STEROID RECEPTORS IN THE HUMAN PROSTATE.
2. SOME PROPERTIES OF THE ESTROPHILIC MOLECULE OF BENIGN PROSTATIC HYPERTROPHY.

E.F. Hawkins, M. Nijs and C. Brassinne.
Service de Médecine et Laboratoire d'Investigation Clinique
Institut Jules Bordet, Brussels, Belgium.

Received April 6,1976

SUMMARY. Some physicochemical properties of the estrophilic 'receptor' of human benign prostatic hypertrophy were examined by agar gel electrophoresis. 1) Competition analyses revealed the high selectivity of the molecule for the naturally occurring estrogens but not for representatives of other classes of steroid hormones (androgens, corticosteroids, progesterone). This, coupled with the failure of an estrogen 'receptor'-rich extract to exhibit detectable tissue specific binding of (^{3}H) 5α-dihydrotestosterone suggests that prostatic androgen and estrogen receptors may have separate identities. 2) The molecule proved highly resistant to enzyme attack, a stability conferred by estradiol-17β rather than by the thiol reagent dithiothreitol. Its proteinaceous nature was finally demonstrated when extract was exposed to enzymes at 0°C prior to steroid addition. 3) Initial complex formation between estrogen and its 'receptor' protein was rapid and reached a plateau after 4 hours. Binding was greater at 0°C than 37°C.

INTRODUCTION. In a previous paper (1) we described the occurrence of an estradiol-17β[1] 'receptor', detected by agar gel electrophoresis and sucrose gradient ultracentrifugation, in some high speed supernatants prepared from surgically obtained samples of BPH[2] tissue. Since then more 'receptor'-positive samples have become available, enabling us to extend our electrophoretic experiments to an examination of some physicochemical properties of the molecule concerned. It is these which form the basis of this report.

MATERIALS AND METHODS. Collection of tissues and agar gel electrophoresis have previously been described (1). Modified and additional techniques are as follows :

Preparation of Tissue Powder and Extracts : Thinly-sliced, liquid nitrogen frozen tissue was pulverized in a 'Mikro-Dismembrator' (Braun, Melsungen, W. Germany). The tissue was subjected to a single 30 second period of maximum oscillation and the resulting powder stored in liquid nitrogen until use.

---

[1] Trivial names used are : estradiol-17β = estra-1,3,5 (10)-trien-3,17β-diol; estrone = 3-hydroxyestra-1,3,5 (10)-trien-17-one ; estriol = estra-1,3,5 (10)-trien-3,16α,17β-triol ; 5α-dihydrotestosterone or 5α-DHT = 5α-androstan-17β-ol-3-one ; testosterone = 4-androsten-17β-ol-3-one ; progesterone = 4-pregnen-3,20-dione ; corticosterone = 4-pregnen-11β,21-diol-3,20-dione ; cortisol = 4-pregnen-11β,17α,21-triol-3,20 dione.

[2] Abbreviations used : BPH = benign prostatic hypertrophy ; DTT = dithiothreitol.

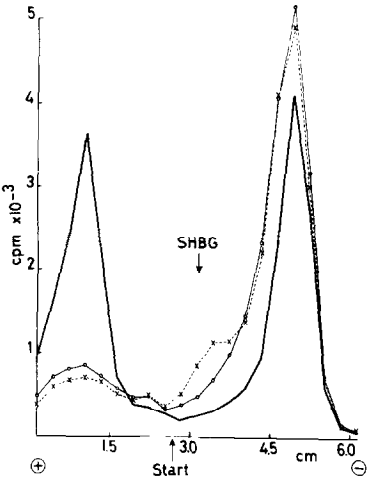

Figure 1. Agar gel electrophoresis : profiles of [^{3}H] estradiol-17β binding to a BPH extract rich in estrogen 'receptor' in the absence (——) and presence of either a 100 (o——o) or a 1000 (x– –x)-fold molar excess of radioinert estradiol-17β. 'Start' denotes the sample well and 'SHBG' the position of sex hormone-binding globulin.

For extract preparation, powder was transferred to ice-cooled 'Eppendorf' 1.5 ml-capacity micro test tubes (Eppendorf, Hamburg, W. Germany) and weighed. A volume of buffer (50 mM-Tris, 0.1 mM-EDTA, 0.5 mM-DTT) equivalent to 1.5x the powder weight was added and the mixture stirred until the buffer thawed. The mixture was gently shaken mechanically for 15 min in ice. The tubes were then centrifuged in a Beckman Microfuge B (Beckman-Spinco, Palo Alto, Ca, USA) for 4 min at approx. 10000xg and 4°C to obtain the extracts used in these experiments.

Steroids : Tritiated estradiol-17β (oestradiol 2,4,6,7-^{3}H, 85 Ci/mmol) and 5α-dihydrotestosterone (5α-dihydro[1,2,4,5,6,7(n)-^{3}H] testosterone, 130 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, U.K.. Radiochemical purity was monitored on Sephadex LH-20 columns.

Radioinert steroids were obtained from the following sources : estrone, Sigma Chemical Co., St. Louis, USA; estriol (A grade), Calbiochem, San Diego, USA; diethylstilbestrol, Koch-Light labs., Colnbrook, Bucks, U.K.; nafoxidine through the courtesy of the Drug Development Branch, National Cancer Institute, Bethesda, USA and all others from Steraloids Inc., Pawling, N.Y., USA. They were used as obtained from the manufacturers.

Incubation and Competition Analysis : All incubations were performed in 'Eppendorf' 1.5 ml micro test tubes for 2 hr at 0°C without shaking (except in the time/temperature experiment; for details see Results). The volume of extract used was 50 µl and ^{3}H -steroids were at a final concentration of 1.2×10^{-8} M. Unspecific binding was computed by the inclusion of tubes containing 1.2×10^{-6} M appropriate radioinert competitor in addition to radiosteroid. Tritiated and unlabeled steroids were always added simultaneously to the extract. Details of their concentrations in the competition analysis are given in Table 1.

Selective Estradiol-17β Binding to 'Receptor' : In all experiments, binding of [^{3}H] estradiol -17β to its anodally migrating 'receptor' was determined by agar gel electrophoresis. This component, usually located in anode gel samples 3 to 9 from the sample well, was estimated by subtracting the radio-

Table 1 : Selective (^{3}H) estradiol-17β binding to BPH extract in the presence of various radioinert steroids.

Steroid	Molar excess	%loss selective (^{3}H) estradiol-17β binding
C_{18}		
Estra-1,3,5 (10)-trien-3,17β-diol	100	100
Estra-1,3,5 (10)-trien-3,17β-diol	1000	106
3-hydroxyestra-1,3,5 (10)-trien-17-one	100	80
3-hydroxyestra-1,3,5 (10)-trien-17-one	1000	96
Estra-1,3,5 (10)-trien-3,16α,17β-triol	100	96
C_{19}		
5α-androstan-17β-ol-3-one	100	no effect
4-androsten-17β-ol-3-one	100	19
5α-androstan-3α,17β-diol	100	10
5α-androstan-3β,17β-diol	100	1
C_{20}		
4-pregnen-3,20-dione	100	no effect
C_{21}		
4-pregnen-11β,21-diol-3,20-dione	100	16
4-pregnen-11β,17α,21-triol-3,20-dione	100	15
Others		
Nafoxidine	100	3
Nafoxidine	500	no effect
Diethylstilbestrol	100	no effect
Diethylstilbestrol	1000	12

Anodally migrating complexes of (^{3}H) estradiol-17β with 'receptor' were detected by agar gel electrophoresis following the simultaneous incubation of extract for 2 hr at 0°C with 1.2×10^{-8} M radiosteroid and unlabeled compounds at the molar excesses specified above. Unspecific binding was computed as described in Materials and Methods. The percentage loss of (^{3}H) steroid-'receptor' complexes in the presence of 1.2×10^{-6} M unlabeled estradiol-17β was set at 100.

activity in this area in the presence of 1.2×10^{-6} M unlabeled estradiol (albumin binding) from the counts over a similar gel region in the absence of unlabeled hormone.

Enzyme Study : Enzymes were incubated with extract according to the scheme detailed in Table 2. Pronase (from Streptomyces griseus, B grade) was purchased from Calbiochem (San Diego, USA), DNase (from beef pancreas, crystallized and lyopholized) and RNase (from beef pancreas, type III-A) from Sigma Chemical Co (St. Louis, USA). All were stored, tightly sealed, in a freezer at minus 28°C.

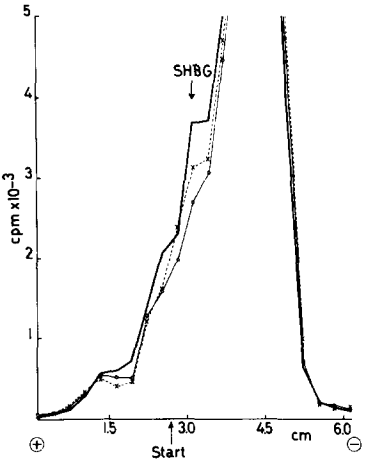

Figure 2. Agar gel electrophoresis : profiles of (^{3}H) 5α-dihydrotestosterone binding to the same extract as depicted in Fig.1 in the absence (——) and presence of a 100-fold molar excess of either unlabeled 5α-DHT (o——o) or estradiol-17β (x– – –x).

RESULTS Figure 1 illustrates the electrophoretic profile of (^{3}H) estradiol -17β binding to BPH extract rich in anodally migrating estradiol -17β 'receptor', the latter distinguished from similarly migrating albumin-bound steroid in the presence of radioinert competitor. Both 100- and 1000-fold molar excesses of unlabeled estradiol-17β were equally effective in eliminating the radiosteroid-'receptor' complexes (Fig.1 and Table 1); the former concentration was chosen for the estimation of albumin binding in all experiments.

In contrast, selective, saturable binding of (^{3}H) 5α-dihydrotestosterone was absent in a similar gel region of the same extract (Fig.2). The small amount of radioactivity observed was unaffected by the presence of either radioinert 5α-DHT or estradiol-17β in a 100-fold molar excess.

The specificity of the 'receptor' was examined in more detail in competition experiments, the results of which are summarized in Table 1. The molecule concerned exhibited marked selectivity for the naturally occurring estrogens with unlabeled estradiol-17β itself as the most effective inhibitor of binding of the radioligand. Other steroids tested including 5α-dihydrotestosterone, testosterone, progesterone, corticosterone and cortisol, were largely or completely ineffective as were two synthetic compounds, nafoxidine and diethylstilbestrol.

Exposure of BPH extracts to enzymes was performed in order to de-

Table 2 : Stability to enzymes of estradiol 'receptor' in BPH extract : analysis by agar gel electrophoresis.

a) Incubation : 2 hr at 0°C with steroid followed by enzyme addition and further incubation for 1 hr at 37°C.

Enzyme (concn)	'Receptor' binding of (^{3}H) estradiol-17β as % of control	
	With DTT (0.5 mM)	Without DTT
Control (+ buffer)	100	100
Pronase (0.8 mg/ml)	98	94
DNase (0.8 mg/ml)	95	108
RNase (0.8 mg/ml)	99	111
Pronase (3.8 mg/ml)	73	-
DNase (3.8 mg/ml)	105	-
RNase (3.8 mg/ml)	102	-

b) Incubation : extract pretreatment with enzymes for 30 min at 0°C prior to steroid addition and further incubation for 2 hr at 0°C.

Enzyme (concn)	'Receptor' binding of (^{3}H) estradiol-17β as % of control (+ DTT).
Control (+ buffer)	100
Pronase (3.8 mg/ml)	not detectable
DNase (3.8 mg/ml)	106
RNase (3.8 mg/ml)	105

c) Incubation : no (^{3}H) steroid-'receptor' complexes were detectable in extract (with or without DTT) pretreated with enzymes (0.8 mg/ml) or with buffer for 1 hr at 37°C before steroid addition and further incubation for 2 hr at 0°C.

At all conditions, extract was incubated both with (^{3}H) estradiol-17β at 1.2×10^{-8} M and with a mixture of radioligand and 1.2×10^{-6} M unlabeled estradiol-17β so that selective binding could be computed (see Materials and Methods). Buffer substituted for enzyme in the control tubes. Enzyme concentrations represent the final in the extract.

termine the chemical nature of the 'receptor'. Initially the molecule proved to be highly resistant since all enzymes at final concentrations of 0.8 and 3.8 mg/ml had negligible effects on preformed radioligand-'receptor' complexes, irrespective of the presence or absence of DTT in the incubate (Table 2a). When the conditions were reversed and extract + DTT was exposed to the lower concentration of enzymes for 1 hr. before steroid addition (Table 2c), selective (^{3}H) estradiol-17β binding could no longer be demonstrated.

The proteinaceous nature of the binder was finally revealed when BPH extract + DTT was incubated with 3.8 mg enzymes/ml. for 30 min at 0°C

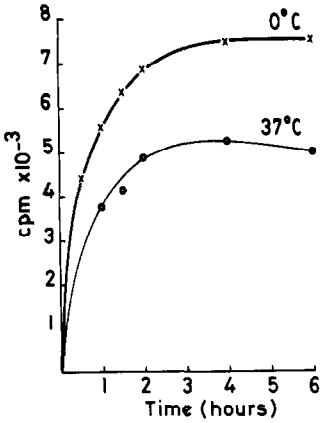

Figure 3. Effect of temperature and time on the selective binding of (^{3}H) estradiol-17β to a 'receptor'-rich BPH extract. Data obtained by agar gel electrophoresis.

before steroid addition (Table 2b). Under these conditions 'receptor' binding of radiosteroid was entirely lost in the presence of pronase but was unaffected by either DNase or RNase.

Temperature dependence of the formation of 'receptor'-steroid complexes was revealed in experiments in which steroids were incubated with 'receptor'-rich BPH extract for various times at 0°C and 37°C (Fig.3). At both temperatures, initial complex formation was rapid and reached a plateau by 4 hours. However, binding was appreciably greater at 0°C than at 37°C throughout the sampling intervals. In all other experiments reported, incubation with steroids was for 2 hr. at 0°C. By this time, selective (^{3}H) estradiol-17β binding rises to more than 90% of the maximum observed over 4 hr at 0°C (Fig.3).

DISCUSSION. In common with other investigators (2) we chose to use tissue extracts rather than cytosols. Our processing methods permit the simultaneous preparation of up to 18 extracts compared with 6 cytosols in the ultracentrifuge. Moreover small extract volumes (eg. 100 µl) may be prepared without danger of centrifuge tube collapse.

The demonstration of more than one class of intracellular steroid hormone receptors in individual target tissues may serve to further our understanding of hormonally dependent disorders of these structures and aid in the therapeusis of such conditions. Evidence for the occurrence of androgen receptors in BPH tissues has been furnished by several laboratories (3-6). The data

contained in this report and in a previous article (1) provide support for the existence of an additional estrophilic protein in at least some BPH samples examined by us. The separate identity of the two molecules is strongly indicated by the presence of saturable estrogen binding in an extract in which selective, tissue-specific uptake of androgen could not be demonstrated (Figs. 1 and 2) and reinforced by the failure of the various androgens tested to affect the coupling of (^{3}H) estradiol-17β to its 'receptor' protein in competition analyses (Table 1).

Competition experiments also demonstrated the high selectivity of the protein for the naturally occurring estrogens since, in common with the androgens, representatives of other classes of steroid hormones (progesterone, corticosterone, cortisol) acted as only minimal antagonists of radioestradiol-17β binding. However the similar ineffectiveness of nafoxidine, a known inhibitor of selective estradiol-17β uptake in several target tissues of the rat and mouse (7) and the human female (8), was unexpected. One group (8) has advanced the theory that nafoxidine may act by an allosteric effect, changing the shape and hence lowering the estradiol-17β affinity of the molecule concerned rather than by direct competition at the binding site itself. If so,the BPH 'receptor' may lack the necessary molecular architecture for this to occur.

The stability of estrogen receptor in human breast cancer tissue has been reported to be markedly improved by the inclusion of thiol reagents such as dithiothreitol in the working buffers (9). This does not appear to be the case for our estrophilic protein. Ligand binding to this molecule was unaltered in the presence or absence of DTT (data not shown) as was complex stability to enzyme exposure (Table 2a). Of far more importance was the protection afforded by estradiol-17β itself, rendering the molecule highly resistant to enzyme attack (Table 2). In intact tissue, the survival of the 'receptor' may well require the continuous presence of estrogen or that it be associated with a cellular organelle so as to prevent its rapid denaturation in the absence of appropriate steroid.

The estrogen receptor of rat uterus binds more steroid at 30°C than at 0°C, the difference representing exchange between bound endogenous hormone and added radioligand (10). In contrast, the 'receptor' described here bound more radioligand at 0°C than at 37°C (Fig. 3). The atypicality of this molecule is further indicated by its failure to precipitate with protamine (unpublished experiments) at concentrations used by other investigators for the solid-phase assay of estrogen receptor (10) as well as its only partial stability to dextran-coated charcoal and its 4S sedimentation coefficient (1). The protein may represent a class of selective, saturable estrogen binder qualitatively different from the classical receptors.

ACKNOWLEDGMENTS. We are grateful to Dr. E. Claereboudt of Institut Jules Bordet and to Dr. E. Bemont for providing the tissue samples used. This study was financially supported by the Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite de Belgique.

REFERENCES.

1. Hawkins, E.F., Nijs, M., Brassinne, C. and Tagnon, H.J. (1975) Steroids 26, 458-469.
2. Hawkins, R.A., Hill, A. and Freedman, B. (1975) Clin. Chim. Acta 64, 203-210.
3. Tveter, K.J., Unhjem, O., Attramadal, A., Aakvaag, A. and Hansson, V. (1971) Advances in the biosciences 7, 193-207.
4. Mainwaring, W.I.P. and Milroy, E.J.G. (1973) J. Endocr. 57, 371-384.
5. Davies, P. and Griffiths, K. (1975) Molec. cell. Endocr. 3, 143-164.
6. Rosen, V., Jung, I., Baulieu, E.E. and Robel, P. (1975) J. clin. Endocr. Metab. 41, 761-770.
7. Terenius, L. (1971) Acta Endocr. 66, 431-447.
8. Hähnel, R., Twaddle, E. and Ratajczak, T. (1973) J. steroid biochem. 4, 687-695.
9. Mc Guire, W.L. and De La Garza, M. (1973) J. clin. Endocr. Metab. 37, 986-989.
10. Chamness, G.C., Huff, K. and Mc Guire, W.L. (1975) Steroids 25, 627-635.