Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 331–339. Pergamon Press. Printed in Great Britain

THE MEASUREMENT OF SEX STEROID BINDING GLOBULIN BY DIFFERENTIAL AMMONIUM SULPHATE PRECIPITATION

S. O'CONNOR, H. W. G. BAKER, A. DULMANIS and B. HUDSON
Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne; and Medical Research Centre, Prince Henry's Hospital, Melbourne, Victoria, Australia

(*Received* 24 *January* 1973)

SUMMARY

The binding parameters of the sex steroid binding globulin (SSBG) for testosterone and estradiol have been measured by a method using differential precipitation of plasma proteins by ammonium sulphate. The method is relatively simple and can be applied in a routine manner for the analysis of large numbers of plasma samples. The values for the binding capacity and association constant are similar to those reported for other methods. The method also enables the calculation of the circulating plasma levels of unbound steroids.

INTRODUCTION

THE presence in human plasma of a protein other than albumin that binds testosterone was first noted by Daughaday[1], and later by Chen *et al.*[2]. Mercier-Bodard *et al.*[3] showed that this protein, a β-globulin could be separated from albumin and transcortin by chromatography on DEAE cellulose. It is now known that this sex steroid binding globulin (SSBG) has a high binding affinity not only for testosterone but for other planar 17β-hydroxysteroids such as dihydrotestosterone and estradiol-17β[4, 5]. The binding capacity of the globulin for such steroids is greater than their endogenous levels in plasma, and is known to vary in different conditions; for instance, it is greater in females than in males, and is increased during pregnancy or following treatment with estrogens.

Several methods have been devised to measure the binding capacity (q) and the association constant (K) of this protein; these include equilibrium dialysis[4], polyacrylamide gel electrophoresis[5], gel filtration[6], batch-wise gel equilibration with Sephadex G-25[7] and ammonium sulphate precipitation[8, 9, 10]. The theoretical and practical advantages of these different techniques have been discussed by Westphal[11]. Many of the methods described are time-consuming and not applicable to the analysis of multiple plasma samples. This criticism applies particularly to equilibrium dialysis which, of all the techniques described, is probably the most valid for the measurement of parameters of binding equilibrium.

The present paper describes a relatively simple method for the measurement of the association constants and binding capacities of SSBG for testosterone and estradiol, and the unbound levels of these steroids in plasma. The method is based on a technique of differential precipitation of plasma proteins by ammonium sulphate following equilibration of the plasma sample with tracer amounts of isotopically labelled steroids.

EXPERIMENTAL

Materials

Blood samples. Venous samples are collected into heparinized tubes. The cells and plasma are separated immediately, and the plasma stored at −20°C until processed.

Isotopes. [1,2-^{3}H-]-testosterone (45 Ci/mmol) and [6,7-^{3}H-] estradiol-17β (40 Ci/mMol) was obtained from The Radiochemical Centre, Amersham, U.K. Small aliquots are purified by t.l.c. (ether–chloroform, 20:80, v/v) at approximately monthly intervals and are stored in suitable dilutions in ethanol at −20°C.

Non-radioactive steroids were obtained from Steraloids Inc., Pawling, New York and from Mann Research Labs. Inc., New York. These were shown to run as a single spot on both t.l.c. (ether–chloroform, 20:80, v/v) and paper chromatography (isooctane–methanol–water, 10:9:1, v/v). They are then used without further purification.

All other reagents and solvents are of analytical grade and are used without further purification.

A toluene-based phosphor, containing 5 g PPO and 0·2 g POPOP/1 is used for liquid scintillation counting.

Methods

Measurement of plasma levels of testosterone and estradiol-17β. Testosterone levels are measured by a competitive protein binding technique similar to that described for the measurement of estradiol[12]; plasma levels of estradiol-17β are measured by radioimmunoassay using an ovine antibody raised against estradiol coupled with bovine serum albumin. Estradiol is isolated from estrone by chromatography using Sephadex G-15 columns, and elution with increasing concentrations of aqueous methanol. This method is similar to that described by Dufau *et al.*[12] with the exception that antibody to estradiol is used in place of pregnancy plasma β-globulin.

Removal of endogenous steroids. Plasma is shaken gently for 1 h with activated charcoal, the concentration of charcoal being 50 mg/ml plasma. The plasma is then centrifuged at 8000 *g* for 15 min, and filtered through filter paper in order to remove charcoal fines.

Equilibration of plasma with isotopic tracer. 2·5 ml plasma samples are diluted to 6·25 ml (1 in 2·5 v/v) with physiological saline (0·15 M) and added to flasks in which isotopic tracer has been dried down. For both testosterone and estradiol the mass of tracer used is between 175 and 185 pg per assay tube (about 60,000 d.p.m.). The diluted plasma is shaken gently and allowed to incubate at room temperature for about 1 h.

Assay tubes. Polystyrene tubes (10 ml) are used. These are pre-coated with a 0·2% solution of human serum albumin (HSA) in physiological saline in order to minimize adsorption of steroid to the tubes. About 4 ml of HSA solution is added to each assay tube. These are shaken and allowed to stand at room temperature overnight. Before use, they are rinsed with distilled water and dried.

The assay

The binding characteristics of individual plasma samples are measured in duplicate. To each assay tube 0, 2·5, 5·0 or 7·5 ng of non-isotopic steroid (testosterone or estradiol) is added in 0·5 ml of physiological saline. Thereafter, 0·5 ml

of plasma that has been equilibrated with isotopic tracer is added to each tube, mixed thoroughly and incubated at +4°C for 6 h.

At the end of the incubation period, 0·5 ml of the plasma mixture is removed and added to 2·0 ml of saturated ammonium sulphate, the final concentration with respect to ammonium sulphate being 80%. The resulting precipitate of albumin and globulins is removed by centrifugation at 1450 *g* for 30 min. Radioactivity in the supernatant is that fraction of steroid not protein-bound.

SSBG is precipitated by the addition of 0·5 ml of saturated ammonium sulphate to the residual plasma (0·5 ml) in each assay tube, the final concentration with respect to ammonium sulphate being 50%. The precipitate of globulins is removed by centrifugation at 1450 *g* for 30 min. Radioactivity in the supernatant represents the fraction bound to smaller molecular weight proteins, for example: albumin, transcortin and α_1-acid glycoprotein (AAG) and that which is not protein-bound[10].

Control tubes are used to estimate the extent to which steroids may be non-specifically bound to assay tubes, and to measure the total amount of radioactivity in each sample, so that the fractions of non-protein bound, albumin-bound and globulin-bound steroid may be determined. These tubes, which contain 0·5 ml of diluted plasma and 0·5 ml of physiological saline are treated in the same manner as the assay tubes, except that after incubation saline is used instead of ammonium sulphate. These are subsequently referred to as 'saline' tubes.

In each assay, a standard plasma pool, the binding characteristics of which have been repeatedly measured, is used as a quality control sample. This standard plasma is treated such that ratio of SSBG bound, non-SSBG bound and free radioactivity is measured in the absence of steroid.

Radioactivity is measured in 0·5 ml of the supernatant in ammonium sulphate treated fractions and in the same volumes from saline tubes. Aqueous samples are transferred directly to counting vials, to which 5 ml of counting phosphor is added. Both testosterone and estradiol are extracted quantitatively into the toluene phosphor. Radioactivity is measured in a Packard liquid scintillation counter (Model 3375), sufficient time being allowed such that the counting error is less than ±1·5%.

Calculations

The mathematical approach described by Vermeulen and Verdonck[4] has been used to calculate binding parameters. The equation described by these authors has the form:

$$\frac{B}{U} = \frac{K}{N}(q - B)$$

where B/U is the ratio between steroid bound to SSBG and that which is free and albumin bound; K is the association constant (l/mol); q is the total concentration of binding sites (mol/l) and B is the amount of steroid bound at any given steroid concentration (mol/l). N is a factor derived from albumin binding where:

$$N = 1 + S_a$$

S_a is the ratio between bound and free steroid when SSBG has been removed from the system.

The bound–unbound fraction B/U can be calculated from the results of the 50% ammonium sulphate precipitation

$$\frac{B}{U} = \frac{\text{Total c.p.m. (saline)} - \text{c.p.m. in 50\% supernatant}}{\text{c.p.m. in 50\% supernatant}}.$$

Likewise, B can be calculated from the results of the 50% ammonium sulphate precipitation

$$B = \frac{\text{Total c.p.m. (saline)} - \text{c.p.m. in 50\% supernatant}}{\text{Total c.p.m. (saline)}} \times \text{mol steroid/l.}$$

Plotting the observed values for B/U (ordinate) against B (abscissa) yields a Scatchard plot[13]. The intercept on the abscissa gives a value for q which must be corrected for the 1 in 5 dilution of the plasma sample. The slope of the line is $-K/N$. In order to calculate K, the binding of steroid to albumin is determined experimentally for each plasma sample. The fraction bound to albumin (S) is determined as follows:

$$S = \frac{\text{c.p.m. in 50\% supernatant} - \text{c.p.m. in 80\% supernatant}}{\text{c.p.m. in 50\% supernatant}}$$

$$S_a = \frac{S}{1-S} + 1.$$

Measurement of free and non-SSBG bound steroids. The fraction of non-protein bound steroid may be calculated as follows:

$$\text{Steroid fraction non-protein bound} = \frac{\text{c.p.m. in 80\% supernatant}}{\text{total c.p.m. (saline)}}.$$

In order to calculate the concentration of non-protein bound steroid in the

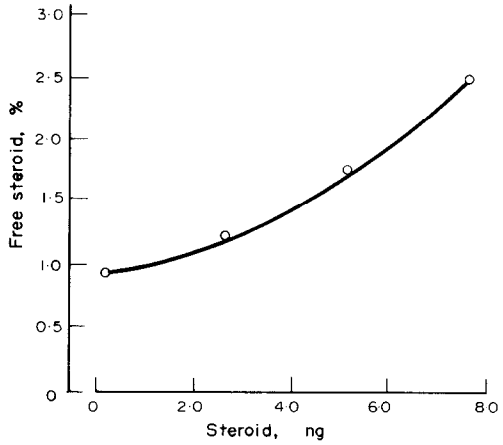

Fig. 1. Plot of free steroid (%) vs ng of steroid (estradiol-17β) added to incubation mixture, 0·5 ml of 1 in 5 dilution of plasma.

plasma sample, the fraction of free steroid is plotted against the mass of steroid added to each assay tube. From a knowledge of the steroid concentration in each plasma sample, the endogenous fraction of non-protein bound steroid can be determined by reference to the plot shown in Fig. 1. This enables the absolute concentration of non-protein bound steroid to be measured. In a similar way, the fraction of non-SSBG-bound steroid may be calculated. This is illustrated in Fig. 2.

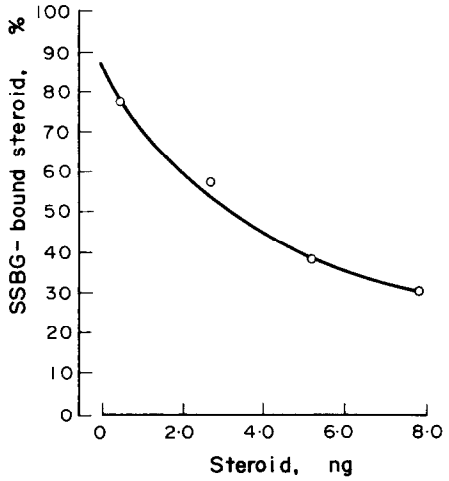

Fig. 2. Plot of steroid bound (%) to SSBG vs ng of steroid added to incubation mixture, 0·5 ml of 1 in 5 dilution of plasma.

RESULTS

Evidence for the separation of plasma proteins by ammonium sulphate. This was investigated by examining immunoelectrophoretically the protein content of the supernatant solutions and precipitates of plasma samples treated with 50 and 80% ammonium sulphate. The results are shown in Figs. 3–6 in which it can be seen that the only protein species present in the 50% supernatant is albumin, that very little albumin is present in the 80% supernatant and that only traces of albumin are present in the 50% precipitate.

Normal values. The binding capacities and association constants for testosterone and estradiol of plasma from normal males and females are shown in Table 1. These values are compared with those obtained by other methods in Table 2.

The mean binding capacity for testosterone (q_T) for 15 normal males aged between 18 and 35 years was $4{\cdot}65 \times 0{\cdot}38$ (sem) $\times 10^{-8}$ M/L, and for 8 females aged between 19 and 30 years was $8{\cdot}17 \pm 1{\cdot}33 \times 10^{-8}$ M/L. These values are significantly different ($p < 0{\cdot}001$). The mean association constant (K_T) for testosterone in males were $4{\cdot}97 \pm 0{\cdot}45 \times 10^9$ L/M, and in the females $6{\cdot}34 \pm 0{\cdot}66 \times 10^9$ L/M. These values are not significantly different.

For estradiol, the mean binding capacity (q_E) for the same 15 males was $4{\cdot}57 \pm 0{\cdot}46 \times 10^{-8}$ M/L and for the same 8 females $6{\cdot}62 \pm 1{\cdot}07 \times 10^{-8}$ M/L ($P < 0{\cdot}01$). The mean association constant for estradiol (K_E) for males was $3{\cdot}15 \pm 0{\cdot}36 \times 10^9$ L/M; for females this value was $3{\cdot}3 \pm 0{\cdot}37 \times 10^9$ L/M ($P > 0{\cdot}1$).

Free steroids. Values for free testosterone and estradiol levels in normal male

Table 1. Values for q and K in normal males and females for testosterone and estradiol

Subjects	Testosterone		Estradiol-17β	
	Binding capacity mol/l ($\times 10^8$)	Association constant l/mol ($\times 10^{-9}$)	Binding capacity mol/l ($\times 10^8$)	Association constant l/mol ($\times 10^{-9}$)
Normal males n = 15	4·65 ± 0·38	4·97 ± 0·45	4·57 ± 0·46	3·15 ± 0·36
Normal females n = 8	8·17 ± 1·33	6·34 ± 0·66	6·62 ± 1·07	3·37 ± 0·37

Mean ± S.D.M.

and female plasma are shown in Table 3, which also shows values for non-SSBG steroid. The free values are similar to those reported by Vermeulen *et al.* [14] and Marshall *et al.* [15].

Precision. This has been determined by making 10 replicate determinations of the binding capacity and association constant for the same pooled plasma. The mean values for this pool were: $q_T = 6{\cdot}86 \pm 0{\cdot}8$ (S.D.) $\times 10^{-8}$ M/L and $K_T = 6{\cdot}40 \pm$

Table 2. Values for q and K for testosterone and estradiol-17β obtained by other authors

Authors	Temperature (°C)	Sex	Association constant (l/mol ($\times 10^{-9}$))		Binding capacity (mol/l ($\times 10^8$))	
			T	E_2	T	E_2
Vermeulen and Verdonck [4]	20	M	0·44–1·22	—	—	—
	25	M	0·6–1·4	—	3·6–6·4	—
	25	F	0·4–1·6	—	4·4–6·4	—
	37	M	0·28–0·8	—	—	—
Corvol *et al.* [5]	20	M	—	—	1·65*	—
		F	—	—	5·0*	—
Vermeulen *et al.* [6]	25	M	0·5–1·0	—	2·3–5·4	—
	25	F	0·3–0·9	—	4·4–13·0	—
Rosner *et al.* [27]	4	M	—	4·0	—	—
			—	0·69	—	—
			—	0·11	—	—
Rosner [10]	4	M	—	—	3·2*	—
	4	F	—	—	6·4*	—
Pearlman *et al.* [24]	—	M	—	—	1·6–4·4	—
	—	F	—	—	5·6–8·3	
Mercier-Bodard *et al.* [25]	25	—	0·5	0·04	—	—
Lebeau *et al.* [26]	4	M	1·2	0·46	—	—
This paper	4	F	6·34	3·37	8·17	6·62
	4	M	4·97	3·15	4·65	4·57

T = testosterone; E_2 = estradiol-17B.

*These values are for dihydrotestosterone.

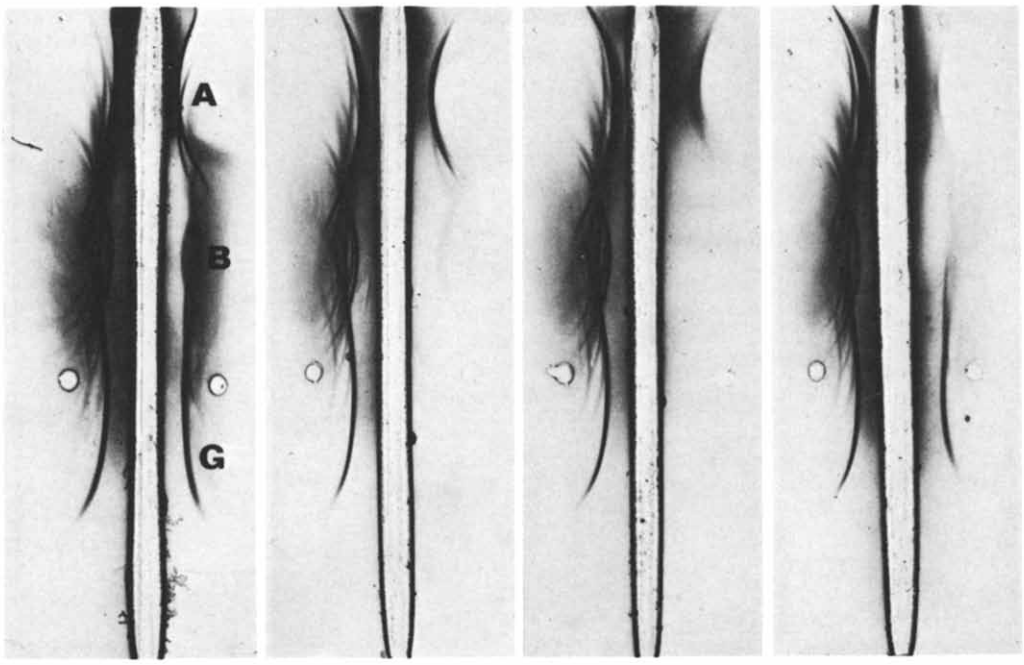

Fig. 3. Immunoelectrophoresis of a 1 in 5 dilution of a normal plasma sample. In this and subsequent figures of immunoelectrophoretic measurements, normal human plasma has been placed in the left hand well and test samples in the right hand well. A = albumin; B = β-globulin; G = γ-globulin. In order to make valid quantitative comparisons equivalent amounts of 1 in 5 plasma and supernatants and precipitate have been used.

Fig. 4. Immunoelectrophoresis of supernatant from 50% ammonium sulphate fraction.

Fig. 5. Immunoelectrophoresis of supernatant from 80% ammonium sulphate fraction.

Fig. 6. Immunoelectrophoresis of precipitate from 50% ammonium sulphate fraction, following reconstitution of this precipitate to the original volume.

(*Facing page* 336)

Table 3. Values (± S.D.M.) for free and non-SSBG bound testosterone and estradiol-17β in normal subjects

Steroid	Testosterone				Estradiol-17β			
Normal subjects	% Free	Free ng/100 ml	% non SSBG bound	non SSBG bound ng/100 ml	%Free	Free ng/100 ml	% non SSBG bound	non SSBG bound ng/100 ml
Males (n = 10)	1·85 ± 0·27	10·17 ± 1·52	46·6 ± 4·6	235·7 ± 23	2·06 ± 0·29	0·02 ± 0·003	41·6 ± 2·5	0·60 ± 0·11
Females (n = 12)	1·04 ± 0·14 **	0·92 ± 0·16 ***	21·5 ± 2·20 ***	17·8 ± 2·8 ***	1·51 ± 0·20	0·02 ± 0·006	35·3 ± 2·2 *	0·63 ± 0·22

*$P < 0·05$, **$P < 0·01$, ***$P < 0·005$.

Table 4. Binding values for testosterone and estradiol in selected clinical disorders. Values given are the means and range values. Figures in parentheses are the numbers of subjects studied

Patients	Testosterone				Estradiol-17β			
	$K_T \times 10^{-9}$ (l/mol)	$q_T \times 10^8$ (mol/l)	Free T (%)	Free T ng/100 ml	$K_E \times 10^{-9}$ (l/mol)	$q_E \times 10^8$ (mol/l)	Free E_2 (%)	Free E_2 ng/100 ml
Thyrotoxic females (3)	3·8 3·23–4·15	24·6 16·3–33·3	0·8 0·5–1·0	0·8 0·4–0·7	4·3 1·8–8·8	17·7 13·0–23·7	1·3 1·0–1·6	1·1 0·08–0·12
Thyrotoxic males	3·3–7·8 (3)	6·3–25·1 (3)	1·3 (1)	7·5 (1)	—	—	—	—
Klinefelter's syndrome	8·3 3·2–17·4 (5)	7·3 3·5–12·9 (5)	1·2 0·8–1·5 (5)	3·4 1·8–5·4 (5)	4·5 4·0–5·0 (3)	1·3 0·3–2·1 (4)	2·3 2·0–3·1 (3)	0·1 0·07–0·13 (3)
Cirrhotic males	4·6 ± 0·6 (28)	11·5 ± 1·3 (30)	1·3 ± 0·2 (27)	5·8 ± 1·2 (27)	3·3 ± 0·4 (19)	9·7 ± 1·2 (19)	2·4 ± 0·4 (19)	0·1 ± 0·03 (18)
Alcoholic males (19)	7·0 ± 0·6	2·8 ± 0·6	1·4 ± 0·7	7·8 ± 1·2	5·3 ± 0·6	6·2 ± 0·9	1·5 ± 0·2	0·06 ± 0·01

$0{\cdot}9 \times 10^9$ L/M. Precision, expressed in terms of a coefficient of variation, was $\pm 12{\cdot}1$ and $\pm 14{\cdot}1\%$ respectively.

Abnormal values. Binding parameters of patients with hepatic cirrhosis, chronic alcoholism, thyrotoxicosis and Klinefelter's syndrome are shown in Table 4.

DISCUSSION

There is good evidence that the sex steroid binding globulin is a β-globulin with a high affinity for testosterone, dihydrotestosterone and estradiol. SSBG has been purified by Mercier-Bodard *et al.*[16] and has been assigned a molecular weight of between 100,000 and 110,000 on the basis of gel filtration on Sephadex G-200[17]. The possible significance of this protein in a number of clinical states has also been discussed by Vermeulen *et al.*[6] and Rosenfield[18].

Although a number of different methods have been used for the measurement of the binding parameters of this protein, few of these are readily adaptable to the routine assay of a large number of plasma samples. The method described has been devised because of our interest in making these measurements as a routine procedure in different clinical states; about 18 plasma samples can be processed by one technician during the course of a working week.

As shown in Table 2 the values derived by this procedure are similar to those obtained by other methods.

The interference in this system by transcortin is negligible. This α-globulin is precipitated by ammonium sulphate at a concentration of 80%. Were the binding to transcortin significant, it could be anticipated that with an increasing mass of steroid the fraction of steroid bound to albumin (and transcortin) would fall. In all plasma samples examined this value has remained constant over the range of steroid concentrations used, which is a characteristic of binding between albumin and steroids.

The finding of elevated values of q_T and q_E in patients with hepatic cirrhosis is of interest and has been reported previously[19–21]. Whether these increases are the result of increased plasma levels of estradiol in this condition, or a part of the abnormal protein biosynthesis that occurs in patients with severe liver disease, or a combination of both these factors is not known. The patients with alcoholism were studied in order to determine whether changes in the binding parameters of SSBG occurred prior to the development of overt cirrhosis. Although some patients with alcoholism showed higher than normal values for q_T and q_E, as a group the values for these parameters were not significantly different from normal.

The high levels of q_T and q_E in patients with thyrotoxicosis has also been reported previously[22] and reflects the abnormally high levels of testosterone in plasma that have been observed in patients with this disease. The mechanism by which these elevations occur is not known.

Using an equilibrium dialysis technique, Clarke *et al.*[23] found an increase in testosterone binding in patients with Klinefelter's syndrome which is consistent with observations reported in this paper. The abnormally low values for q_E in this disorder are somewhat surprising and have not, as far as we are aware, been reported previously. It is possible this abnormality may be one of the genetic determinants of the disorder. As a consequence of this low binding capacity, values for free estradiol are high and may account for some of the feminizing features of the disorder.

REFERENCES

1. Daughaday W. H.: *J. clin. Invest.* **37** (1958) 511.
2. Chen P. S., Mills I. H. and Bartter F. C.: *J. Endocr.* **23** (1961) 129.
3. Mercier-Bodard C., Alfsen A. and Baulieu E. E.: Proc. 2nd Symp. on Steroid Hormones, Ghent, Belgium, 1965; *Excerpta Med. Int. Congr. Ser.* **101** (1966) 212.
4. Vermeulen A. and Verdonck L.: *Steroids* **11** (1968) 609.
5. Corvol P. L., Chrambach A., Rodbard D. and Bardin C. W.: *J. biol. Chem.* **246** (1971) 3435.
6. Vermeulen A., Verdonck L., Van der Straten M. and Orie N.: *J. clin. Endocr.* **29** (1969) 1470.
7. Pearlman W. H. and Crepy O.: *J. biol. Chem.* **242** (1967) 182.
8. O'Connor S., Baker H. W. G. and Hudson B.: *Proc. Endocr. Soc. Australia* **14** (1971) 21.
9. Heyns W. and De Moor P.: *Steroids* **18** (1971) 709.
10. Rosner W.: *J. clin. Endocr.* **34**, (1972) 983.
11. Westphal U.: *Steroid-Protein Interactions. Monographs on Endocrinology*, Vol. 4. Springer, Berlin, Heidelberg, New York (1971) 15.
12. Dafau M. L., Dulmanis A., Catt K. J. and Hudson B.: *J. clin. Endocr.* **30** (1970) 351.
13. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660.
14. Vermeulen A., Stoica T. and Verdonck L.: *J. clin. Endocr.* **27** (1967) 1012.
15. Marshall J. C., Anderson D. C., Burke C. W., Galvao-Teles A. and Fraser R. T.: *J. Endocr.* **53**, (1972) 261.
16. Mercier-Bodard C., Alfsen A. and Baulieu E. E.: *Acta Endocr. (Kbh.) suppl.* 147, **64** (1970) 204.
17. Gueriguian J. and Pearlman W. H.: *J. biol. Chem.* **243** (1968) 5226.
18. Rosenfield R. L.: *J. clin. Endocr.* **32** (1971) 717.
19. Rosenbaum, W., Christy N. P. and Kelly W. G.: *J. clin. Endocr.* **26** (1966) 1399.
20. Tarvernetti R. R., Rosenbaum W., Kelly W. G., Christy N. P. and Roginsky M. S.: *J. clin. Endocr.* **27** (1967) 920.
21. Breuer Von J., Schneider H. Th. and Breuer H.: *Z. Klim. Chem. klin. Biochem.* **8** (1970) 626.
22. Crepy O., Dray F. and Sebaoun J.: *C.R. Acad. Sci (Paris)* **246** (1967) 2651.
23. Clarke A. F., Calandra R. S. and Bird C. E.: *Can. Biochem.* **4** (1971) 89.
24. Pearlman W. H., Crepy O. and Murphy M.: *J. Clin. Endocr.* **27** (1967) 1012.
25. Mercier-Bodard C. and Baulieu E. E.: *Ann. Endocr.* **29** (1968) 159.
26. Lebeau M. C., Mercier-Bodard C., Olds J., Baerquin D., Brecy T., Raynaud J. P. and Baulieu E. *Ann. Endocr.* **30** (1969) 183.
27. Rosner W., Christy N. P. and Kelly W. G.: *Biochemistry* **8** (1969) 3100.