance of the 17β-hydroxy metabolites.

Moreover, we found a significant correlation between the free T fraction and the conversion of testosterone to 5α androstane-3α, 17β-diol ($r = 0{\cdot}76$, $n = 22$).

As there is strong evidence that at least 50 per cent of 5α-androstane-3α, 17β-diol recovered in urine of males arises from extrahepatic metabolism[15], our results suggest that only non-specifically-bound testosterone can be taken up

Table 2. Urinary metabolites of testosterone in % of administered radioactivity

	Young adults 20–50 yr ($n = 6$)	Old adults 68 yr ($n = 14$)
Glucuronide fraction		
Etiocholanolone	$12{\cdot}5 \pm 1{\cdot}4$	$16{\cdot}1 \pm 1{\cdot}3$
Androsterone	$11{\cdot}8 \pm 2{\cdot}1$	$10{\cdot}1 \pm 1{\cdot}5$
Etioch/Androst	$1{\cdot}1 \pm 0{\cdot}2$	$1{\cdot}85 \pm 0{\cdot}25$
5β-anhrostane-3α 17β-diol	$3{\cdot}7 \pm 0{\cdot}4$	$1{\cdot}9 \pm 0{\cdot}5$
5α-androstane-3α, 17β-diol	$1{\cdot}8 \pm 0{\cdot}3$	$0{\cdot}7 \pm 0{\cdot}1$
5β-diol + 5α-diol	$5{\cdot}5 \pm 0{\cdot}5$	$2{\cdot}6 \pm 0{\cdot}7$
5β-diol/5α-diol	$2{\cdot}3 \pm 0{\cdot}3$	$4{\cdot}0 \pm 0{\cdot}9$

and metabolized by target tissues. Mauvais-Jarvis[16] arrived at a similar conclusion on the basis of results obtained in patients with testicular feminization. This is also in accordance with *in vitro* experiments of Lasnitzky (personal communication), who observed that the testosterone binding protein inhibits the effect of testosterone on prostatic tissue *in vitro*.

It seems therefore that the hypothesis that TeBG might have a specific carrier function rendering *T* available only to target tissues[17] should be abandoned.

Finally, we believe that our results suggest that "the apparent free testosterone concentration" is a close approximation of free testosterone *in vivo*, and that they offer further evidence that free testosterone represents the biologically active fraction.

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DISCUSSION

Crabbé: Alex, not knowing much about the field, I must say I was surprised by the fact that the fraction of unbound testosterone as you have determined it, is, if I got it correctly, the same for men and for women, i.e. about 1%.

Vermeulen: No, in young men between 20 and 50 yr old, it's 2%; in females, it's 1%. In male hypogonadism it's about 1·25%; between 50 and 70 yr it's about 1·75% and decreases to 1·40%. Before puberty, the values are more or less similar to the value in females.

De Moor: Do you have any data on the hepatic extraction in relation to the free fraction?

Vermeulen: No, we didn't determine this.

Munck: Have any cases been discovered in which there is a genetic inability to make a testosterone-binding protein, and if so, does it make any difference?

Vermeulen: I know of no patients who have a defect in the testosterone-binding globulin. For transcortin, De Moor and others have described some families. Pieter, do you have families with no testosterone-binding capacity?

De Moor: No, we measured many samples without finding one.

Martini: What is the evidence for saying that the 5α-reduced metabolites are not made in the liver?

Vermeulen: These are studies where differently labelled testosterone is administered both orally and intravenously, so comparing the metabolic pattern, and another series of experiments which have been done by Mauvais-Jarvis where testosterone is administered as a paste on the skin and at the same time administered orally or intravenously and comparing the metabolites. So it appears that under these conditions both the intravenously administered testosterone and the percutaneously administered results in more 5α-metabolites than orally administered testosterone.

Slater: Could you tell us at what temperature the equilibrium dialysis was carried out?

Vermeulen: All dialysis was performed at 37°C overnight (i.e. 15 h).