Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 345–350. Pergamon Press. Printed in Great Britain

PROGESTERONE-BINDING PROPERTIES OF MICROSOMES FROM RAT LIVER

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(*Received* 18 *August* 1975)

SUMMARY

The progesterone binding properties of microsomal suspensions from rat liver have been studied using a Dextran-coated charcoal method for the determination of the progesterone binding capacity. Microsomes isolated from the livers of adult female, male and pregnant female rats all exhibited progesterone binding of high affinity and limited capacity ($K_a \sim 2 \times 10^7 M^{-1}$), in addition to non-specific binding of progesterone.

The high affinity progesterone binding components of the hepatic microsomes were partly solubilized by treatment with the detergent Triton X-100. These solubilized progesterone binding components were progesterone saturable ($K_a \sim 3 \times 10^7 M^{-1}$) and specific for progesterone. Of the 11 steroids tested progesterone showed the highest competition for microsome-bound [^{3}H]-progesterone.

The progesterone binding capacity of female rat liver microsomes was decreased after protease and RNase treatment, suggesting that both protein and RNA were involved, directly or indirectly, in progesterone binding to the microsomes. Treatment of the liver microsomes with EDTA (0–17 mM) or KCl (0.4 M) had no effect on the binding of progesterone. The microsomal suspensions of rat liver resisted freezing without loss of their progesterone binding activity, whereas approximately one half of their progesterone binding capacity was lost after incubation at +37°C for 15 min.

INTRODUCTION

Sex specific binding of oestradiol and testosterone to the microsomal membranes of rat liver has been reported by Blyth *et al.*[1–3]. This high affinity binding of oestradiol and testosterone to male and female rat liver microsomes, respectively, is known to reside in the smooth reticular membranes [2, 3]. The plasma membrane fraction of rat liver shows no such specific binding of high affinity for oestradiol or testosterone [4].

Rat uterine cytosol contains a progesterone-specific receptor of high affinity [5–7]. This receptor fulfills the criteria for organ specificity, and progesterone binding of this type is not found in rat liver cytosol [5]. The rat uterus also contains microsomal progesterone binding components which differ distinctly from the cytosolic receptor in steroid specificity [7, 8].

In the present paper the binding of progesterone by rat liver microsomes is reported. This type of binding seems to have several features in common with the binding of oestradiol and testosterone by rat liver microsomes [1–3], whereas it clearly differs from the microsomal and cytoplasmic binding of progesterone found in the rat uterus [5–8].

MATERIALS AND METHODS

Chemicals. The chemicals used were the same as described previously [7]. The tissue samples were prepared and the progesterone binding studies carried out in a medium containing 25 mM Tris–HCl, 1.25 mM EDTA and 250 mM sucrose, pH 7.4 (TES buffer). In some experiments EDTA was omitted from the medium (TS buffer). Purified Triton X-100 was obtained from Koch–Light Laboratories Ltd., Colnbrook, England.

Steroids. 1,2,6,7-[^{3}H]-Progesterone (S.A. 96 and 105 Ci/mmol) and the non-radioactive steroid standards used were the same as described previously [7–9]. For the trivial and systematic names of the steroid standards used, see Table 2.

Microsomes. Adult male and female Sprague–Dawley rats were used. Of the 36 female rats, 9 were pregnant. During preparation the samples were kept in ice-cold TES buffer, pH 7.4. The rats were killed by decapitation, the livers removed, immersed in ice-cold TES buffer and cut into pieces. They were then washed, weighed and sliced into 2–3 mm pieces which were again washed with 3 volumes of TES buffer (weight/vol.). A 20% homogenate was prepared in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 12,000*g* for 15 min at +2°C, and the 12,000*g* supernatant was then centrifuged at 105,000*g* for 60 min at +2°C. The supernatant was decanted, the tube and the surface of the 105,000*g* pellet rinsed twice with TES buffer, after which, the pellets were gently suspended in 5.0–10.0 ml of TES per liver in a homogenizer. The protein concentration of the resulting microsome suspensions was 5–12 mg/ml. Electron microscopy of the

105,000*g* pellet revealed no contamination of the membrane fraction by nuclei or mitochondria. The microsomal suspension was usually used immediately for progesterone binding studies, and only occasionally stored at −20 C overnight.

Progesterone binding capacity assay. The progesterone binding capacity of the microsomes was determined using a dextran-coated charcoal (DCC) method, which has been described in detail previously [7, 8]. The incubation time was 1 h at +6 C and the reaction time with DCC (0.5% Norit A, 0.005% Dextran T 70) usually 15 min, but occasionally 10 min. All samples were run in duplicate. Protein determinations showed that the DCC did not adsorb the microsomes during the assay. The charcoal was sedimented by centrifugation at 12,000*g* for 2 min, conditions under which the microsomes remained in the supernatant. Scatchard plots were drawn, and the "reduced Scatchard plot" method [10, 11] was used to determine the approximate number of high affinity progesterone binding sites and the association constant (K_a) of these binding sites.

Protein assay. Protein was determined according to the method of Lowry *et al.*[12].

Solubilization of the progesterone binding components by treatment with Triton X-100. The liver microsome suspensions were treated with the detergent Triton X-100 by adding ice-cold 5% (v/v) Triton X, in 25 mM Tris buffer pH 7.4, to the microsome preparations to give a final Triton X concentration of 0.1–1.0% (v/v). Alternatively, after the optimal Triton X concentration had been found, the microsomal pellet was directly suspended in 0·1% Triton X in 25 mM Tris and 250 mM sucrose, pH 7.4 (TTS buffer), using 5 ml of TTS per liver. The Triton X treated microsomes were centrifuged in capped polycarbonate tubes at 105,000*g* for 60 min at +2 C and the supernatant containing the solubilized components was used in the subsequent binding studies. The pellet was suspended in TES or TS buffer by homogenization and then assayed for its progesterone binding capacity. Triton X-100 was found to increase the amount of radioactivity left in the supernatant in the progesterone binding assay, thus showing some "binding" of steroid to the detergent. Therefore in the assay, buffer controls containing similar concentrations of Triton X and steroids were run parallel with the microsomal samples to determine any background radioactivity after DCC adsorption. Background values increased with increasing concentrations of Triton X-100. The respective background values were subtracted before the calculation of the data.

The steroid specificity of the Triton X-solubilized progesterone binding components was studied by determining the approximate relative affinity (R.A.) of the solubilized binder for progesterone and other steroids. In the determination, 1.9 nM [^{3}H]-progesterone and 0–2000 nM non-radioactive steroid were used, and the R.A. were calculated from the 50% binding level of radioactive progesterone [13].

Thin-layer chromatography (t.l.c.) of microsome-bound [^{3}H]-labelled steroid. To investigate whether progesterone was metabolized when bound to the liver microsomes, microsomal suspensions were prepared from the livers of one male and one female rat, and incubated with tritiated progesterone at +6 C for 1 h, after which the free steroids were adsorbed onto DCC and the microsome-bound steroid was extracted with diethylether:ethyl acetate as described in detail elsewhere [8]. The extracts were then analyzed by t.l.c. using the solvent system chloroform:acetone 9:1 (v/v) [8].

Enzyme treatment. The liver microsomes of 3 female rats were treated with protease and RNase as described previously [8]. The enzyme treatment was performed at +37 C using an incubation time of 15 min. After the incubation the samples were cooled and assayed for their progesterone binding capacity. Control microsome suspensions were kept at 0 C and +37 C for the same period.

Treatment by EDTA and KCl. To study any effect of increasing concentrations of EDTA on the total microsomal progesterone binding capacity, microsomes were prepared in TS buffer from the liver of a female rat and divided into four equal portions, which were suspended in four different media: (a) TS buffer containing 25 mM Tris–HCl and 250 mM sucrose, (b) TES_1 buffer containing 1.1 mM EDTA in addition to Tris and sucrose, (c) TES_2 buffer containing 4.2 mM EDTA in addition to Tris and sucrose and (d) TES_3 buffer containing 17 mM EDTA in addition to Tris and sucrose. These four different microsomal suspensions were kept for 30 min at 0 C, after which, their progesterone binding capacity was determined. The results were calculated per microsomal protein content, which varied between 4.9–6.9 mg/ml.

To investigate whether any microsomal progesterone binding components of rat liver could be extracted by 0.4 M KCl, microsomes were prepared in TS buffer from the liver of a female rat in the usual way, and then divided into two portions which were suspended in TS buffer and a buffer containing 0.4 M KCl and 25 mM Tris pH 7.4. The suspension containing KCl was then recentrifuged for 60 min at 105,000*g*, the pellet resuspended in TS buffer and the supernatant containing KCl used as such in the progesterone binding studies. The progesterone binding capacity and the appropriate protein concentrations of the three preparations obtained were then measured.

Measurement of radioactivity. Radioactivity was measured in an LKB Wallac liquid scintillation counter 81 000 (LKB Wallac Inc., Turku, Finland) using Insta-Gel (Packard Instrument International S.A., Zürich, Switzerland) as the scintillation solution. No correction for quenching was made as standards were counted simultaneously with all analytical samples in an identical medium. The counting efficiency was 34%.

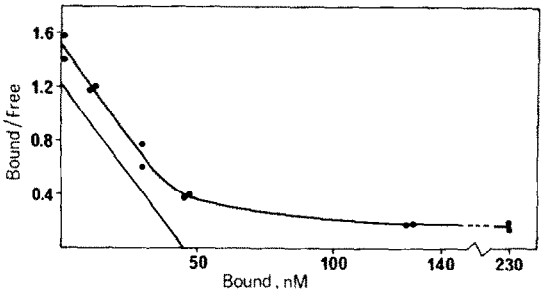

Fig. 1. The Scatchard plot obtained using microsomes prepared from the liver of a male rat in TES buffer, pH 7.4. The concentration of [^{3}H]-progesterone was 2 nM, and the final protein concentration 2.1 mg/ml. The incubation time was 1 h at +6°C, and the reaction time with DCC 15 min. The straight line is the reduced Scatchard plot obtained as described in the references no. 10 and 11 and gives a K_a of 2.7 × 10^7 M^{-1} for progesterone in this case.

RESULTS

Binding of progesterone by rat liver microsomes. Progesterone was bound with high affinity and limited capacity by the liver microsomes of male, female and pregnant female rats. High amounts of nonspecific binding (~ 50% of total) were also present. Figure 1 shows a Scatchard plot obtained for the liver microsomes of a male rat. The K_a of the high affinity progesterone binding components was ~ 1.7 ± 0.8 (mean ± S.D.) × 10^7 M^{-1} (n = 9) for progesterone when calculated by the "reduced Scatchard plot" method [10, 11]. The approximate concentration of high affinity progesterone binding sites varied between 15–35 pmol/mg microsomal protein, and tended to be slightly higher in male rats.

T.l.c. of microsome-bound [^{3}H]-steroid. The microsome-bound progesterone was not metabolized under the conditions used in the binding studies, as 92% of the [^{3}H]-steroid bound to female microsomes and 94% of that bound to male liver microsomes were located in the area of the progesterone standards on the t.l. plates. The purity of the standard [^{3}H]-progesterone was 94–96% when assessed in the same chromatographic system.

Solubilization of the microsomal progesterone binding components by Triton X-100. The progesterone binding components of female rat liver microsomes were partly solubilized by treatment with the detergent Triton X-100 (Table 1). In the presence of 0.1–0.2% (v/v) Triton X, optimal solubilization was achieved. At this concentration of Triton X, 50% of the total progesterone binding capacity was found in the 105,000*g* supernatant after centrifugation of the detergent-treated microsomal suspension. This concentration of detergent slightly affected the binding of progesterone by reducing the total amount of bound steroid (solubilized + pellet). With Triton X-100 concentrations of 0.5 and 1.0%, progesterone binding was absent when determined by the DCC method.

The results of the Triton X treatment of microsomes from the liver of a 17 day pregnant rat are shown in Fig. 2. The solubilized binding components residing in the 105,000*g* supernatant are saturable with progesterone, whereas, the binding of progesterone by the resuspended 105,000*g* pellet is nonsaturable. The K_a of the solubilized progesterone binding components was ~ 2.8 ± 1.1 × 10^7 M^{-1} (n = 4).

Steroid specificity. The steroid specificity of the Triton X-100 solubilized progesterone binding components is given as the relative affinity (R.A.) for various steroids in Table 2. Of the 11 steroids tested, progesterone had the highest R.A., but other C-21 steroids and C-19 steroids had R.A. greater than 10%. The steroids with an intact 4-ene-3-keto group, except cortisol, showed the highest binding while a reduction of the 4,5-double bond reduced affinity both in the androstan and pregnan series. The next potent competitors after progesterone were 17-hydroxyprogesterone (R.A. 93%) and the synthetic progestins medroxyprogesterone acetate (R.A. 25%) and norethisterone (R.A. 21%). Testosterone displayed quite a good competition with an R.A. of 18%, whereas, oestradiol and cortisol were not bound to any significant degree. The steroid specificity of the microsomal progesterone binding components was also studied using 2 nM radioactive progesterone and one level, 167 nM, of test steroid. Progesterone competed best for the microsome-bound [^{3}H]-progesterone in these conditions, also.

Enzyme treatment. The total progesterone binding capacity of female rat liver microsomes was reduced by 46% and 43% (mean of three experiments) after protease and RNase treatment, respectively. The results were not corrected for the non-specific binding exhibited by the protease solution itself, which increased the progesterone binding percentage of protease-treated microsomes by 10–20%. After treatment by protease there was still some progesterone-specific binding left, as a 100- and 1000-fold excess of progesterone reduced binding of [^{3}H]-progesterone (Fig. 3). In the RNase-treated microsomes the remaining binding of [^{3}H]-progesterone was less affected by excess non-radioactive progesterone, indicating that the progesterone-specific binding components had been mostly degraded as seen in Fig. 3. These results showed that both protein and RNA were involved directly or indirectly in the progesterone binding to the microsomal binding sites of rat liver.

Treatment by EDTA and KCl. The progesterone binding capacity of liver microsomal suspensions containing 0–17 mM EDTA in addition to Tris–sucrose, pH 7.4, did not vary significantly. The binding of progesterone was the same in all four preparations, and the competitive protein binding curves obtained were similar, indicating that the high affinity binding of progesterone was not affected by EDTA under the conditions used.

Treatment with 0.4 M KCl was also without effect, as the high affinity progesterone binding components were retained in the pellet fraction and only nonspecific binding of progesterone was evident in the KCl solubilized proteins found in the supernatant fraction.

Table 1. Solubilization of rat liver microsomal protein by treatment with Triton X-100 and the binding of [^{3}H]-progesterone by the solubilized progesterone binding components and the nonsolubilized pellet fraction. The concentration of [^{3}H]-progesterone was 2.1 nM, the incubation time was 1 h at +6°C and reaction time with dextran-coated charcoal 15 min

	Control Microsome suspension		Solubilized 105,000*g* supernatant		Resuspended 105,000*g* pellet		Supernatant + pellet (total)
Concentration of Triton X-100 (%)	Protein concentration $\frac{mg}{ml}$	[^{3}H]-progesterone bound $\frac{c.p.m.}{mg\ protein} \times 10^{-3}$	Protein concentration $\frac{mg}{ml}$	[^{3}H]-progesterone bound $\frac{c.p.m.}{mg\ protein} \times 10^{-3}$	Protein concentration $\frac{mg}{ml}$	[^{3}H]-progesterone bound $\frac{c.p.m.}{mg\ protein} \times 10^{-3}$	[^{3}H]-progesterone bound $\frac{c.p.m.}{mg\ protein} \times 10^{-3}$
0	5.03	19.5	—	—	—	—	19.5
0.1	—	—	2.52	10.0	4.66	7.9	17.9
0.2	—	—	3.41	11.4	4.11	5.4	16.9
0.3	—	—	3.81	9.2	3.37	4.9	14.1

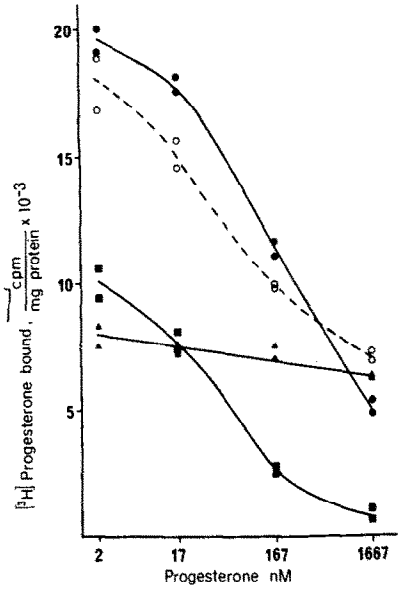

Fig. 2. The effect of Triton X-100 treatment on the binding of progesterone by liver microsomes from a 17 day pregnant rat. Triton X-100 concentration 0.1% (v/v) and [^{3}H]-progesterone 2 nM. The steroid incubation time was 1 h at +6°C, and the reaction time with DCC 15 min. ●——●: control microsome suspension, protein concentration 1.7 mg/ml, ■——■: 105,000*g* supernatant from the Triton X-treated microsome suspension, protein concentration 0.8 mg/ml, ◀——▶: 105,000*g* pellet (resuspended) from the Triton X-treated microsomal suspension, protein concentration 1.6 mg/ml, O---O: the curves of the 105,000*g* supernatant and resuspended pellet fraction added together.

Effect of storage and temperature. The freezing of microsomal suspensions, in TES buffer pH 7.4, slightly reduced their progesterone binding capacity. After 24 and 72 h at −20°C binding was reduced to 96% and 83% of the original capacity, respectively. When stored at +6°C the binding components were degraded more quickly. During the short 15 min incubation at +37°C used in the enzyme treatment 45% of the progesterone binding capacity was lost in one experiment.

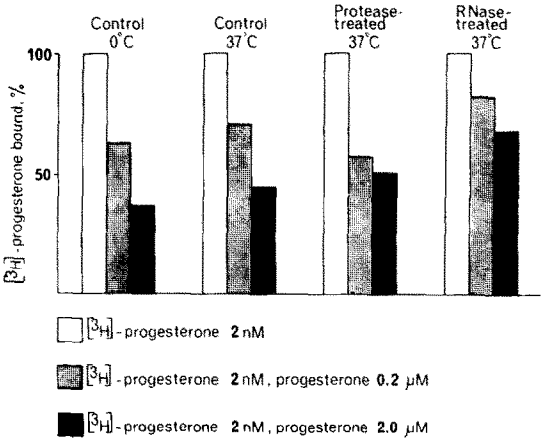

Fig. 3. The relative progesterone binding capacity of female rat liver microsomes treated with protease and RNase. The values given represent the mean of three different experiments performed on separate days. The ability of each preparation to bind 2 nM [^{3}H]-progesterone is given as 100% and the decrease in binding caused by the addition of excess nonradioactive progesterone has been calculated relative to the binding at the 2 nM concentration. The incubation time with the enzymes was 15 min at +37°C. The absolute progesterone binding capacity of the treated microsomal suspensions was decreased by 46% and 43% by protease and RNase treatment, respectively, when compared to the +37°C control.

DISCUSSION

Certain features of oestradiol and testosterone binding to rat liver microsomes reported by Blyth *et al.*[1–3] resemble the present results. The affinity of the high affinity binding sites called "tight" sites by Blyth *et al.*[1] is of the same magnitude, the K_d being 34 nM for oestradiol and 36 nM for testosterone [1], whereas, a K_d of ~ 36 nM was calculated for the Triton X-100 solubilized progesterone binding components. Furthermore, the concentration of microsomal high affinity steroid binding sites is of the same order [1–3], and all three steroids are still bound after the freezing of microsomal preparations [1, 2].

Table 2.

Trivial name	Systematic name	Relative affinity %
progesterone	4-pregnene-3,20-dione	100
17-hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione	93
medroxyprogesterone acetate	6α-methyl-3,20-dioxo-4-pregnen-17α-yl-acetate	25
norethisterone	17α-ethynyl-17β-hydroxy-4-estren-3-one	21
testosterone	17β-hydroxy-4-androsten-3-one	18
20α-dihydroprogesterone	20α-hydroxy-4-pregnen-3-one	16
5α-pregnanedione	5α-pregnane-3,20-dione	13
5β-pregnanedione	5β-pregnane-3,20-dione	12
5α-dihydrotestosterone	17β-hydroxy-5α-androstan-3-one	12
cortisol	11β,17α,21-trihydroxy-4-pregnene-3,20-dione	2
oestradiol	1,3,5(10)-oestratriene-3,17β-diol	<3

The relative affinity (R.A.) of the Triton X-100 solubilized microsomal progesterone binding components from rat liver for various steroids. Microsomes from female rat livers were prepared in TS buffer pH 7.4, suspended in TTS buffer pH 7.4 and recentrifuged at 105,000*g*. The supernatant thus obtained was used for the determination of the R.A., which were calculated as described in the text and reference no. 13.

It has been shown that steroid hormones can promote binding of polysomes to smooth microsomal membranes *in vitro* [14]. Blyth *et al.*[2] suggested that the microsomal high affinity binding sites for oestradiol and testosterone are involved in such polyribosome-membrane interactions. In the present study it was found that RNA may be involved in progesterone binding to liver microsomes, as both protease and RNase treatment decreased the microsomal progesterone binding capacity. Approximately one half of the total progesterone binding capacity remained after treatment with both enzymes. The reason for this might be either incomplete enzymatic digestion, or, that the progesterone binding sites residing in the membrane structure are partly protected against enzyme action. The idea that the steroid binding sites may be components of the membrane structure itself is further supported by the fact that 0.4 M KCl was unable to extract these binding components from the membranes. Only the detergent Triton X-100 solubilized the progesterone binding components from the membranes.

Treatment of rough microsomal membranes with EDTA causes a stepwise removal of the ribosomal subunits from the membrane surface [15, 16]. Such degranulated rough membranes contain no high affinity binding sites for oestradiol or testosterone [3]. In the present study treatment with EDTA had no effect on the microsomal progesterone binding capacity.

The microsomes of rat myometrium contain high affinity progesterone binding sites [8]. The microsomal progesterone binding components of rat liver differ from those of rat myometrium in progesterone binding affinity ($K_a \sim 6 \times 10^7\ M^{-1}$ in myometrium), and in steroid specificity. Testosterone, 5α-dihydrotestosterone and 5α-pregnanedione compete well with [^{3}H]-progesterone bound to myometrial microsomes, whereas, 17-hydroxyprogesterone and the progestins norethisterone and medroxyprogesterone acetate, which have a relatively high affinity for the liver microsomal binding components, have a low affinity for the myometrial microsomes. The microsomal progesterone binding components of rat liver and myometrium are both distinctly different from the cytosolic progesterone receptor of rat uterus [5–7].

The relative affinities of various steroids for the Triton X-solubilized microsomal binding proteins would suggest that the microsomal progesterone binding protein is an enzyme, and that steroid binding represents a formation of the enzyme-substrate complex. C-19 and C-21 steroids with a 4-ene-3-keto group, except cortisol, are bound. Thus the binding protein might be a microsomal 4-ene-5α-reductase. It is also known from the substrate specificity pattern of the microsomal 4-ene-5α-reductase of rat liver that progesterone, testosterone and 17-hydroxyprogesterone are efficiently metabolized by the enzyme [17]. However, the microsomal 4-ene-5α-reductase of rat liver is controlled by androgens and has lower activity in adult male than female rats [18], whereas, the activity of several other steroid metabolizing enzymes is higher in male rats [19]. In the present study, the progesterone binding capacity of liver microsomes was slightly but not significantly higher in male rats, which is in disagreement with the information concerning the steroid metabolizing enzymes mentioned above.

In conclusion, the high affinity progesterone binding components of rat liver microsomes are described. These are different from progesterone binding proteins found in other rat tissues, but they resemble the previously reported microsomal oestradiol and testosterone binding components of rat liver. Progesterone is bound with the highest affinity, but many other 4-ene-3-keto steroids are also bound. From the results of the present paper it cannot be concluded whether this progesterone binding protein is an enzyme or is in some way involved in membrane-ribosome interaction with a functional significance other than enzyme action.

Acknowledgements—The technical assistance of Mrs. Helena Lindgren is gratefully acknowledged. The work was supported by a grant from the Ford Foundation.

REFERENCES

1. Blyth C. A., Freedman R. B. and Rabin B. R.: *Nature New Biol.* **230** (1971) 137–139.
2. Blyth C. A., Freedman R. B. and Rabin B. R.: *Eur. J. Biochem.* **20** (1971) 580–586.
3. Blyth C. A., Cooper M. B., Roobol A. and Rabin B. R.: *Eur. J. Biochem.* **29** (1972) 293–300.
4. Blyth C. A., Clark R. P., Freedman R. B., Hammond J., James D. W., Rabin B. R., Ridge D., Vinter J. and Williams D.: *Eur. J. Biochem.* **32** (1973) 57–62.
5. Feil P. D., Glasser S. R., Toft D. O. and O'Malley B. W.: *Endocrinology* **91** (1972) 738–746.
6. Terenius L.: *Steroids* **19** (1972) 787–794.
7. Haukkamaa M.: *J. steroid Biochem.* **5** (1974) 73–79.
8. Haukkamaa M. and Luukkainen T.: *J. steroid Biochem.* **6** (1975) 1311–1317.
9. Haukkamaa M. and Luukkainen T.: *J. steroid Biochem.* **5** (1974) 447–452.
10. Měšter J., Robertson D. M., Feherty P. and Kellie A. E.: *Biochem. J.* **120** (1970) 831–836.
11. Sandberg A. A., Rosenthal H., Schneider S. L. and Slaunwhite W. R.: In *Steroid Dynamics* (Edited by G. Pincus, T. Nakao and J. F. Tait). Academic Press, New York (1966) pp. 36–39.
12. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
13. Korenman S. G.: *Endocrinology* **87** (1970) 1119–1123.
14. Sunshine G. H., Williams D. J. and Rabin B. R.: *Nature New Biol.* **230** (1971) 133–136.
15. Sabatini D. D., Tashiro Y. and Palade G. E.: *J. molec. Biol.* **19** (1966) 503–524.
16. Rabin B. R., Sunshine G. H. and Williams D. J.: In *Chemical Reactivity and Biological Role of Functional Groups in Enzymes. Biochemical Society Symposia*, No. 31 (Edited by R. M. S. Smellie). Academic Press Inc., London, Ltd. (1970).
17. Nozu K. and Tamaoki B-J.: *Acta endocr., Copenh.* **76** (1974) 608–624.
18. Schriefers H., Hoff H-G. and Ghraf R.: *Hoppe-Seyler's Z. physiol. Chem.* **354** (1973) 501–506.
19. Ghraf R., Lax E. R., Hoff H-G. and Schriefers H.: *Acta endocr., Copenh.*, **77** (1974) 287–297.