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PRODUCTION OF TESTOSTERONE AND CORTICOSTEROIDS BY THE RAT ADRENAL GLAND INCUBATED *IN VITRO* AND THE EFFECTS OF STIMULATION WITH ACTH, LH AND FSH

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SUMMARY

Using techniques of *in vitro* incubation it was found that the Wistar rat adrenal has the capacity to synthesise testosterone from added radioactive precursors and endogenous precursors.

With [^{14}C]-progesterone as added precursor the formation of labelled testosterone was demonstrated by methods including chromatography in various systems, the formation of derivatives and isotope dilution. In 2 h incubations of female glands the yield of labelled testosterone was 0.01–0.04% of the added precursor, i.e. about 1% of that of corticosterone.

From endogenous precursors, testosterone production was measured by RIA. Confirmation of the values was obtained by cross checking with values obtained with the samples in another laboratory (using RIA with a different antiserum), and by comparison with a g.l.c. method based on the diheptofluorobutyrate derivative with electron capture detection.

The capacity of the female gland to produce testosterone was approximately twice that of the male (per mg tissue), and yields were approximately 1% of those of total corticosteroids as measured by CPB. Addition of FSH or LH to the incubation media in varying concentrations up to 0.6 and 0.4 μg/ml respectively had no sustained effect on testosterone output, whereas ACTH (0.2 μg/ml) tripled the output from female glands. Corticosteroid output was stimulated by all three tropic hormones similarly.

The results offer an explanation for reports in the literature showing the partial dependence of circulating testosterone levels on the presence of the adrenal and the variation of corticosteroid output with the stage of the oestrous cycle in rats.

INTRODUCTION

It has long been accepted that the mammalian adrenal cortex is a source of androgens as well as corticosteroids. In the human adrenal for example, dehydroepiandrosterone (DHA) is a prominent secretory product especially as the sulphate [1], and in the human female, androstenedione secretion by the adrenal cortex may contribute significantly through peripheral conversion to the total pool of circulating testosterone [2] and oestrogens [3]. Other C_{19} steroids which may be secreted by the adrenal cortex in man and other species include 11β-hydroxyandrostenedione and, in lower yields than the others, testosterone [4–7].

Secretion of androgens by the rat adrenal cortex has received little direct study. This may have occurred because most reports state that 17α-hydroxylation was not detected when using rat adrenal preparations [8, 9] and this process is normally regarded as essential in androgen biosynthesis [10]. However, strong circumstantial evidence for androgen production by the rat adrenal cortex stems from the studies of Kniewald *et al.* [11]. They found that circulating plasma levels of testosterone were elevated in the period immediately after castration and only later decreased to the very low values normally found. On the other hand, circulating testosterone levels were reduced rapidly after adrenalectomy. These findings suggest either that the adrenal cortex secretes testosterone directly or possibly that there may be some interaction between adrenals and testis in testosterone formation.

Studies on the production of testosterone by rat adrenal tissue from radioactive precursors have yielded conflicting evidence. In some experiments, no formation of C_{19} steroids was obtained from cholesterol, pregnenolone or 17α-hydroxyprogesterone [12] whereas in others, transformations of pregnenolone into dehydroepiandrosterone, androsterone, testosterone and 11β-hydroxyandrostenedione was reported [13, 14].

This paper re-examines the capacity of the rat adrenal gland to secrete testosterone *in vitro*, and the effect of trophic hormones on testosterone and corticosteroid production.

MATERIALS AND METHODS

Incubations. White Wistar rats (200–250 g body wt) were killed by cervical dislocation and the adrenals quickly removed, cleaned and stored on ice until

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required for incubation, which invariably followed within 1 h. Three series of incubations were performed.

Experiment 1. Conversion of [^{14}C]-progesterone. Ten pairs of female rat adrenals were minced and incubated in 10 ml Krebs bicarbonate Ringer containing [4-^{14}C]-progesterone (1 μCi: S.A. 193 μCi/mg) for 2 h at 37° C. Incubation medium and tissue were then exhaustively extracted with ethyl acetate and the products were fractionated in the systems of chromatography previously described [15]. Further procedures with this material are given in Results.

Experiment 2. Production of steroids from endogenous precursors and the effects of trophic hormones. Individual pairs of adrenals were incubated in 5 ml Krebs bicarbonate Ringer (containing 0.33 M Ca^{2+}) for 30 min at 37° C. At the end of this period the incubation medium was discarded and fresh medium added, with trophic hormones when appropriate (see below). Incubation then proceeded for a further 2 h. Tissue and medium were then homogenised and in some experiments samples were taken for the estimation of protein [16]. Steroids were exhaustively extracted with ethyl acetate and appropriate aliquots taken for assay.

Experiment 2a. Adrenals from six male rats, six control female rats and three groups of six female rats with addition to each 5 ml incubation medium of either 0.4 μg LH, 2 μg FSH, or 1 μg ACTH were incubated as described above.

Experiment 2b. Adrenals from female rats were incubated with varying doses of LH (0, 0.1, 0.2, 0.5 and 2 μg/5 ml) and FSH (0, 0.1, 0.2, 0.5, 2.0 and 3.0 μg/5 ml) (six incubations per dose).

Experiment 3. Interaction of adrenal and testis tissue. Interstitial tissue was prepared from rat testes by microdissection [17]. Six 10 mg batches were separately incubated in 5 ml Krebs bicarbonate Ringer for 2 h at 37° C. In another series of incubations six pairs of adrenal glands from the same animals were incubated separately and in a third series the two tissue types were incubated together. Steroids were extracted at the end of the incubation period with ethyl acetate.

Steroid assay. A competitive protein binding assay based on dog plasma transcortin was used to give an index of total corticosteroid output [18]. In one set of experiments (Experiment 2a) a radioimmunoassay, based on an antiserum raised to the 3-carboxymethoxime derivative of corticosterone, was used to test the validity of the competitive protein binding assay. The antiserum was generously donated by Drs. V. Martin and C. W. Edwards and is described by Gross *et al.* [34]. It shows cross reactivity with deoxycorticosterone (24%) but no significant reactivity with other steroids likely to be produced by rat adrenals. The antiserum was used at a concentration of 1:6000 and was equilibrated with the extracts and standards overnight at 4° C. Separation of free and bound steroid was performed on Sephadex minicolumns [18].

For testosterone a radioimmunoassay based on an antiserum raised to the 3-carboxymethoxime derivative of testosterone was used as previously described [18]. The antiserum was generously donated by Dr. D. Exley. It has properties exactly similar to other testosterone-3-carboxy-methoxime derived antisera [35] and has negligible cross reactivity with a wide variety of C_{21}, C_{19} and C_{18} steroids, except for a 20% reaction with 5α-dihydrotestosterone. To provide verification of the results, duplicate samples of the extracts from Experiment 3 were estimated in the laboratory of Dr. W. P. Collins using an antiserum raised to the carboxyethyl thioether derivative of 1α-hydroxytestosterone, which was characterised by the usual methodology [36], and gives negligible cross reactivity with a wide variety of other steroids, with the exception of 5α-dihydrotestosterone (10–15% cross-reactivity). In addition, total extracts in Experiment 1 were treated with heptafluorobutyric anhydride in acetone [19] and samples were subjected to g.l.c. using a Packard chromatograph (model 7839). The glass columns used were 2 ft long with a packing of 0.3% XE-60 on Gas Chrom. Q. Chromatograms were run at a temperature of 210° C. Peaks corresponding to testosterone diheptafluorobutyrate were measured and augmented without loss of symmetry by the addition of authentic material.

RESULTS

Formation of radioactive steroids (Experiment 1). In extracts of samples incubated with [4-^{14}C]-progesterone, material corresponding to testosterone on paper chromatography in the systems toluene: 70% methanol and light petroleum (B.P. 60–80°): 75% methanol was acetylated with acetic anhydride and pyridine, and rechromatographed in the light petroleum: 75% methanol system. It was then eluted, hydrolysed with NaOH, oxidised with *tert.* butyl chromate, and rechromatographed in the same system. Authentic [^{3}H]-androstenedione was added to the material corresponding to androstenedione, and the mixture rechromatographed in thin layer silica gel films (Eastman chromagram) in the systems light petroleum–benzene–ethylacetate (1:1:4 by vol.); chloroform–methanol (97:3 v/v) and toluene–methanol (96:4 v/v). Successive ^{3}H/^{14}C values were 28.3, 30.1 and 26.0, which (in view of the low yields obtained) were regarded as being sufficiently close to indicate homogeneity of the biosynthetic and authentic materials. Corticosterone was isolated as previously described [20] and both products were estimated after chromatography as the acetates.

Yields of testosterone were approximately 0.01–0.04% of the added [^{14}C]-progesterone precursor, compared with 1–4% yields for corticosterone.

Formation of testosterone from endogenous precursors (Experiments 2a–b). Using samples of incubations of female adrenals (controls and stimulated by ACTH and gonadotrophin), values obtained by the competitive protein binding for corticosteroids show

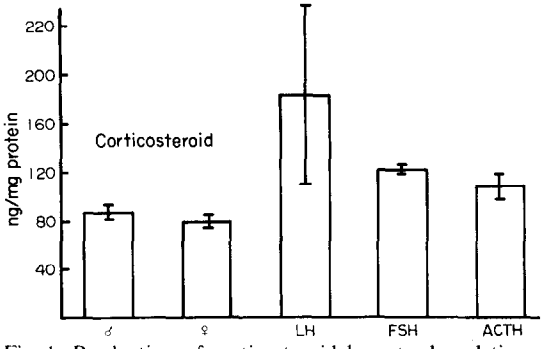

Fig. 1. Production of corticosteroid by rat adrenal tissue incubated *in vitro* for 2 h (1 pair glands/5 ml Ringer/flask). Values expressed as ng corticosteroid produced/mg protein, are means of 6 incubations ± S.E. Experiments with additions of trophic hormones were performed with female tissue only and the additions were either 1 μg ACTH, 0.5 μg LH or 2 μg FSH/flask. Comparison of ACTH addition with female controls, $P < 0.02$; comparison of FSH addition with female controls, $P < 0.001$.

satisfactory correlation ($r = 0.7$) with the radioimmunoassay for corticosterone although the CPB data were systematically higher than the RIA data by a factor of at least 1.5. This may be anticipated in view of the affinity of transcortin for many types of corticosteroid.

Values for testosterone obtained by use of the routine radioimmunoassay gave a good correlation with values obtained by Dr. W. P. Collins ($r = 0.93$; slope 0.95). Similarly, values obtained from the peak heights in the g.l.c. procedures gave a correlation coefficient of 0.7 with the RIA (the loss of correspondence here when compared with the RIA is almost certainly due to the lower accuracy of the g.l.c. method in which internal standards were not routinely used).

Both male and female adrenals produced testosterone from endogenous precursors (Fig. 2). In these experiments it should however be noted that the female glands had a greater capacity to produce testosterone than the male. When expressed per mg protein, the female capacity was approximately twice that of the male (570 pg/mg vs 210 pg/mg). It should also be borne in mind that the female gland is at least 25% larger than the male, hence the overall capacity of the female adrenal to produce testosterone *in vitro* was greater still.

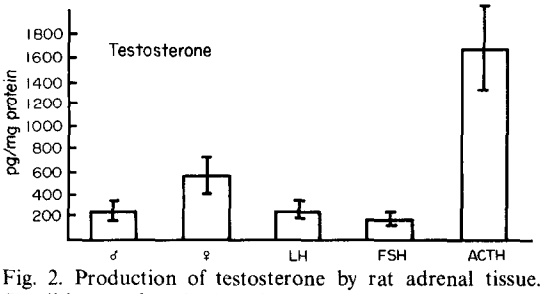

Fig. 2. Production of testosterone by rat adrenal tissue. Conditions as for Fig. 1. Values, expressed as pg testosterone produced/mg protein, are means of 6 incubations ± S.E. Comparison of male and female, $P < 0.05$; comparison of ACTH addition with female controls, $P < 0.02$.

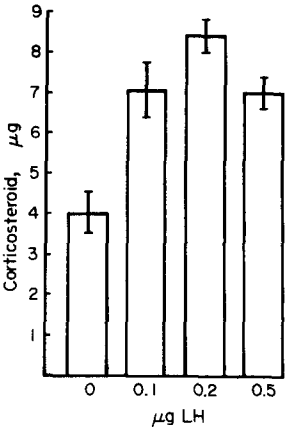

Fig. 3. Production of corticosteroid by female rat adrenal tissue. Conditions as for Fig. 1. Additions were 0.1, 0.2 or 0.5 μg LH/flask. Values expressed as total corticosteroid produced/incubation, are means of 6 incubations ± S.E. Comparison of maximum stimulation with controls, $P < 0.001$.

Effects of stimulation. From Fig. 1 (Experiment 2a) it can be seen that when expressed per mg protein the production of corticosteroid (measured by CPB) by male and female adrenals is similar. When 0.1 μg/ml LH or 0.4 μg/ml FSH or 0.2 μg/ml ACTH was added to female tissue similar stimulation of corticosteroid production was observed. (Although the mean values between control and LH stimulated incubations appear different, these differences are in this case not statistically significant, but see Fig. 3). In contrast, no effect of the gonadotrophins was seen on testosterone production (Fig. 2) which was greatly stimulated by ACTH.

Maximal output of corticosteroid production was obtained under gonadotrophin stimulation with additions of about 0.2 μg LH (Fig. 3) and 2 μg FSH (Fig. 4) (Experiment 2b). In contrast no sustained stimulation of testosterone was found with either gonadotrophin at any concentration (Figs. 5 and 6).

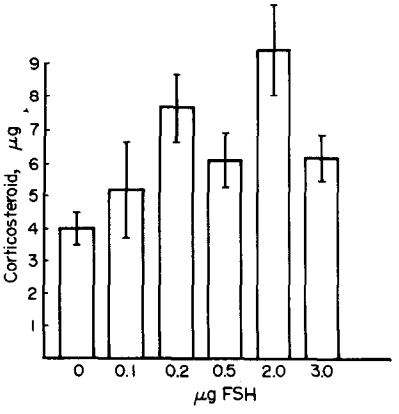

Fig. 4. Production of corticosteroid by female rat adrenal tissue. Conditions as for Fig. 1. Additions were 0.1, 0.2, 0.5, 2.0 or 3.0 μg FSH/flask. Values, expressed as total corticosteroid produced/incubation, are means of 6 incubations ± S.E. Comparison of maximum stimulation with controls, $P < 0.01$.

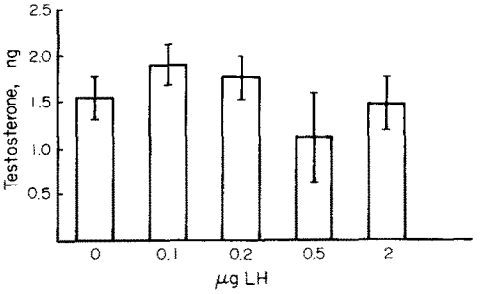

Fig. 5. Production of testosterone by female rat adrenal tissue. Conditions as for Figure 1. Additions were 0.1, 0.2, 0.5 or 2 μg LH/flask. Values, expressed as total testosterone produced/incubation, are means of 6 incubations ± S.E.

Mixed tissue incubations. (Experiment 3). In Fig. 7 it can be seen that the incubation of adrenal tissue and interstitial testis tissue together gave no increases in testosterone production beyond that expected by the addition of the products of the two tissues incubated separately.

DISCUSSION

It now seems clear that the rat adrenal cortex, like those of other mammalian species, has the capacity to synthesise and secrete testosterone *in vitro*. The results reported here not only show the formation of testosterone from radioactive progesterone but also its secretion under different conditions of stimulation from endogenous precursors. This confirms and extends the results of other workers [13, 14, 21] who have demonstrated the production of androgens from radioactive pregnenolone by rat and also mouse adrenal tissue. The pathway for formation of testosterone has not been studied and requires investigation but the yields of testosterone relative to other steroids are so small as to make this difficult. However the formation of androgens presumably proceeds via 17α-hydroxylation and it has been suggested this process may be increased when the 11β-hydroxylase system is inhibited [22], which may prove a useful experimental approach.

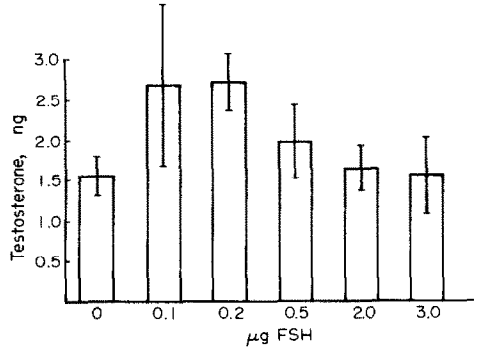

Fig. 6. Production of testosterone by female rat adrenal tissue. Conditions as for Fig. 1. Additions were 0.1, 0.2, 0.5, 2.0 and 3.0 μg FSH/flask. Values, expressed as total testosterone produced/incubation, are means of 6 incubations ± S.E.

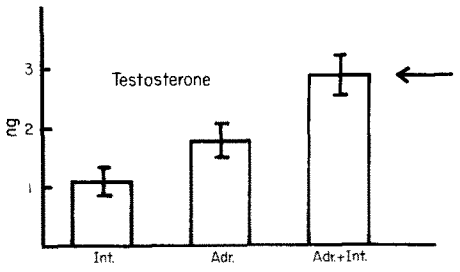

Fig. 7. Production of testosterone by rat testis interstitium (10 mg tissue/flask) and rat adrenal tissue (1 pair glands/flask) incubated *in vitro* for 2 h both separately and together. Values, expressed as total testosterone produced/incubation, are means of 6 incubations ± S.E. Arrow indicates the sum of the amounts of testosterone produced by the individual tissues incubated separately.

Effects of ACTH. The fact that testosterone production is stimulated by the addition of ACTH to the incubation medium is in agreement with the studies of other authors on the production of adrenal androgens in other species. In man for example, ACTH increased DHA and DHAS secretion [23–25] *in vivo* and *in vitro* [26]. Higher concentrations of androstenedione and testosterone (and oestrogen) were found in human adrenal vein blood than in circulating plasma [6] and these too were increased by ACTH administration. On the other hand, *decreased* levels of circulating testosterone after ACTH treatment has been shown in man [27, 28] and the guinea-pig [29]. It is possible that peripheral conversions of the androgens may complicate the issue here. However, in one marsupial species, *Trichosurus vulpecula*, ACTH administration caused a decline in circulating androgen which in this case was consistent with changes *in vitro* [18]. In another, *Didelphis virginiana*, ACTH was without effect either on circulating androgens or on androgen production *in vitro* [30].

It is therefore possible that species variation may account for some of these anomalies (see below).

The relative lack of effect of ACTH on corticosteroid production in these experiments is somewhat unexpected in view of the literature on ACTH effects *in vitro*. Studies in progress suggest this arises from the nature of the methodology (currently many authors use either superfusion or cell suspension techniques which can modify the response) and the precise source of the tissue (Vinson, unpublished observations).

Effects of gonadotrophins. The stimulation of corticosteroid output by both LH and FSH in these experiments is unexpected. While evidence based on morphological and histological studies has in the past suggested the possibility of such an effect [31, 32], direct evidence has been lacking. The phenomenon offers an explanation for the increases in corticosteroid secretion seen in prooestrous rats [33]. In experiments with *Didelphis virginiana*, stimulation of corticosteroid production by the addition of LH *in vitro* has already been reported [30]. The lack of effect of

the gonadotrophins on testosterone production by rat adrenals is consistent with the general body of literature which cites ACTH as the main controlling agent for adrenal androgen secretion (see above). However, other authors report that gonadotrophins stimulate androgen formation in human adrenal tissue [26] and in two marsupial species PMS or HCG treatment stimulated testosterone production [18, 30]. In view of the differences between these species and the rat with regard to stimulation of testosterone formation by both ACTH (see above) and gonadotrophins, it appears that there may be wide species variation in the control mechanisms.

Finally it appears that there is no indication that the adrenal and testis interact in any way in the production of testosterone *in vitro* (see Fig. 7). An interpretation of the findings of Kniewald *et al.* [11] could therefore be that after castration, testosterone is produced by the adrenal during the period of post-surgical trauma.

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REFERENCES

1. Baulieu E. E.: *J. clin. Endocr. Metab.* **22** (1962) 501–510.
2. Horton R. and Tait J. F.: *J. clin. Invest.* **45** (1966) 301–313.
3. Grodin J. M., Siiteri P. K. and MacDonald P. C.: *J. clin. Endocr. Metab.* **36** (1973) 207–214.
4. Kase N. and Kowal J.: *J. clin. Endocr. Metab.* **22** (1962) 925–928.
5. Ward P. J. and Grant J. K.: *J. Endocr.* **26** (1963) 139–147.
6. Baird D. T., Uno A. and Melby J. C.: *J. Endocr.* **45** (1969) 135–136.
7. Vinson G. P. and Whitehouse B. J.: In *Advances in Steroid Biochemistry and Pharmacology* (Edited by M. H. Briggs). Academic Press, New York, Vol. 1, (1970) pp. 163–342.
8. Birmingham M. K. and Kurlents E.: *Can. J. Biochem.* **37** (1959) 510.
9. Laplante C., Giroud C. J. P. and Stachenko J.: *Endocrinology* **75** (1964) 825–827.
10. Dorfman R. I. and Ungar F.: In *Metabolism of Steroid Hormones.* Academic Press, New York (1965) p. 126.
11. Kniewald Z., Danisi M. and Martini L.: *Acta endocr. (Copenh.)* **68** (1971) 614–624.
12. Koref O., Steczek K. and Feher T.: *Acta endocr. (Copenh.)* **66** (1971) 727–736.
13. Askari H. A., Monette G. and Leroux S.: *Endocrinology* **87** (1970) 1377–1380.
14. Milewich L. and Axelrod L. R.: *J. Endocr.* **54** (1972) 515–516.
15. Vinson G. P., Phillips J. G., Chester Jones I. and Tsang W. N.: *J. Endocr.* **49** (1971) 131–140.
16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
17. Christensen A. K. and Mason N. R.: *Endocrinology* **76** (1956) 646–656.
18. Vinson G. P.: *Gen. Comp. Endocr.* **22** (1974) 268 276.
19. Dehennin L. A. and Scholler R.: *Steroids* **13** (1969) 739–761.
20. Vinson G. P. and Whitehouse B. J.: *Acta endocr. (Copenh.)* **61** (1969) 695–708.
21. Rosner J. M., Charreau E., Houssay A. B. and Epper C.: *Endocrinology* **79** (1966) 681–686.
22. Young R. B. and Sweat M. L.: *Archs. biochem. Biophys.* **121** (1967) 576–579.
23. Vaitukaitis J. L., Dale S. L. and Melby J. C.: *J. clin. Endocr. Metab.* **29** (1969) 1443–1447.
24. Wieland R. G., de Courcy C., Levy R. P., Zala A. P. and Hirschmann H.: *J. clin. Invest.* **44** (1965) 159–168.
25. Antonini F. M., Porro A., Serio M. and Tinti P.: *Exp. Gerontol.* **3** (1968) 181–92.
26. Terasawa M.: *Yonago Igaku Zasshi* **19** (1968) 477–487.
27. Rivarola M. A., Saez J. M., Meyer W. J., Jenkins M. E. and Migeon C. J.: *J. clin. Endocr. Metab.* **26** (1966) 1208–1218.
28. Beitins I. Z., Bayard F., Kowanski A. and Migeon C. J.: *Steroids* **21** (1973) 553–563.
29. Bullock L. P. and New M. I.: *Endocrinology* **88** (1971) 523–526.
30. Vinson G. P. and Renfree M. B.: *Gen. comp. Endocr.* **27** (1975) 214–222.
31. Chester Jones I.: *Br. Med. Bull.* **11** (1955) 156–159.
32. Lanman J. T. and Dinerstein J.: *Endocrinology* **67** (1961) 1–8.
33. Hinsull S. M. and Crocker A. D.: *J. Endocr.* **48** (1970) lxxix.
34. Gross H. A., Ruder H. J., Brown K. S. and Lipsett M. B.: *Steroids* **20** (1972) 681–695.
35. Midgley A. R. and Niswender G. D.: *Acta endocr. Copenh. suppl.* **147** (1970) 320–331.
36. Tyler J. P. P., Hennam J. F., Newton J. R. and Collins W. P.: *Steroids* **22** (1973) 871–889.

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BINDING OF THE ESTRADIOL RECEPTOR TO HEN OVIDUCT NUCLEI AND CHROMATIN

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SUMMARY

A method has been developed for a rapid determination of estradiol receptor binding to nuclei and chromatin based on precipitation with $CaCl_2$ and sedimentation through concentrated sucrose. The method is applied to hen oviduct nuclei and chromatin. Both types of material show optimal binding at 0.15 M NaCl at 25°C. The extent of binding increases linearly with increasing concentration of radioactive cytosol indicating the absence of detectable high affinity binding sites. A linear concentration curve is observed also with chromatin from chicks with very low levels of endogenous estradiol.

Receptor binding sites on chromatin are characterized using DNA binding substances. Intercalating and minor groove reagents inhibit binding 10–30% respectively. The mode of action of these compounds suggests that both hydrophilic and hydrophobic bonds are involved in the binding of estradiol receptor to chromatin. Non-ionic detergents are potent inhibitors of receptor binding. Some 60% of bound receptors may be removed with Triton X-100, further implicating hydrophobic binding.

INTRODUCTION

The nuclear binding sites of steroid hormones have been an object of studies attempting to establish the mechanism of action of steroid hormones (for review, see [1, 2]). Yet the intranuclear sites of steroid hormone action remain unestablished. The recent reports on the non-saturable binding of steroid receptors into nuclei[3] and on the massive low affinity binding of estradiol receptors into any kind of double stranded DNA[4, 5] are particularly puzzling and certainly obscure efforts to find high affinity binding sites. This is further complicated by the high aggregation tendency of the receptors[4, 6].

In the present study a method is worked out to investigate the binding of estradiol receptors to nuclei and chromatin. Artifacts caused by receptor aggregation are minimized in this procedure without sacrificing simplicity. The method is applied to show that hen oviduct nuclei and chromatin differ only slightly in their receptor binding and display no high affinity binding. The binding reaction is further characterized using detergents and chemicals reacting with DNA. Evidence is presented implicating the presence of multiple acceptor sites on chromatin.

MATERIALS AND METHODS

Preparation of cytosol and chromatin. Laying hens were killed by decapitation. The oviduct was removed and washed in ice-cold saline. The ligaments were removed and the tissue cut in small pieces with scissors. It was suspended in about 1.5 vol. of buffer TEGN containing 10 mM Tris–HCl, 1 mM Na_3 EDTA, 10% glycerol, 0.15 M NaCl and 1 mM 2-mercaptoethanol, pH 8.1[4] and homogenized with 20 strokes of a loosely fitting Teflon–glass homogenizer. The suspension was centrifuged at 700 *g* for 10 min. The supernatant was the source of cytosol which was prepared by centrifugation at 140,000 *g* for 45 min. The 700 *g* pellet was the source of chromatin.

Oviduct cytosol was charged with 1.2×10^{-8} M [2,4,6,7-^{3}H]-estradiol-17β (45 or 82 Ci/mmol, Amersham) at 4°C for at least 90 min. Dextran-coated charcoal (0.1 vol., 3% charcoal and 0.1% dextran) were added and incubation was continued for 10 min. The mixture was centrifuged at 8000 *g* for 15 min and the supernatant was always used freshly.

Oviduct nuclei were purified from the 700 *g* pellet. It was homogenized with 20 strokes in 0.1 M NaCl containing 1 mM $MgCl_2$ (medium NM) and passed through four layers of a cheese cloth. The filtrate was centrifuged at 700 *g* for 10 min. The pellet was homogenized with 10 strokes in medium NM and 1.75 M sucrose in medium NM (1:1 v/v), passed through four layers of a cheese cloth and centrifuged at 700 *g* for 10 min. The pellet was homogenized with 10 strokes in 1.75 M sucrose in medium NM; the homogenate was layered over 1/2 vol. of the same medium in 40 ml plastic tubes and the gradients were centrifuged in a Sorvall HS 4 swing-out rotor at 5000 *g* for 15 min. The pellet was washed twice with 0.15 M NaCl and then used as the nuclear preparation.

Chromatin was purified by homogenizing the 5000 *g* pellet in 70 mM NaCl containing 20 mM EDTA, pH 7.0, using 15 strokes followed by sedimentation at 8000 *g* for 10 min. This procedure was repeated and the resulting pellet was homogenized in 0.15 M NaCl and layered over an equal vol. of 1.6 M sucrose. The gradients were centrifuged in a Spinco SW-25 rotor at 63,000 *g* for 30 min. The pellet was used as chromatin.

Assay for estradiol receptor binding to chromatin. The binding assays normally contained 3 to 10 A_{260} units (absorbance at 260 nm) of the nuclear or chromatin preparations and 10 to 1000 μl of charged, charcoal treated estradiol cytosol in a total vol. of 1.5 ml of 0.15 M NaCl. In some assays a constant volume of cytosol was added from mixtures of radioactive and nonradioactive cytosol[5]. The incubation was carried out in tubes coated with bovine serum albumin at 25 C for 90 min with constant shaking.

At the end of the incubation the samples were made 10 mM in $CaCl_2$ and they were kept on ice for 5 min. They were layered on 2 ml of 1.6 M sucrose containing 2 mM $CaCl_2$ in plastic tubes coated with bovine serum albumin to reduce adsorption of material onto the walls. The tubes were immediately centrifuged at 4500 *g* for 20 min in a HS 4 Sorvall swing-out rotor. The tubes were inverted and allowed to stand upside-down at room temperature. Any remaining drops of sucrose were wiped dry with paper tissue; the chromatin pellet was taken up in 0.5 ml of ethanol and mixed with 5 ml of Bray's scintillation fluid for the determination of radioactivity.

Other procedures. Protein was determined according to Lowry *et al.*[7] and DNA according to Giles and Myers[8].

Total histones were prepared from oviduct chromatin according to Bolund and Johns[9] by extracting with 0.25 M HCl. Histone F 1 was prepared by using 0.5 M NaCl[9].

RESULTS

Optimation of the binding assay. Lability and aggregation[4, 6] of the oviduct estradiol receptor may seriously bias cell-free binding studies with nuclear components and particularly with fractionated chromatin which is not readily sedimentable. Such sources of artifact are inadequately attended in some published binding assays[4]. For this reason a procedure was developed which would enable a rapid and reliable assessment of the binding of estradiol receptor to nuclei and chromatin. This included centrifugation of the bound receptor through concentrated sucrose. However, as estradiol receptor is relatively unstable in sucrose, only a short-term exposure to sucrose could be allowed.

To obtain a prompt sedimentation of chromatin, precipitation with ions including Na^+, Mg^{2+}, and Ca^{2+} was tested. All of these ions are known to change the sedimentation properties of chromatin[10, 11]. In the case of oviduct chromatin $CaCl_2$ was shown to be the most effective precipitant (Fig. 1). At a concentration range between 1 and 10 mM this ion almost quantitatively precipitated oviduct chromatin. $MgCl_2$ and NaCl were not as effective as $CaCl_2$ and, moreover, they had a fairly narrow range for optimal precipitation. For this reason $CaCl_2$ was used in the binding assays, in which oviduct chromatin was sedimented through 1.6 M sucrose containing 2 mM $CaCl_2$.

Oviduct nuclei and chromatin used in the present study were characterized by protein/DNA ratios of 4.6 and 2.4, respectively.

The effect of temperature and incubation time on the binding of (^{3}H) estradiol receptor to nuclei and chromatin was studied in an incubation medium containing 0.15 M NaCl (Fig. 2). The most rapid binding was observed at 37°C but in this case the receptor–chromatin complex was not very stable as the extent of binding started to decline at longer incubation times. By contrast, no such instability was noted at 25 C recommending this temperature and an incubation time of 90 min for the assay conditions of choice. Chromatin bound initially more estradiol receptor as compared to nuclei but the level attained at 90 min was somewhat higher in nuclei than in chromatin.

The ionic requirements of the binding reaction were tested (Table 1). The most active binding occurred at 20 mM or 0.15 M NaCl. KCl at a 0.15 M concentration promoted binding somewhat less than NaCl.

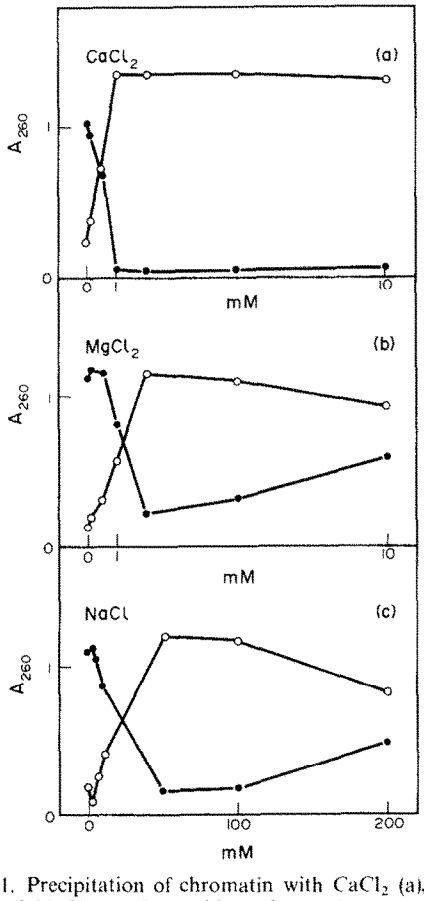

Fig. 1. Precipitation of chromatin with $CaCl_2$ (a), $MgCl_2$ (b) and NaCl (c). Hen oviduct chromatin was sonicated in H_2O and centrifuged at 10,000 *g* for 10 min. The supernatant was collected, and salts were added to a constant concentration of chromatin (14 A_{260} units/ml) at 0 C. The samples were centrifuged at 4500 *g* for 20 min and absorbance of the supernatant (—●—) and the pellet (—○—) was measured. Mean of two experiments.

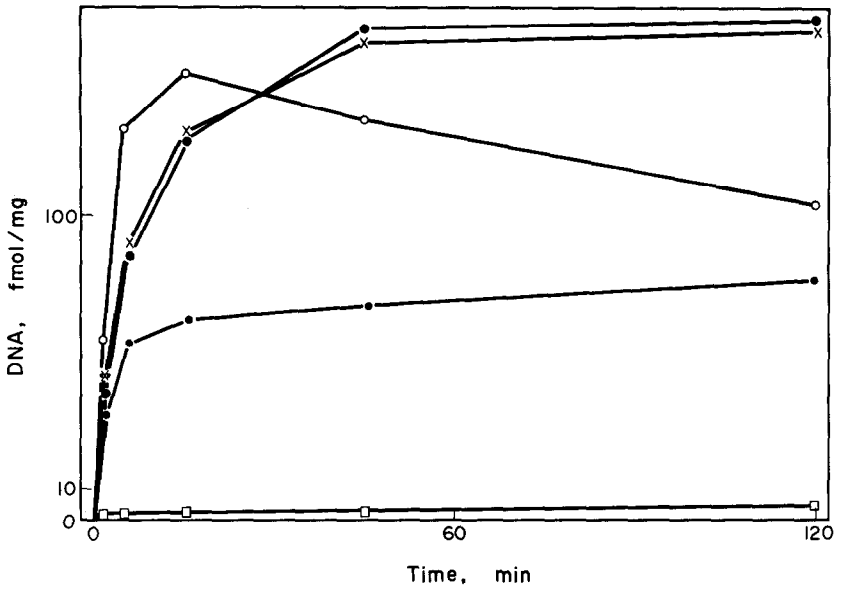

Fig. 2. Effect of incubation time and temperature on the binding of estradiol receptor to oviduct nuclei and chromatin. Each assay contained about 5 A_{260} units of nuclei or chromatin and 0.2 ml of oviduct cytosol in a total vol. of 1.5 ml, (—●—) nuclei at 25°C; (—○—) chromatin at 37°C, (— x —) 25°C, (—●—) 0°C; (—□—) cytosol alone. Mean of 2 experiments.

The addition of $CaCl_2$ or $MgCl_2$ decreased the extent of binding at 2 mM concentrations. The major difference between nuclei and chromatin was observed in the presence of ATP. ATP slightly increased binding to nuclei (difference statistically insignificant at $P < 0.05$) whereas no change was noted with chromatin. The ionic requirements of the oviduct system have similarities to those of the uterine system from calf[12].

A number of control experiments were performed to demonstrate the reliability of the assay developed. These included (not shown) demonstration that the receptor–chromatin complex remained stable during the sucrose gradient centrifugation, that the bound receptor was not readily exchangeable for an excess of soluble calf thymus DNA or *E. coli* tRNA, that radioactivity extracted from chromatin at 0.5 M NaCl behaved as a macromolecular complex in sucrose and glyserol gradient centrifugations and in ammonium sulfate precipitation, and that negligible radioactivity was registered in assays where chromatin was omitted. Furthermore, identical results were obtained with cytosols treated with charcoal, ammonium sulfate or charcoal + ammonium sulfate. These data confirm that the radioactivity registered was bound to receptor.

Table 1. Effect of ions and ATP on the binding of estradiol receptor to oviduct nuclei and chromatin

Additions	Bound receptor fmol/mg DNA Nuclei	Chromatin
NaCl, 20 mM	278 ± 18	270 ± 38
NaCl, 150 mM	280 ± 41	268 ± 33
NaCl, 300 mM	168 ± 58	142 ± 36
KCl, 150 mM	218 ± 11	241 ± 16
NaCl, 150 mM, $CaCl_2$ 2 mM	247 ± 51	216 ± 53
NaCl, 150 mM, $MgCl_2$ 2 mM	246 ± 45	215 ± 20
NaCl, 150 mM, ATP 2 mM	315 ± 46	256 ± 43

About 5 A_{260} units of oviduct nuclei or chromatin was incubated in the presence of 0.2 ml of labelled cytosol prepared in 0.15 M NaCl at 25°C for 90 min.

Means ± SEM of at least 4 determinations.

Concentration dependence of receptor binding. Binding of estradiol receptor to oviduct nuclei and chromatin was studied using increasing concentrations of radioactive receptor but a constant amount of cytosol. The cytosols were prepared by mixing [^{3}H]-estradiol labelled and non-radioactive cytosol. Oviduct nuclei bound slightly more estradiol receptor than chromatin and the extent of binding was directly proportional to the amount of radioactive receptor added (Fig. 3a). Thus no saturable component could be detected in agreement with reports on nuclei[3] and DNA[5]. Moreover, the identity of the nuclear and chromatin binding curves suggest that no major binding component was lost during purification of chromatin.

The binding assays described in Fig. 3a were carried out with nuclei and chromatin form laying hens, which produce endogenous estrogens. The endogenous receptors could be so tightly bound to chromatin that they would not be replaced with exogenous receptors used in the binding assays. This would then result in the apparent absence of high affinity binding. Such a possibility was investigated with newborn chicks treated with estradiol for 10 days and withdrawn for at least 2 weeks[13]. The production of hormone-inducible oviduct proteins is known to be negligible in such animals[14]. The

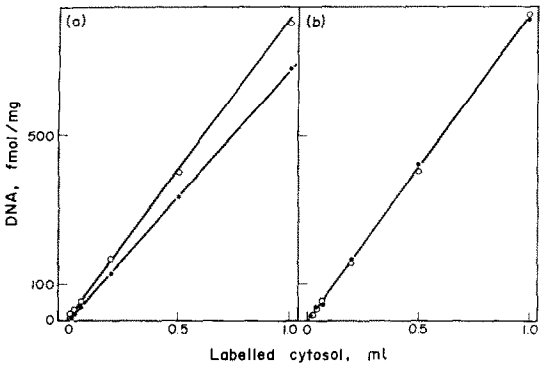

Fig. 3. Binding of estradiol receptor to hen (a) or chick (b) nuclei and chromatin as a function of radioactive receptor concentration. (a) nuclei (—○—) and chromatin (—●—); (b) nuclei from estradiol primed chicks after 2 weeks withdrawal (—●—) or 2 h after a secondary stimulation with 2 mg of estradiol (—○—). Mean of 4 experiments.

binding assay was carried out with chromatin from primed chicks before and after a secondary stimulation with estradiol (Fig. 3b). No difference was observed in the extent of binding nor in the shape of the concentration curve between the two chromatins, suggesting absence of high affinity binding in chick oviduct for estradiol receptors.

Characteristics of the binding reaction. DNA is a possible acceptor molecule for estradiol receptors in nuclei and chromatin. There are a number of chemicals that interact with DNA and bind more or less specifically to some chemical or topographic structures on DNA. Some of these substances were used to characterize the acceptor structures of estradiol receptors on chromatin. Two types of assays were performed (Table 2). One involved a pretreatment of chromatin with the substances and removal of unbound material by sedimentation. In the other type of assay the substances were present in the estradiol binding assay. In this case appropriate controls were necessary because some of the compounds used precipitated the estradiol receptor.

In Table 2 the compounds are classified according to their postulated major mode of action with DNA. Compounds which are believed to bind to the minor groove of DNA, including polylysine and histone F 1[15, 16], reduced the binding of the receptor by 12 to 30%. An equally large inhibition was caused by intercalating drugs ethidium bromide, chloroquine and actinomycin D[17, 18]. By contrast, an excess of total histones, believed to bind to the major groove[19] failed to change estradiol receptor binding. Although all combinations of the substances were not tested, at least some of the effects of minor groove and intercalating reagents were additive, suggesting that 25 to 60% of the estradiol receptor binding may be ascribed to DNA. Furthermore, the acceptor sites may be heterogeneous. The results have to be viewed with some caution since chromatin contains, in addition to DNA, protein and RNA, which may influence the specificity of the probes applied. This may explain why ethidium bromide is less effective with chromatin than with DNA[20].

Another group of compounds, which were tested in the receptor binding assay, included detergents. When chromatin was pretreated with non-ionic detergents such as Triton X-100, only a small reduction was observed in receptor binding (not shown). However, when the detergents were included in the binding assay, the extent of binding decreased drastically (Table 3). At 0.3% concentration non-ionic detergents including Triton X-100, Non-idet P-40 and Lubrol W reduced binding to 14–24% of the control. The total concentration of protein in the binding assays was 5–10 mg/ml. At 0.3% concentration sodium deoxycholate was not as effective as the non-ionic detergents. Ionic detergents were not tested further.

Table 2. Effects of DNA active compounds on the binding of estradiol receptor to chromatin

Compounds added	Bound receptor % of control (n: 3–6) Chromatin pretreated with compounds[1]	Compounds present in the binding assay[2]
Control	100	100
Minor groove reagents[3]		
Polylysine, 1 mM	75 ± 8	70 ± 2
Histone F 1, 0.5 mg/ml	88 ± 3	
Major groove reagents[3]		
Total histones, 0.5 mg/ml	101 ± 4	—
Intercalating reagents[3]		
Ethidium bromide, 1 mM	85 ± 6	81 ± 2
Chloroquine, 1 mM	88 ± 1	—
Actinomycin D, 1 mM	71 ± 7	—

[1] Chromatin (0.5–1 mM in DNA bases) was incubated with added compounds for 15 min at room temperature. About 200 μg of bovine serum albumin was added and the chromatin was collected by centrifugation followed by the binding assay.

[2] Blank values (compounds added, chromatin omitted) were subtracted.

[3] Proposed mode of action with DNA not excluding combined modes of action.

Table 3. Effects of detergents on the binding of estradiol receptor to chromatin

Detergent		Bound receptor % of control
Triton X-100	0.3%	16
	1%	7
Nonidet P 40	0.3%	14
	1%	9
Lubrol W	0.3%	24
	1%	3
Sodium deoxycholate	0.3%	61

The detergents were present in the binding assay at the indicated final concentration.
Means of 2 experiments.

because they change the sedimentation properties of chromatin, complicating the binding assay.

The dependence of receptor binding on the concentration of Triton X-100 was tested in Fig. 4. Binding was reduced to 50% at Triton X concentration of 0.05%. If Triton X was added at the end of the binding assay, about 40% of receptor remained bound (Fig. 4). Triton X appeared to interfere with the binding of estradiol receptor to chromatin by making the receptor unable to attach to chromatin as well as by removing a fraction of the bound receptor.

One possible explanation for the effects of detergents would be that the detergents cause dissociation of estradiol from its receptor. An absolute exclusion of such a possibility is difficult because the bound detergent changes the molecular weights of estradiol and its receptor. However, two experiments were carried out suggesting that the estradiol-receptor complex remains intact in the presence of Triton X-100. One of them included precipitation with activated charcoal. Triton X did not remove radioactivity from the supernatant (not shown). Another test involved Sephadex G 25 chromatography (Fig. 5). Independent of Triton X, the bulk of estradiol radioactivity was eluted in the void volume. These experiments lend

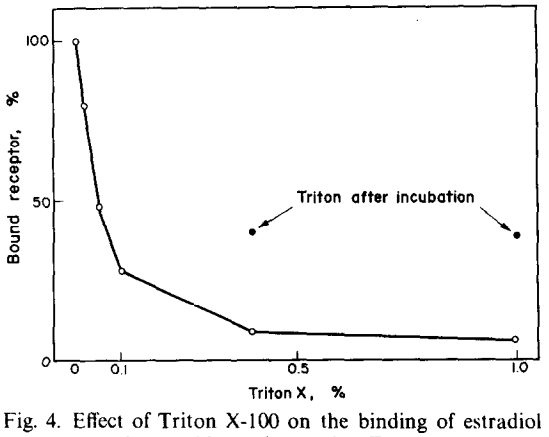

Fig. 4. Effect of Triton X-100 on the binding of estradiol receptor to hen oviduct chromatin. The two separate points indicate the extent of binding when Triton was added after the binding assay. The total protein concentration was 5 to 10 mg/ml. Mean of 2 experiments.

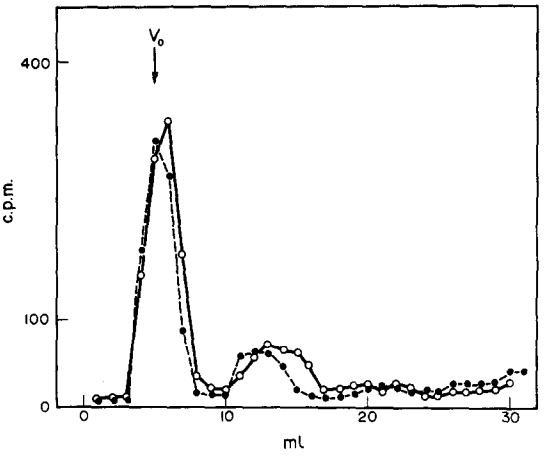

Fig. 5. Sephadex G 25 chromatography of labelled cytosol with (—○—) or without (- - - -) 1% Triton X-100. The columns were equilibrated with 0.15 M NaCl. 1 ml fractions were collected.

no support on the detergent induced dissociation of the estradiol receptor complex.

DISCUSSION

The procedures previously described for studying the binding of steroid receptors to chromatin, including repeated sedimentations, co-sedimentations in gradients, trapping on membrane filters and gel chromatography, may not be suitable in case of labile and aggregating receptors such as the hen oviduct estradiol receptor[4]. More elaborate methods such as sedimentation partition chromatography[4] may not be applicable because of insolubility and variable particle size of chromatin. By contrast, the procedure described in this paper should not be obscured by lability or aggregation of the receptor. $CaCl_2$ precipitated chromatin is sedimented through 1.6 M sucrose so rapidly that even large aggregates of protein have no time to pellet. Furthermore, $CaCl_2$ is so effective in precipitating chromatin that the method can be applied even to sonicated fractions of chromatin.

The binding assay developed was used in the present study to optimize the conditions for hen oviduct nuclei and chromatin. No appreciable differences were observed between nuclei and chromatin, both displaying optimal binding at 0.15 M NaCl. Any additions appeared to decrease the extent of binding. However, ATP was shown to stimulate binding slightly to nuclei only.

A number of contradictory reports have been published on the chemical nature and affinity of nuclear acceptor sites for estradiol receptors. Such discrepancies have been suggested to be due to the ready aggregation and lability of the estradiol receptor[3, 4]. The present study demonstrated, in agreement with some previous reports[5], that no apparent high affinity binding was observed if constant amounts of cytosol were used in the binding assay. This was true of nuclei and chromatin from laying hens and of chromatin

from chicks with apparently very low levels of endogenous estradiol. These results suggest that the inability to demonstrate a high affinity acceptor component is not due to losses during preparation of material or to masking of acceptor sites by endogenous hormone. However, high affinity acceptor sites may have so low a capacity as to remain undetected in the present kind of assay calling for fractionation of chromatin components.

It was shown in the present study that receptor binding to chromatin was reduced by 10–30% with compounds known to bind to the minor groove of DNA or to intercalate with DNA. The effects of some of these compounds were shown not to overlap, suggesting that at least two different binding sites are involved. A minor groove probe, polylysine is known to interact mainly with phosphate groups of DNA. It may cover ionic groups which may bind estradiol receptor. These bonds could be broken by salt, which is commonly applied to extract steroid receptors from nuclei[21]. By contrast, intercalating drugs are likely to probe non-ionic bonds, which may be susceptible to hydrophobic reagents such as detergents. Triton X was shown to remove a fraction of estradiol receptor from chromatin. Although this may imply unspecific binding[22], it can also demonstrate breakage of hydrophobic bonds. The interference of low concentration of non-ionic detergents with the receptor binding assay, demonstrated in this study, provides evidence on the existence of hydrophobic sites on the estradiol receptor possibly involved in its binding to chromatin. This may implicate that both hydrophilic and hydrophobic bonds are involved in the binding of estradiol receptor to chromatin. Their further characterization and biological significance remain to be established.

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REFERENCES

1. O'Malley B. W. and Means A. R.: *Science* **183** (1974) 610–620.
2. Williams D. L.: *Life Sci.* **15** (1974) 583–597.
3. Chamness G. C., Jennings A. W. and McGuire W. L.: *Biochemistry* **13** (1974) 327–331.
4. Yamamoto K. R. and Alberts B.: *J. biol. Chem.* **249** (1974) 7076–7086.
5. Andre J. and Rochefort H.: *FEBS Lett.* **50** (1975) 319–323.
6. Harrison R. W. and Toft D. O.: *Endocrinology* **96** (1975) 199–205.
7. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
8. Giles K. W. and Myers A.: *Nature, Lond.* **206** (1965) 93.
9. Bolund L. and Johns E. W.: *Eur. J. Biochem.* **35** (1973) 546–553.
10. Marushige K. and Bonner J.: *Proc. natn. Acad. Sci., U.S.A.* **68** (1971) 2941–2944.
11. Arnold E. A. and Young K. E.: *Archs Biochem. Biophys.* **164** (1974) 73–89.
12. Sala-Trepat J. M. and Vallet-Strouvé C.: *Biochim. biophys. Acta* **371** (1974) 186–202.
13. Oka T. and Schimke R.: *J. Cell Biol.* **43** (1969) 123–136.
14. Palmiter R. D., Christensen A. K. and Schimke R. T.: *J. biol. Chem.* **245** (1970) 833–845.
15. Clark R. J. and Felsenfeld G.: *Nature new Biol.* **229** (1971) 101–106.
16. Ciba Foundation Symposium 28: *The Structure and Function of Chromatin*, Elsevier (1975) p. 91.
17. LePecq J. B. and Paoletti C.: *J. molec. Biol.* **27** (1967) 87–106.
18. Kleeman L. and Huang R.-C. C.: *J. molec. Biol.* **64** (1972) 1–8.
19. Shih T. and Bonner J.: *J. molec. Biol.* **48** (1970) 469–487.
20. Pfeiffer A., André J. and Rochefort H.: 10th Meeting of FEBS, Paris 1975, 1399.
21. Buller R. E., Schrader W. T. and O'Malley B. W.: *J. biol. Chem.* **250** (1975) 809–818.
22. Brumbaugh P. F., Haussler D. H., Bursac K. M. and Haussler M. R.: *Biochemistry* **13** (1974) 4091–4097.