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CHOLESTEROL, HAEMOLYSIS AND METALS INFLUENCE ON TESTOSTERONE-PROTEIN BINDING

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SUMMARY

Testosterone was determined by the competitive protein-binding method using as the source of testosterone binding globulin (SBP) plasma from pregnant women giving birth to single, twins or triplets. Comparison of the standard curves obtained shows that the slope is optimal with plasma from women pregnant with twins or triplets. In the last case the curve was almost linear over the range of 0 to 4 ng of testosterone. Cholesterol in amounts of 1–20 μg and metal ions (Hg^{2+}, Ag^{+}, Cu^{2+}, Fe^{2+}) in a molar ratio of 0·7–14·0 to plasma proteins gave falsely increased testosterone values.

INTRODUCTION

BASED on the principle of competitive protein binding a number of methods have recently been presented[1–6] for the determination of testosterone in female plasma. These techniques offer a practical approach to routine, clinical studies. However, the specificity and sensitivity of the procedures described are not perfect, and need improvements.

In these methods, the source of the so-called testosterone-binding globulin (SBP)*, first reported by Mercier *et al.*[7], is late pregnancy plasma. The plasma is diluted to about 1% in order to minimize the non-specific binding of testosterone by albumin and to obtain a standard curve in a range adequate for the determination of the testosterone in plasma samples. It has been suggested that oestrogens play an important role in the increase of testosterone binding capacity (TBC) in plasma[8]. It has been previously reported that urinary oestriol in patients with twin pregnancies is increased considerably[9, 10] and that in general changes in the concentration of plasma oestrogens during pregnancy are reflected by changes in urinary levels[11]. For a more detailed study of oestrogen output in twin pregnancy see Adlercreutz and Luukkainen[12]. This led us to compare the testosterone binding capacity of late twin and single pregnancy plasma and to suggest that the former is preferable for the testosterone determinations[13]. In the present report the standard curves obtained with such plasmas are presented.

SBP is not entirely specific for testosterone, since it binds a number of other steroids as well to different degrees[14–16]. This is not only of academic interest but also of great practical importance since such steroids may be present in normal or pathological plasma samples, may interfere with testosterone binding

*During the IV Meeting of the International Study Group for Steroid Hormones in Rome, December 1969, it was agreed to use this abbreviation (instead of the previously used TBG) following the suggestions of Drs. E. E. Baulieu and U. Westphal (S = sex steroid, B = binding, P = plasma protein).

and give false results. In normal individuals the plasma cholesterol concentration is very high relative to testosterone, and cases of hypercholesterolaemia are not uncommon. The testosterone concentration in female plasma is approximately 50 ng per 100 ml, the ratio of testosterone to total cholesterol being consequently about 1 to 4,000,000. (Total cholesterol: range 135–260 mg/100 ml of plasma. Free cholesterol: range 40–160 mg/100 ml of plasma).

Since inadequate care in drawing blood samples and subsequent blood sample handling frequently give haemolysis, and since it has been reported that steroid binding occurs to some extent by different erythrocyte fractions (total haemolysate, isolated "ghosts", "ghost free" haemolysate)[17], the effect of haemolysis on SBP binding capacity was determined. It was found that provided no visible haemolysis was present in the original plasma sample used for standard testosterone binding curves the shape of the curve was not significantly affected.

Metal ion inhibition of the association between progesterone and α_1-acid glycoprotein (orosomucoid) has recently been reported[18]. Considerable interference of such binding was caused by Hg^{2+}, Ag^{+} Cu^{2+}, Fe^{2+}. In the present report, these metals have been found to interfere with the testosterone-protein association.

MATERIALS AND METHODS

(1,2-^{3}H)-Testosterone with a specific activity of 153 mC/mg was obtained from Amersham, England. Before use, its radiochemical purity was verified by thin-layer and paper chromatography. Non-labelled testosterone was purchased from Ikapharm, Ramat-Gan, Israel. The purity of the compound was verified by gas–liquid chromatography (F & M, Model 400, Avondale, Pa. U.S.A.) on a 1% SE-30 liquid phase, using the free steroid and its trimethylsilyl ether[19]. Florisil (magnesium silicate) 60–100 mesh (AB Kistner, Sweden) was washed four times with deionized water and the finer particles were decanted. The remaining particles were dried overnight at 100°C. Cholesterol (Sigma Chem. Corp., St. Louis, Miss., U.S.A.) was used after its purity had been verified by gas chromatography of the compound itself and its trimethylsilyl ether on a 1% SE-30 liquid phase. All chemicals employed in this work were of reagent grade. The metal salts employed in the inhibition studies were of reagent grade ($HgCl_2$, $AgNO_3$, $CuCl_2 \cdot 2\,H_2O$, $FeSO_4 \cdot 7\,H_2O$, Merck A. G., Darmstadt, Germany). All solvents were of analytical grade and used without further purification.

The plasma samples were obtained from normal healthy women pregnant with single or twins after overnight fasting. All women were in the 38th week of gestation. Blood samples from one woman with triplets in the 7th month of gestation were also used. Heparinized blood samples were drawn from a peripheral vein at 8 a.m. The blood was centrifuged immediately and the plasma separated and either assayed on the same day or kept frozen at −20°C. The pregnancy plasma was diluted with deionized water. Total serum proteins were measured with the biuret reaction, using the Technicon N-method and an Auto-Analyzer. Determination of albumin was performed electrophoretically with a Beckman–Spinco apparatus (Cellulose acetate slides in barbiturate buffer of pH 8·6.)

To determine binding, 1 ml of solution containing the dilute pregnancy plasma and the tracer steroid (approximately 15,000 dis/min) were pipetted into a small disposable plastic tube containing the testosterone for the standard curve, shaken

for 30 sec in a Vortex Junior mixer and cooled at 10°C for 30 min. Florisil, 40 mg, was added with a small plastic spoon, and the tube was mixed again 30 sec and placed in the water bath for 10 min (10°C). The radioactivity in a 0·2 ml aliquot of the supernatant was determined in a WALLAC NTL 314 (Turku, Finland) liquid scintillation spectrometer after addition of 10 ml of Bray's scintillation mixture (naphtalene 60·0 g, PPO 4·0 g, POPOP 0·2 g, methanol 100 ml, ethylene–glycol 20 ml, dioxan ad 1000 ml). The efficiency for tritium in unquenched samples in the WALLAC NTL 314 was 48%, with a background of 25 counts/min. The quenching effect due to the presence of the biological material was corrected by the external standard channels ratio method, the difference in counting efficiency being less than 0·5% in all experiments. All the experiments were done in duplicate and the figures presented are mean values. The difference between duplicates did not exceed 5%.

In the experiments with cholesterol, this sterol was added to the tubes together with testosterone in methanolic solution, the solvent was evaporated and the procedure continued as described above. In some experiments, different amounts of cholesterol were studied without added cold testosterone.

In the studies on the effect of haemolysis, erythrocytes from a late single pregnancy blood sample, with a mean corpuscle haemoglobin concentration of 32 g/100 ml, were washed three times with a double volume of normal saline, then haemolysed by the addition of deionized water (1 : 1, v : v), and the resultant haemolysate was added to the dilute protein-tracer mixture to a final haemoglobin concentration of 1–10 mg Hb/100 ml. The same pregnancy plasma was also the source of the SBP for the standard testosterone curves. One experiment was performed in the same way but with the haemolysate after removal of the membranes by high speed centrifugation.

To test the effect of metals, the dilute protein tracer solution was added to the metal salt dissolved in deionized water, just before the experiment. When cuprous and ferrous ions were tested, mM ascorbic acid was included in the incubation.

RESULTS

(1) Typical standard curves obtained with plasma from pregnant women giving birth to single or twins are shown in Figs. 1 and 2. The plasma was diluted to 1% and to 0·5%. The diluted plasma from women with twin pregnancy gave a steeper slope in the range of 1–6 ng of testosterone. The difference was especially pronounced in the range of 0–3 ng. Dilution of the plasma from 1% to 0·5% did not improve the curve obtained with plasma from women giving birth to singles, whereas with plasma from women giving birth to twins this change was pronounced giving a much more favourable slope of the curve. The results obtained with the 0·5% dilution of plasma from a triplet pregnancy in the seventh month of gestation are shown in Fig. 2.

(2) When 1, 2, 5, 10, 15, 20 μg of cholesterol were added to the tubes containing testosterone an effect approximately corresponding to 0·6–1·0 ng of testosterone was noted with respect to testosterone–protein interaction. Figure 3 shows the interference of 5 μg of cholesterol on the standard curve of testosterone.

(3) Addition of the red cell haemolysate containing the red cell membranes to the diluted protein–tracer mixture (final haemoglobin concentration of 1–5 mg/100 ml) did not change appreciably the testosterone binding curve, but with a

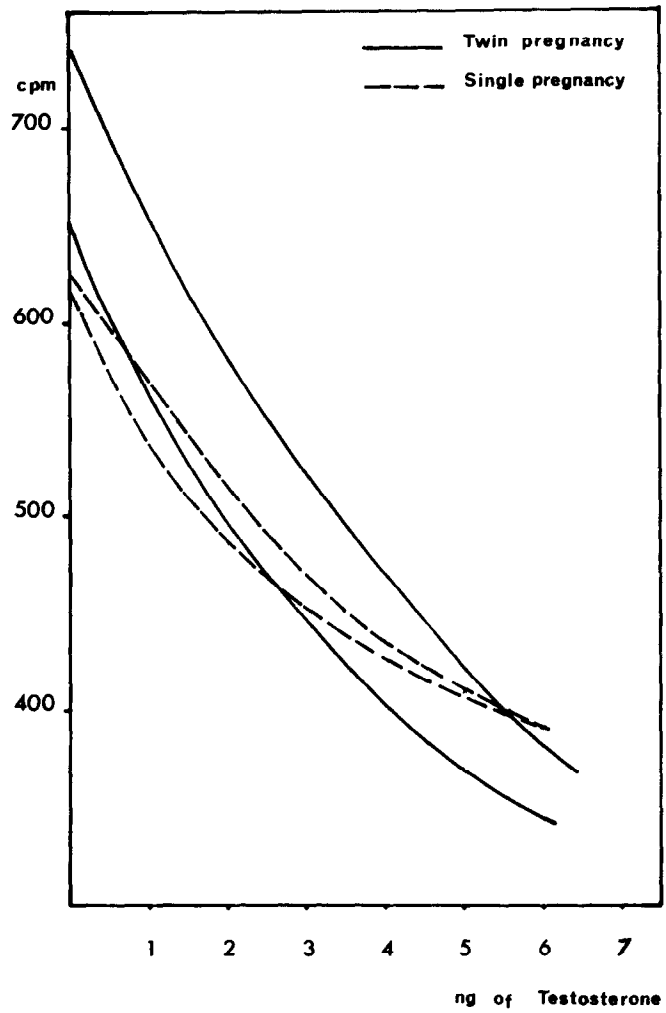

Fig. 1. Standard curves for testosterone binding obtained with plasma from two pregnant women giving birth to single and two to twins. The dilution of the plasma was 1%. Net counts/min of 0·2 ml aliquot of these plasma samples are plotted against ng of unlabelled testosterone added per tube.

higher red cell haemolysate concentration (5–10 mg/100 ml), the standard testosterone binding curve obtained was flattened out. Membrane-free haemolysate gave the same results.

(4) Metal ions were added to the protein–tracer solution in increasing amounts. On the assumption that the mean molecular weight of the plasma proteins was 100,000, the molar ratio of the metal ions to proteins ranged from 0·7 to 14·0. Throughout this range, the four metal salts tested would interfere with testosterone determination. The magnitude of their interference was $Hg^{2+} > Ag^{+} > Cu^{2+} > Fe^{2+}$ and in the presence of these ions the testosterone standard curve changed, giving falsely elevated results. With higher concentration of these metals, the curve was destroyed owing to denaturation of the protein(s). Figure 4 shows the curves obtained with molar ratio of metal ions to proteins 3·5/1.

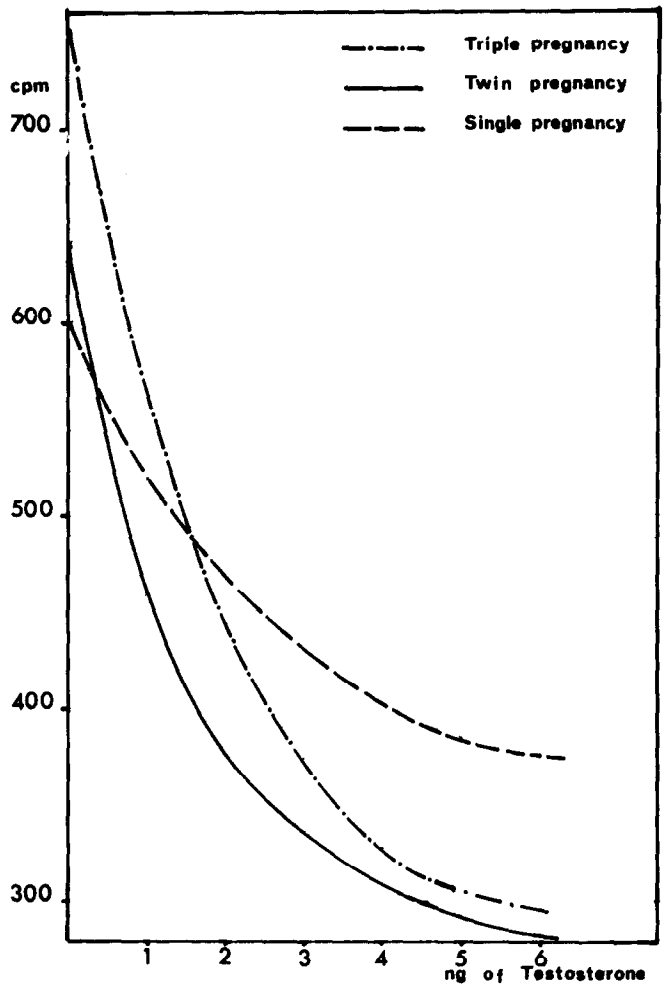

Fig. 2. Standard curves for testosterone binding obtained with plasma from women pregnant with single, twins, triplets. The dilution of the plasma was 0·5%. Experimental details are given in the text.

DISCUSSION

In trying to determine testosterone by the protein-binding technique one main problem is to find a suitable source of SBP. Adrenalectomized-hypophysectomized, oestrogen-treated patients have been used, but obviously blood from such patients is not easy to obtain. Albumin-free plasma from women in late pregnancy was tried[1] as the source of SBP but without better results than with diluted pregnancy plasma, since, owing to the great lability of this globulin, its binding properties are lost following purification[20, 21]. The same experience has been encountered in this laboratory. Blood plasma from women pregnant with twins[22] or pregnant with triplets seems to be a rather adequate source for SBP to be used for testosterone quantification.

The values for the testosterone-binding capacity (determined by the use of a Scatchard-type plot and the equilibrium dialysis technique) were: Single pregnancy plasma (mean of five cases) $4{\cdot}4 \times 10^{-7}$ M/l, twin pregnancy plasma (mean

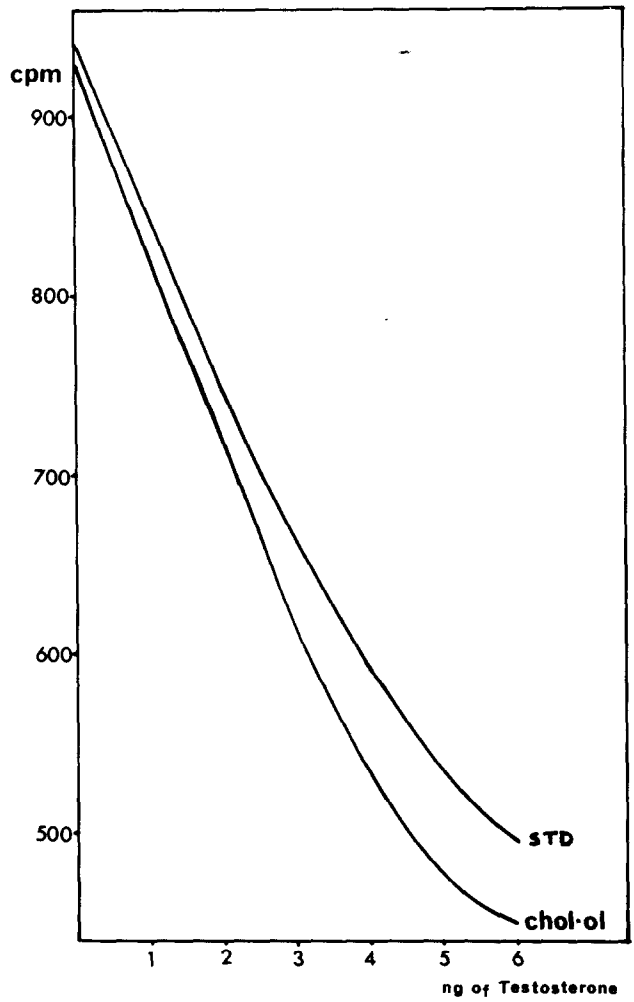

Fig. 3. Influence of the addition of 5 μg of cholesterol to standard binding curve for testosterone. The dilution of the pregnancy plasma was 1%. Experimental details are given in the text.

of five cases) $7{\cdot}0 \times 10^{-7}$ M/1, and triplet pregnancy plasma $8{\cdot}0 \times 10^{-7}$ M/1. Albumin contents were 3·54 g/100 ml (51·9% of total proteins), 3·22 g/100 ml (53·6% of total proteins) and 3·11 g/100 ml (51·8% of total proteins) respectively.

Twin pregnancies are not uncommon – 1/80 of the single pregnancies – and they are generally detected early, so plasma can be drawn in sufficient quantity. One ml of a 0·5% dilution is needed for routine testosterone determination. The TBC of the plasma must be determined in each case by the use of a Scatchard-type plot, because of individual binding variations due to the presence of one or two placentas, endogenous steroid level etc. This plasma is then divided into many portions stored at −20°C to avoid repeated freezing-thawing which eventually destroys the SBP. Confirming previous reports (on single pregnancy plasma), undiluted twin pregnancy plasma did not deteriorate during two to four months storage at −20°C, but with eight times freezing-thawing 50% of its binding capacity was lost.

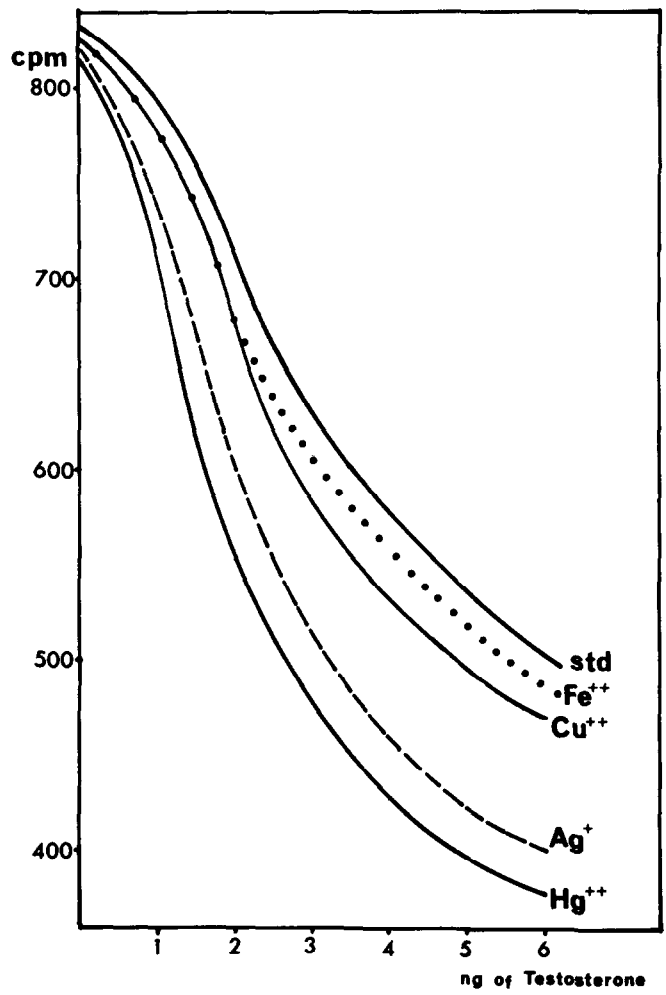

Fig. 4. Influence of Hg^{2+}, Ag^{+}, Cu^{2+}, Fe^{2+} on the testosterone binding curve when the metals were present in 3·5/1 molar ratio to the protein content. Dilution of the pregnancy plasma was 1%. Experimental details are given in the text. Std: no metals added.

The interference on the SBP-testosterone association due to a steroid is dependant not only upon steroid affinity, but also upon steroid concentration in the plasma and its presence in the final dry extract used for the assay. Strongly competing steroids such as oestradiol-17β are removed by alkaline washing; others by chromatography. The percentage binding of a great number of naturally occurring and synthetic steroids to SBP has been reported[14–16]. It is surprising that cholesterol, though present in blood plasma in such great amounts, has not been much studied. To our knowledge only two reports on this subject, with conflicting results, have been published. Murphy[23] reports a competition of 0·14% and Heyns *et al.*[16] no competition at all. In our experiments, using the florisil technique, competition in the order of 0·08–0·1% was found. Even if such a figure looks insignificant, 1–20 μg of cholesterol gives a value equivalent to 0·6–1·0 ng of testosterone, which if present in the assay system will alter the results. Since total cholesterol levels are commonly around 250 mg/100 ml of

plasma and since this compound is extracted by the solvents used, comparatively small amounts of cholesterol must be present in the final dry extract of the unknown plasma sample after the chromatographic separation. The mechanism by which cholesterol interferes is not clear, but this sterol will interfere with progesterone–protein interaction too[24]. It seems that the possible interference of other plasma lipids should also be studied. If haemolysis of the blood occurs to a visible degree, it is advisable not to use the plasma from such samples as source for SBP.

Metal ion inhibition of the association between progesterone and α_1-acid glycoprotein has been studied[18], using relatively pure α_1-acid glycoprotein, a well-characterized compound which is commercially available. Since SBP cannot be purchased, the present study has necessarily been performed on diluted late pregnancy plasma. Although with such plasma, physicochemical data cannot be obtained, our qualitative approach may be useful because such plasma is used in all methods for testosterone determination employing the competitive protein-binding technique. The interference of the standard testosterone binding curve reflects the influence of the metals on all the proteins contained in the plasma. With Hg^{2+} in very small amounts an effect is indicated by the more sigmoid shape of the curve probably due to protein denaturation. The influence of Ag^{+} is stronger than that of Cu^{2+} and Fe^{2+}, which have approximately the same effect. The presence of any of these metals would give increased testosterone values. It is possible that high blanks or unexpectedly high values in samples can be due to interference by these metal ions which are frequently present in low concentrations in solvents, water etc.

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