Steroid–protein interactions in bovine plasma

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Summary. The binding of testosterone, corticosterone and 17 α -hydroxyprogesterone by bull plasma protein was studied by equilibrium dialysis. Testosterone in bovine plasma was bound by a CBG-like protein and by a specific protein (testosterone-binding protein or TBP) of limited capacity and high affinity. The TBP was specific for C₁₈ and C₁₉ steroids with a 17 β -hydroxy group. Precision of the steroid–protein binding measurements was tested and was satisfactory. The testosterone-binding capacity in bull plasma samples did not seem to be related to testosterone levels in peripheral plasma. Significant differences between bulls and cows with regard to the binding capacity were observed.

Introduction

Testosterone is the most important androgen secreted by the testicular interstitial tissue of the adult bull (Lindner, 1969). A peripheral plasma concentration of about 4.5 ng/ml may be reached by 9 months of age (Thibier, 1975a). The available evidence indicates that the biosynthesis of testosterone occurs via the Δ^4 pathway: progesterone $\rightarrow$ 17 α -hydroxyprogesterone $\rightarrow$ androstenedione $\rightarrow$ testosterone.

Many steroid hormones are strongly bound to plasma proteins in the blood (Westphal, 1971). In man, two kinds of proteins are known to bind steroids with high affinity and limited capacity: (1) corticosteroid-binding globulin, CBG (Daughaday, 1956, 1958a), or transcortin, which specifically binds C₂₁ steroids, and (2) sex steroid-binding protein, SBP (Daughaday, 1958b; Mercier, Alfsen & Baulieu, 1966; Mercier-Bodard, Alfsen & Baulieu, 1970), which specifically binds C₁₈ and C₁₉ sex steroids. Other components, such as orosomucoid and albumin, are able to bind steroids in man (Sandberg, Slaunwhite & Antoniadis, 1957; Westphal, Ashley & Selden, 1961; Westphal, 1964, 1971), but they possess a much lower affinity for steroids than do CBG and SBP. Little has been published on the steroid-binding proteins in bovine plasma. The presence of a “corticosterone-binding globulin”-like protein which specifically binds cortisol and corticosterone was reported by Lindner (1964) and Seal & Doe (1965), and a high-affinity binding for testosterone has been shown to occur in bovine plasma (Murphy, 1968). Direct evidence for a testosterone-binding protein (TBP) came from polyacrylamide gel electrophoresis which resulted in the separation from both albumin and CBG of a TBP (Corvol & Bardin, 1973). From the amount of testosterone associated during serum fractionation by electrophoresis, the binding affinity was classified as ‘moderate’ by these authors.

The purpose of the present investigation was: (1) to analyse the testosterone-binding specificity in bull plasma, (2) to determine the concentrations and dissociation constants of the various steroids found in bovine plasma, and (3) to study the testosterone-binding activity in bulls in different physiological states.

Materials and Methods

Animals

A blood sample was taken from each of sixteen French Friesian bulls at 52 $\pm$ 0.5 weeks of age by puncture of the jugular vein. The bulls were individually penned in a performance test station and

had all been maintained under similar conditions since they were 8 days old. Their mean ($\pm$ S.D.) weight at 1 year of age was 434 ± 44 kg. After 9 months of age they were trained to ejaculate into an artificial vagina once a week. Libido and sperm production were normal. Plasma obtained from the blood samples was allocated to one of two groups according to the testosterone concentrations. The eight bulls in Group I had a testosterone concentration <2 ng/ml (mean = 1.19 ng/ml), and those in Group II had testosterone concentrations >5 ng/ml (mean = 7.47 ng/ml). Blood samples were also taken from the jugular vein of six French Friesian cows 21 days after artificial insemination. Three of the cows were pregnant and the peripheral plasma progesterone levels in the other three were <0.5 ng/ml, suggesting that they were in the follicular phase (Thibier & Saumande, 1974). The blood was centrifuged immediately after sampling (2800 g for 10 min) and plasma was decanted and stored at -20°C until assayed.

The steroid-protein binding measurement

The radioinert (Roussel-Uclaf) and labelled (N.E.N.) steroids used were: $[1,2,6,7\text{-}^3\text{H}]$ testosterone (81 Ci/mmol), $[1,2\text{-}^3\text{H}]17\alpha$ -hydroxyprogesterone (50.3 Ci/mmol), $[1,2\text{-}^3\text{H}]$ corticosterone (50 Ci/mmol). Steroids were shown to be homogeneous by thin-layer chromatography. A 0.01 M tris-HCl buffer, pH 7.4, was used. The protein binding of steroids was determined by an equilibrium dialysis method. Dialysis tubing was washed with distilled water and tris-HCl buffer. For all experiments, 1 ml diluted whole serum (1/20, 1/40, and 1/80) was placed inside the bag and the radioactive steroid was placed outside the bag in 4 ml tris-HCl buffer. In competitive experiments the unlabelled steroid was placed with the labelled steroid outside the bag. To each dialysing system were added various amounts of non-radioactive steroid ranging from 10^{-10} to 10^{-7} M and a fixed amount (1×10^{-10} M) of labelled steroid. All binding experiments were performed at 4°C with stirring, for a set time of 48 hr. In some experiments, in order to remove most of the endogenous steroids, plasma was subjected to charcoal (Norit A) treatment (Heyns, van Baelen & de Moor, 1967). The percentage of steroid binding was determined by the following equation: $\% \text{ bound} = 100 [1 - (D \cdot V_r)/(R \cdot V_d)]$, where R and D are the total amounts of radioactivity present inside and outside the dialysis bag, respectively, and V_r and V_d are the corresponding volumes (Sandberg, Rosenthal, Schneider & Slaunwhite, 1966). Steroid binding constants, binding capacity (N) and constant of dissociation (K_D) were determined from results obtained by the equilibrium dialysis binding experiments, in which a fixed quantity of labelled steroid and various amounts of the unlabelled steroids were used. Estimation and statistical evaluation of binding parameters were analysed on the assumption that each binding system was independent, the sites of each system were identical, there was no co-operative effect, the system obeyed the law of mass action, and proteins did not affect the activity of steroids except by binding. These binding constants were calculated (1) by a computational method (C II 10070 computer; Raynaud, 1973) and (2) by a graphic analysis according to Scatchard (1949) as modified by Rosenthal (1967). Similar results were obtained by both methods.

Steroid assays

Testosterone and progesterone were measured by radioimmunoassay with antisera raised in rabbits. The procedures were very similar for the two steroid assays and the reliability of the method was satisfactory (Thibier, Castanier, Tea & Scholler, 1973; Leymarie, Roger, Castanier & Scholler, 1974; Thibier, 1975a). Inter-assay precision for duplicate assays was 7.3% for testosterone and 11% for 17α -hydroxyprogesterone and progesterone. The limits of sensitivity for the assays of these three steroids calculated as described by Currie (1968) were 0.03, 0.05 and 0.07 ng/ml, respectively.

Results

Steroid-protein interactions

Binding constants. Bull plasma bound testosterone, corticosterone and 17α -hydroxyprogesterone with high affinity and limited capacity. The mean $\pm$ S.D. binding capacities (N) and dissociation

constants (K_D) $\times 10^{-9}$ M were: testosterone, $N=204.0\pm 20.8$ (see Text-fig. 1), $K_D=2.71\pm 0.28$; corticosterone, $N=85.8\pm 5.2$, $K_D=2.43\pm 0.14$; 17α -hydroxyprogesterone, $N=34.6\pm 9.8$, $K_D=1.86\pm 0.54$. The fact that the binding capacity for testosterone is 2.5 times greater than that for corticosterone suggests the existence of two binding proteins for testosterone. When bull plasma was saturated with a high concentration of corticosterone (3×10^{-4} M) for 48 hr, the binding capacity for testosterone was $114\pm 19.8\times 10^{-9}$ M, and this binding system was considered to be the TBP.

Accuracy of the binding constant values. This was estimated by the coefficient of variation of results obtained by repeated determinations ($n=12$) of K_D and N from the same plasma samples. The mean values of K_D and N in these series were not significantly different. The mean precision and

Text-fig. 1. Binding of testosterone (—) to bull plasma as determined by equilibrium dialysis experiments with plasma diluted 1/80. The broken line represents the Scatchard-type plot as modified by Rosenthal (1967).

Text-fig. 2. Competition for the bound [^3H]corticosterone (a), [^3H]testosterone (b) and [^3H]17 α -hydroxyprogesterone (c) in bull plasma diluted 1/20 as a function of the unlabelled steroid concentration. Competitive steroids were 17 α -hydroxyprogesterone ($\blacktriangle$), testosterone ($\bullet$) and corticosterone ($\blacksquare$).

confidence limits ($P < 0.05$) was 2.32×10^{-9} M ($1.80 < K_D < 2.84$) and 166.4×10^{-9} M ($134.9 < N < 197.8$) for the inter-assay determinations and 2.11×10^{-9} M ($1.80 < K_D < 2.42$) and 172.8×10^{-9} M ($152.5 < N < 193.1$) for the intra-assay determination.

Specificity studies. Competition experiments were performed between testosterone, corticosterone and 17α -hydroxyprogesterone by increasing the amount of unlabelled steroids (Text-fig. 2). The competition of testosterone and [3 H]testosterone, and corticosterone and [3 H]corticosterone were used as controls. Testosterone was able to compete with [3 H]corticosterone, although corticosterone was a weak inhibitor of [3 H]testosterone-binding proteins, and with [3 H] 17α -hydroxyprogesterone. The results of competition experiments with several steroids against the [3 H]testosterone binding by bull plasma diluted 1/80 are shown in Table 1. The steroids could be assigned to one of three groups according to their specificity. The ability to displace [3 H]testosterone from the high-affinity binding sites was a property of four C_{19} steroids (Group 1). Less displacement by the seven steroids of Group 2 was observed. The remaining steroids (Group 3) competed either poorly or not at all. The competitive binding ability of several steroids against [3 H]testosterone-binding protein was further studied with bull plasma which had been saturated with corticosterone (3×10^{-4} M). From the results in Table 2, it appears that 5α -androstane- 17β -ol,3-one competed more strongly than testosterone and 5α -androstane- 3β , 17β -diol was also a good competitor whereas oestradiol- 17β and 5α -androstane- 3α , 17β -diol were poor competitors. Deoxycorticosterone, 17α -hydroxyprogesterone and progesterone did not exhibit any competitive effect.

Table 1. Testosterone-binding specificity in bull plasma diluted 1/80

Unlabelled steroids	[3 H]testosterone bound (%)
—	54.3 ± 1.53
Group 1	
5α -Androstan- 17β -ol,3-one	21.3 ± 1.52
Testosterone	21.6 ± 0.57
5α -Androstan- 3α , 17β -diol	22.6 ± 3.51
5α -Androstan- 3β , 17β -diol	24.0 ± 1.73
Group 2	
Deoxycorticosterone	32.3 ± 1.51
Oestradiol- 17β	32.3 ± 1.53
Progesterone	35.6 ± 2.3
17α -Hydroxyprogesterone	36.0 ± 1.73
Dehydroepiandrosterone	36.0 ± 1.0
Androstenedione	38.0 ± 3.6
Epiandrosterone	38.0 ± 1.0
Group 3	
Testosterone- 17α	42.0 ± 3.0
Oestradiol- 17α	43.3 ± 2.08
Oestrone	46.0 ± 2.0
5β -Androstan- 3α , 17β -dio	47.6 ± 0.57
Cortisone	49.0 ± 1.0
Corticosterone	49.6 ± 4.04
Cortisol	52.3 ± 3.20
5β -Androstenedione	52.7 ± 3.05
Etiocholanolone	56.6 ± 1.52

Competition studies by equilibrium dialysis with unlabelled steroid (1×10^{-7} M) added to the plasma with 1.7×10^{-10} M-[3 H]testosterone. Values are means $\pm$ S.D. for three measurements.

Influence of peripheral plasma testosterone concentration on TBP activity

The testosterone concentrations and binding constant values for bull plasma are shown in Table 3. The coefficients of variation of K_D and N were around 30%. Comparison of mean binding constant

Table 2. Competition studies with various steroids in bull plasma diluted 1/40 and saturated with corticosterone (3×10^{-4} M)

	Unlabelled steroid concentration		
	1 nM	10 nM	100 nM
Testosterone	—	21	34
5 α -Androstan-17 β -ol,3-one	10	32	38
5 α -Androstan-3 β ,17 β -diol	4	17	32
Oestradiol-17 β	1	8	26
5 α -Androstan-3 α ,17 β -diol	2	4	18
Deoxycorticosterone	0	4	15
17 α -Hydroxyprogesterone	1	3	13
Progesterone	0	3	7

The unlabelled steroid was added to the plasma with 1 nM-[3 H]testosterone and the figures indicate the percentage decrease in the bound radioactive testosterone.

values of Group I and II bulls showed no significant differences for N , but a significant one for K_D . The plasma samples from Group II bulls had a higher K_D . Intra-group correlation between either K_D or N and testosterone level was not significant ($P > 0.05$). The binding constant values for TBP were similar in pregnant and non-pregnant cows (Table 3), and the coefficients of variation were again around 30%. The mean K_D values in cows were intermediate between those of the bulls in Groups I and II ($P > 0.05$). The N constant values of cows were significantly higher ($P < 0.05$) than those of bulls. For all animals, the N and K_D correlations were highly significant ($P < 0.01$).

Table 3. The mean ($\pm$ S.D.) testosterone-binding capacities (N) and dissociation constants (K_D) in the plasma of bulls and cows, and plasma testosterone concentrations in bulls (not detectable in cows)

	$N \times 10^{-9}$ M	$K_D \times 10^{-9}$ M	Testosterone (ng/ml)
Bulls			
Group I + Group II (16)	$202 \pm 54.1^*$	2.88 ± 0.92	4.33 ± 3.99
Group I (8)	182 ± 51.2	$2.38 \pm 0.32^\dagger$	1.19 ± 0.50
Group II (8)	221 ± 52.7	$3.37 \pm 1.07^\dagger$	7.47 ± 1.38
Cows			
Pregnant + non-pregnant (6)	$282 \pm 75.0^*$	2.68 ± 0.63	
Pregnant (3)	286 ± 95.9	2.73 ± 0.41	
Non-pregnant (3)	279 ± 69.8	2.62 ± 0.91	

* Significant, $P < 0.05$ (Student's t test).

† Significant, $P < 0.05$ (Student's t test).

Discussion

The presence of two specific proteins binding testosterone with high affinity and limited capacity has been confirmed in bull plasma. Displacement studies, as well as the binding capacities determined for corticosterone and testosterone in whole plasma and in whole plasma saturated with corticosterone, show that CBG and TBP molecules are both involved in the binding of testosterone. The competition experiments suggest that a 17 β -hydroxy group in a C_{19} steroid structure (with Δ^4 or 5 α ,3 keto or 3 α - or 3 β -hydroxy) is necessary for interaction with the TBP. When the binding of testosterone with testosterone-17 α or with androstenedione is compared, it is seen that the 17 α -hydroxy group and the 17-keto group cause a large decrease in binding activity. The 5 α -reduced

17 β -hydroxy metabolites of testosterone, such as 5 α -androstan-17 β -ol,3-one and 5 α -androstan-3 β ,17 β -diol, are bound to an extent similar to that of testosterone. The binding is lowered by the presence of a 5 β structure as indicated by comparing 5 α -androstan-3 α ,17 β -diol with 5 β -androstan-3 α ,17 β -diol. As far as the various C₁₉ steroids are concerned, TBP seems to bind them with the same affinity as does SBP in man (Kato & Horton, 1968; Vermeulen & Verdonck, 1968; Mercier-Bodard *et al.*, 1970). For C₁₈ steroids, oestradiol-17 β is bound to the TBP with a lower affinity than the C₁₉ steroids and competes with the testosterone-TBP complex more strongly than do oestradiol-17 α and oestrone.

The precision of the steroid-protein binding measurement was considered satisfactory. The coefficient of variation of the site concentration was about 20% for the intra-assay precision and the reliability of this procedure therefore renders it useful for studies of physiological conditions. The mean testosterone concentration found in the bulls in the present study was very similar to that of randomly sampled bulls of that age (Thibier, 1975a). It is therefore reasonable to assume that the mean binding constants of 1-year-old bulls would be about 200×10^{-9} M for N and 3×10^{-9} M for K_D . The testosterone-binding capacity (N) does not seem to be related to testosterone levels, but binding affinity was significantly higher in plasma samples with low levels of testosterone. As K_D and N are quite closely related, this may appear rather illogical and the reason for this discrepancy is unknown. It has been shown by Katongole, Naftolin & Short (1971) and Thibier (1975b) that peripheral plasma testosterone levels in bulls fluctuate during the day. Since only one sample was taken from each bull, it was not known whether daily testosterone patterns of the bulls in Groups I and II were significantly different (Thibier, 1975b). Comparison of the binding constants for the bulls and cows seems to be more useful in this respect. The mean values of testosterone-binding capacity appeared to be significantly different in males and females. This suggests that plasma samples with very low levels of testosterone have a higher mean value of N . In our study, N was 40% higher in cows than in bulls and testosterone levels in the females were below 0.05 ng/ml, i.e. less than $\frac{1}{30}$ those in the young bulls. It may be concluded from the differences between N in males and females that increased plasma testosterone concentration reduces the TBP activity. Similar results have been reported for human serum by Dray (1969) and de Moor, Steeno, Heyns & van Balaen (1969). After puberty in humans, values for males are significantly lower than those for females, but in females with hirsutism 'male' values are observed and in males with hypogonadism 'female' values are found (Vermeulen, Verdonck, van der Straeten & Orie, 1969). It is interesting to correlate these findings with the observations that testosterone also decreases the levels of other plasma proteins such as transcortin in the rat (Gala & Westphal, 1965).

In cows, there was no significant difference in the values of the constants between early pregnant and non-pregnant females. Several authors (Shemesh, Ayalon & Lindner, 1968; Stabenfeldt *et al.*, 1969, 1970; Edqvist, Ekman, Gustafsson & Astrom, 1970; Donaldson, Bassett & Thorburn, 1970) have shown that progesterone levels in the cow are much higher in pregnancy than in the follicular phase. Progesterone levels seem therefore to have no influence on the binding constants of TBP.

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