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# IN VITRO STUDY ON ESTROGEN-BINDING MACROMOLECULES IN CASTRATED RAT PROSTATE

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## ABSTRACT

Polyacrylamide gel electrophoresis, dextran-coated charcoal adsorption, Sephadex gel filtration and thin-layer chromatography allowed us to attempt the characterization of estrogen-binding components in the castrated rat prostate. The results are as follows: 1) Among various tissue cytosols examined, the ventral prostate cytosol showed the highest binding of  $^3\text{H}$ -estradiol-17 $\beta$ , followed by the cytosols of the liver, kidney, testis, seminal vesicle and skeletal muscle. 2) After incubation of the ventral prostate cytosol with  $^3\text{H}$ -estradiol-17 $\beta$  in the absence or the presence of various unlabeled steroids, a specific estrogen-binding material was found with low binding capacity and high affinity for this hormone. This binding was found not due to the contamination of blood serum proteins. 3) After incubation of the ventral prostate with  $^3\text{H}$ -estradiol-17 $\beta$ , estrogen-binding material was also found both in the cytosol and in the nucleus of the tissue. 4) Using the thin-layer chromatographic technique it was found that estrone and estradiol-17 $\beta$  occupied most of the radioactivity in various subcellular fractions obtained from the ventral prostate incubated with  $^3\text{H}$ -estradiol-17 $\beta$ , and the ratio of estradiol: estrone was highest in the nucleus, followed by the binding component of the cytosol and the cytosol. These results may suggest that there is a specific low capacity-high affinity estrogen-binding component in the adult rat prostate.

## INTRODUCTION

Since Huggins et al. (1941) reported that estrogen reduced the development of human prostate carcinoma, this hormone has been used for treatments of various prostatic disorders, although it was reported that this hormone has no effect on the benign prostate hypertrophy (Chisholm, 1970).

It is generally thought that estrogen mainly exerts its influence on the prostate through the feedback route where estrogen inhibits LH-secretion from the pituitary, which leads to the reduction of secretion of testicular androgens. On the other hand, the direct effects of this steroid on the prostate have been considered in many studies concerning the alteration of enzymatic activities in the prostatic tissue with special references to androgen metabolism

(Groom et al., 1971; Bonne et al., 1973; Briggs et al., 1973; Lee et al., 1973; Jenkins et al., 1974; Lee et al., 1974). The mechanism of the effect of this hormone on the prostate may be explained as that it inhibits the binding of 5 $\alpha$ -dihydrotestosterone to its receptor protein in the tissue (Fraser et al., 1974). *In vitro* studies, however, have revealed the presence of estrogen in the canine prostate (Lloyd et al., 1975) and significant uptake of estradiol-17 $\beta$  by the prostate of rat (Tveter, 1970) and dog (Sturman et al., 1974). Sinha et al. (1973), using an autoradiographic technique, reported the *in vitro* uptake of  $^3\text{H}$ -estradiol-17 $\beta$  or its metabolites by cell nucleus of the human prostatic carcinoma. These findings suggest the existence of the estrogen-binding macromolecule or the receptor in the cytosol of this tissue. Although there have been

a few reports concerning the estradiol-binding macromolecule in the ventral prostate of the retired rat (Armstrong et al., 1974), in the calf prostate (Jungblut et al., 1975; Wagner et al., 1975) and in the intact and the cancerous prostates of the humans (Wagner et al., 1975), these reports came from the findings in cell-free system. The mechanism of action of estrogen-binding macromolecule in the prostate has, therefore, remained unsolved. As the first step to make clear the above problems, the present study was carried out to demonstrate and characterize the estrogen-binding macromolecules.

### MATERIALS AND METHODS

#### (A) Chemicals

[6, 7-<sup>3</sup>H]-estradiol-17 $\beta$  (sp. act. 47.9 Ci/mmol, <sup>3</sup>H-E<sub>2</sub>), [1, 2-<sup>3</sup>H]-5 $\alpha$ -dihydrotestosterone (sp. act. 50 Ci/mmol, <sup>3</sup>H-DHT) and [1, 2, 6, 7-<sup>3</sup>H]-corticosterone (sp. act. 100 Ci/mmol, <sup>3</sup>H-CC) all were purchased from New England Nuclear (Boston, U.S.A.). Various unlabeled steroids such as estradiol-17 $\beta$  (E<sub>2</sub>), estrone (E<sub>1</sub>), testosterone (T), 5 $\alpha$ -dihydrotestosterone (DHT) and corticosterone (CC) were obtained from Sigma Chemical Co, U.S.A. Diethylstilbesterol phosphate (DESP) was obtained from Kyorin Pharmaceutical Co., Tokyo. Dextran, charcoal, acrylamide, N,N'-methylene-bis (acrylamide) and N,N,N',N'-tetramethylethylenediamine (TEMED) all were purchased from Wako Pure Chemical Industries, Osaka. Kieselgel HF<sub>254+366</sub> was obtained from E. Merck AG, Darmstadt, Germany. Sephadex G-25 and G-100 were purchased from Pharmacia Fine Chemicals, Sweden.

#### (B) Methods in cell-free studies

*Preparation of cytosols* Adult male rats of Wistar strain, weighing 200~250 g, were used throughout the study. They were castrated 18 to 20 hours before sacrifice. The blood was collected from the abdominal aorta, after which the ventral prostate, testis, seminal vesicle, liver, kidney and skeletal muscle were excised. Each tissue was minced in 1 volume of TE buffer (0.01 M Tris-HCl, pH 7.4, containing 0.0015 M

EDTA) and homogenized in a teflon-glass homogenizer. The homogenate was then centrifuged at 800 $\times$ g for 10 minutes, followed by 105,000 $\times$ g for 1 hour in a Hitachi RP-55 rotor. The resulting supernatant, "cytosol", contained about 40 mg of protein/ml. The blood serum was obtained by centrifugation at 1,500 $\times$ g for 20 minutes.

*Estimation of <sup>3</sup>H-E<sub>2</sub> binding to cytosols from various organs* A 0.3 ml aliquot of the cytosol from each tissue (containing 3 mg protein) were incubated with 10<sup>-9</sup> M <sup>3</sup>H-E<sub>2</sub> at 0°C for 16 hours, after which dextran-coated charcoal suspension (0.05% dextran-0.5% charcoal in TE buffer) was used to separate bound from free <sup>3</sup>H-E<sub>2</sub>. Radioactivity of <sup>3</sup>H-E<sub>2</sub>-bound fraction was measured in a Triton-toluene-PPO-POPOP system in a scintillation spectrophotometer (Model 3390, Packard Instrument Co., U.S.A.).

*Polyacrylamide gel electrophoresis (PAGE)* A 0.1 ml aliquot of each cytosol and the diluted serum (each containing 3.6 mg protein) was incubated at 0°C for 16 hours either with 2 $\times$ 10<sup>-8</sup> M <sup>3</sup>H-E<sub>2</sub>, <sup>3</sup>H-DHT or <sup>3</sup>H-CC. Another 0.1 ml aliquot of the cytosol (containing 4 mg protein) was incubated at 0°C for 16 hours with 2 $\times$ 10<sup>-8</sup> M <sup>3</sup>H-E<sub>2</sub> in the absence or the presence of 2 $\times$ 10<sup>-5</sup> M unlabeled hormones such as E<sub>2</sub>, E<sub>1</sub>, DESP, DHT, T and CC. After incubation a 50  $\mu$ l aliquot of the incubate was then layered onto 7.5% polyacrylamide gel (60 mm $\times$ 6 mm) prepared in 0.375 M Tris-HCl buffer, pH 8.9, with 0.06% TEMED (Schuster, 1971) with a marker, bromophenol blue (BPB). The relative mobility (R<sub>f</sub>) of <sup>3</sup>H-E<sub>2</sub>-binding materials was calculated from the mobility of BPB. After electrophoresis, gels were sliced into 1 mm segments, and each slice was offered to radioactivity measurement in a toluene-PPO-POPOP system. In some cases, the diluted prostate cytosol (0.2 ml containing 3 mg protein) was incubated with 3 $\times$ 10<sup>-8</sup> M <sup>3</sup>H-E<sub>2</sub> and filtered through a Sephadex G-100 column. The fraction eluted within void volume was then offered to PAGE.

*Scatchard plot analysis* The diluted pro-

state cytosol (0.2 ml containing 4.6 mg protein) was incubated at 0°C for 16 hours in the various amounts of  $^3\text{H-E}_2$  ( $3.4 \times 10^{-14}$  to  $5.2 \times 10^{-11}$  mole). After incubation bound  $^3\text{H-E}_2$  was separated from free one using the dextran-coated charcoal suspension. Specific binding was estimated by measuring the difference of bound steroid in cytosols incubated with or without an excess of unlabeled  $\text{E}_2$ . Plotting by the method of Scatchard (1949) permitted to calculate the dissociation constant and the molar concentration of binding sites.

### (C) Method in tissue incubation studies

*Preparation of subcellular fractions* The prostate was dissected out from castrated rats, minced and incubated for 1 hour at 37°C in the Eagle's medium containing  $10^{-8}$  M  $^3\text{H-E}_2$ . The incubate was washed with chilled TKM buffer (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M  $\text{MgCl}_2$  containing 0.25 M sucrose) and homogenized in the same buffer containing  $10^{-6}$  M unlabeled  $\text{E}_2$ . The homogenate was centrifuged at  $800 \times g$  for 10 minutes. The  $800 \times g$  pellet was suspended in TKM buffer, filtered through three layers of gauze and then centrifuged at  $800 \times g$ . Subsequent procedures to obtain purified cell nuclear pellet were performed by the method of Blobel-Potter (1966). The nuclear pellet was suspended in a solution of 0.4 M KCl, 0.05 M Tris-HCl, pH 7.5 and 0.005 M  $\text{MgCl}_2$  containing 0.25 M sucrose, and incubated for 45 minutes to obtain the supernatant fraction (nuclear extract). On the other hand, the  $800 \times g$  supernatant was centrifuged at  $105,000 \times g$  for 1 hour to obtain cytosol. The cytosol and the nuclear extract were offered to PAGE in the same way as mentioned above.

*Thin-layer chromatography (TLC)* The prostate cytosol, the binding component of the cytosol obtained from Sephadex G-25 filtration and the nucleus were treated with ethyl ether to extract steroids from them. The extracts were spotted on a thin-layer plate coated with Kieselgel HF<sub>254+366</sub> and chromatographed in a solvent system of benzene: methanol (9:1, v/v). After de-

velopment of the plate, the spots of  $\text{E}_1$ ,  $\text{E}_2$  and estriol were detected under UV-lamp (365 nm wave length, Manaslu-light, Tokyo). These estrogen spots were scraped off, acetylated and finally offered to TLC with a solvent system of benzene: methanol (49:1, v/v). In some cases, appropriate areas of the polyacrylamide gel in which electrophoresis of the prostate cytosols was made were cut and extracted with ethyl ether. TLC of the extract was then carried out to determine bound steroids.

## RESULTS

### *E<sub>2</sub>-binding by cytosols from various organs*

Among the tissues studied, the cytosol of the ventral prostate showed the most prominent binding of  $\text{E}_2$ , followed by those of liver, kidney, seminal vesicle and testis (Fig. 1). The cytosol of muscle showed the lowest binding.

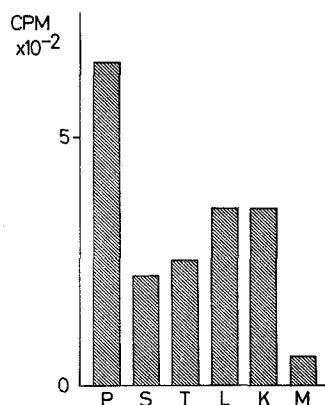


Fig. 1.  $^3\text{H}$ -estradiol-17 $\beta$  binding to cytosols of various organs. P: prostate, S: seminal vesicle, T: testis, L: liver, K: kidney, M: skeletal muscle. A 0.3 ml aliquot of cytosol containing 2 mg protein was incubated with  $10^{-13}$  M  $^3\text{H}$ -estradiol-17 $\beta$  at 0°C for 16 hours. Details are in the text.

*Polyacrylamide gel electrophoresis of  $^3\text{H-E}_2$ -binding materials* Two distinct bound peaks were shown in the rat ventral prostate cytosol after incubation with  $^3\text{H-E}_2$  (Fig. 2). Peak A showed rapid migration ( $R_f=0.97$ ) with only a small amount of  $\text{E}_2$ -binding. On the contrary, peak B showed slow migration ( $R_f=0.65$ ) with a large amount of  $\text{E}_2$ -binding. When the void

volume fractions of the prostate cytosol filtered through Sephadex G-100 column was applied to PAGE, these two  $E_2$ -binding peaks were also detected (Fig. not shown).

It was made clear by PAGE (Fig. not shown) that peak B in the prostate cytosol incubated with  $^3H$ -CC or  $^3H$ -DHT was only scarcely detected. On the other hand, peak A in the cytosol incubated with  $^3H$ -CC was five times larger than that incubated with  $^3H$ - $E_2$ , although peak A in the cytosol incubated with  $^3H$ -DHT was at an equal level to that incubated with  $^3H$ - $E_2$ . There was also a prominent peak with  $R_f=0.17$  in the cytosol incubated with  $^3H$ -DHT.

To estimate an influence of blood serum proteins on  $^3H$ - $E_2$ -binding to the prostate cytosol, the serum was incubated with  $^3H$ - $E_2$  and applied to PAGE (Fig. 2). A conspicuous peak ( $R_f=0.55$ ) was found slightly after the peak of serum albumin.

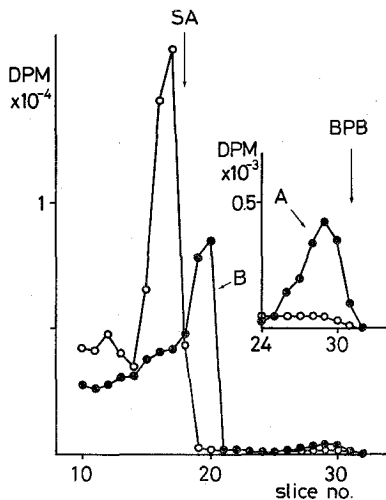


Fig. 2. Binding of  $^3H$ -estradiol-17 $\beta$  to prostate cytosol ( $\bullet$ - $\bullet$ ) and diluted serum ( $\circ$ - $\circ$ ) measured by polyacrylamide gel electrophoresis. A 0.1 ml aliquot of prostate cytosol and blood serum (each containing 3.6 mg protein) were incubated with  $2 \times 10^{-8}$  M  $^3H$ -estradiol-17 $\beta$  at  $0^\circ C$  for 16 hours. BPB: position of bromophenol blue, SA: position of serum albumin. Note two peaks, A ( $R_f=0.97$ ) and B ( $R_f=0.65$ ) in cytosol. More detailed radioactivity values in slice nos. 24 to 32 are shown at the upper right inset.

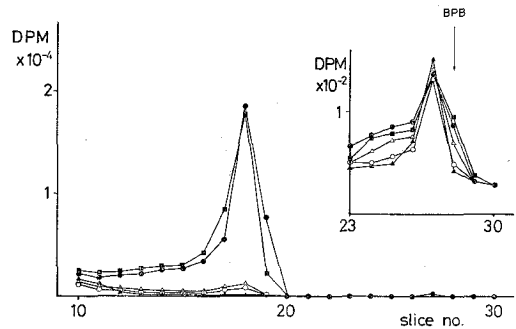


Fig. 3. Polyacrylamide gel electrophoresis of steroid specificity of estradiol-17 $\beta$ -binding material. A 0.1 ml aliquot of prostate cytosol containing 4 mg protein was incubated at  $0^\circ C$  for 16 hours with  $2 \times 10^{-8}$  M  $^3H$ -estradiol-17 $\beta$  alone ( $\bullet$ - $\bullet$ ) or in the presence of  $10^{-5}$  M unlabeled steroids, such as estradiol-17 $\beta$  ( $\circ$ - $\circ$ ), estrone ( $\Delta$ - $\Delta$ ), diethylstilbesterol phosphate ( $\blacktriangle$ - $\blacktriangle$ ) and 5 $\alpha$ -dihydrotestosterone ( $\blacksquare$ - $\blacksquare$ ). BPB: position of bromophenol blue. More detailed radioactivity values in slice nos. 23 to 30 are shown at the upper right inset.

This radioactive peak is clearly distinguished from peak B of the prostate cytosol by their different  $R_f$  values.

The prostate cytosol incubated with  $^3H$ - $E_2$  in a 1000-fold excess of non-radioactive  $E_2$  or  $E_1$  significantly reduced  $E_2$ -binding peak B with slow migration. The addition of DESP abolished this binding to an undetectable level. Same doses of T, DHT or CC, however, did not affect this binding (Fig. 3). On the other hand, a rapidly migrating  $^3H$ - $E_2$ -binding peak A was not affected by any of these unlabeled steroids.

*Scatchard plot analysis* The binding of  $^3H$ - $E_2$  to the prostate cytosol was characterized by Scatchard plot analysis (Fig. 4). The cytosol bound  $^3H$ - $E_2$  with high affinity ( $K_d=2.2 \times 10^{-11}$  M), and the number of binding sites for  $^3H$ - $E_2$  was  $9.3 \times 10^{-16}$  moles/mg protein.

*PAGE of subcellular fractions...tissue incubation studies* Two radioactive peaks, A and B, were found both in the cytosol and in the nuclear extract from the prostate tissue after incubation with  $^3H$ - $E_2$  (Fig. 5). Peak B in the cytosol was larger than that in the nucleus, whereas peak A in

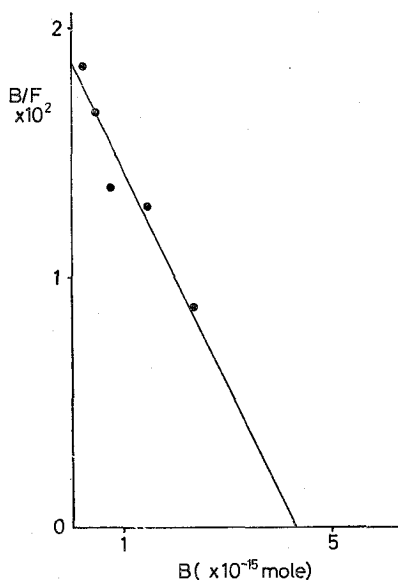


Fig. 4. Scatchard plot of estradiol-17 $\beta$  binding in prostate cytosols. A 0.2 ml aliquot of the cytosol containing 4.6 mg protein was incubated with  $^3\text{H}$ -estradiol-17 $\beta$  ( $1.7 \times 10^{-10}$  to  $2.6 \times 10^{-9}$  M) for 16 hours at 0°C.

the nuclear extract was larger than that in the cytosol. After cutting off peaks A and B from cytosol and nuclear extract, TLC in the solvent system of benzene:methanol (9:1) was performed (Data not

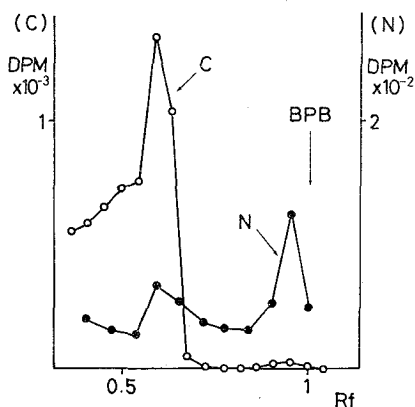


Fig. 5. Estradiol-17 $\beta$  binding in cytosol and nucleus of prostate analysed by polyacrylamide gel electrophoresis. The tissue was incubated in Eagle's medium containing  $10^{-8}$  M  $^3\text{H}$ -estradiol-17 $\beta$  at 37°C for 1 hour. Details are in the text. Cytosol: (○—○), nuclear extract: (●—●). BPB: position of bromophenol blue,  $R_f$ : relative mobility to BPB, C: cytosol, N: nucleus.

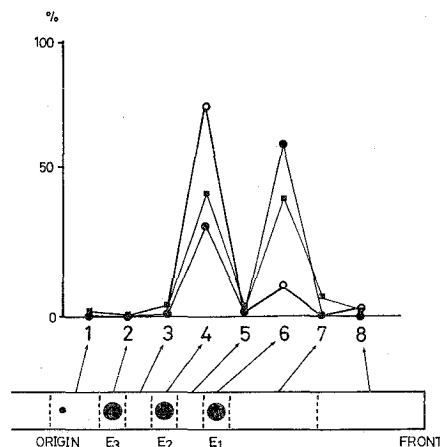


Fig. 6. Steroid analysis by thin-layer chromatography of cytosol and nucleus of the prostate. The tissue was incubated in Eagle's medium containing  $10^{-8}$  M  $^3\text{H}$ -estradiol-17 $\beta$  at 37°C for 1 hour. Ethyl ether extracts were developed in benzene:methanol (9:1). Cytosol: (●—●), binding component of cytosol: (■—■), nucleus: (○—○). Fractions 2 to 6 were of 1 cm length and fractions 1, 7 and 8 were of 2, 3.4 and 4.2 cm length, respectively. Details are in the text.

shown). As a result, the material at peak B in the cytosol rather bound  $^3\text{H}$ -E $_2$  than  $^3\text{H}$ -E $_1$  with a ratio of E $_2$ :E $_1$  (73:27). On the contrary, the material at peak A in the cytosol predominantly bound  $^3\text{H}$ -E $_1$ , and the ratio of E $_2$ :E $_1$  was 32:68.

*Steroid analysis* Thin-layer chromatographic analysis revealed that the major radioactivity was found in both E $_1$  and E $_2$  fractions of the prostate cytosol, binding component of the cytosol and the purified nuclei when the prostate tissue was incubated with  $^3\text{H}$ -E $_2$  (Fig. 6). The cytosol contained much more E $_1$  than E $_2$ , but the binding component of the cytosol contained both estrogens at the same level. The nuclei contained much more E $_2$  than E $_1$ . Extremely high purity of E $_1$  and E $_2$  in their TLC fragments from each subcellular preparations was confirmed by acetylation except E $_2$  in its fragment from nuclei, in which about 40% unknown contaminant existed.

## DISCUSSION

The prostate cytosol fraction used in this

experiment might have extracellular components including the secretion, extracellular fluid and blood plasma. Contamination of the blood plasma has particularly to be taken care of in the study on steroid-binding macromolecules. In this respect, Wagner (1972) reported average 50% contamination of blood plasma to the cytosol of calf prostate. Cowan et al. (1976) also reported 4~7% contamination by volume of the human prostate with benign hyperplasia. In the present study about 5% of the cytosol protein was of plasma origin, when corticosterone-binding material in the cytosol was taken as an indicator. The existence of both cortisol-binding globulin-like and sex hormone-binding globulin-like macromolecules in the human prostate with benign hyperplasia described by Cowan et al. (1976) was not detected in the rat prostate. This may depend on the differences of species, treatments, conditions, and so on.

Liao et al. (1970) described that there are at least two DHT-binding proteins in the cytosol of rat ventral prostate. One of them, beta protein, exhibits an extremely high affinity and specificity toward DHT and several synthetic androgens, but not toward steroids such as estrogens (Liao et al., 1972, 1973). Another protein, alpha protein, binds  $E_2$  as well as DHT (Fang et al., 1971). Armstrong et al. (1974) reported estrogen-binding protein in the rat prostate that was distinguished from DHT-receptor because the addition of unlabeled  $E_2$  had little effect on the  $^3H$ -DHT binding in the rat prostate cytosol and conversely the addition of unlabeled DHT at the same concentration reduced the amount of bound  $^3H$ -DHT. Accordingly, they did not remark the DHT-binding protein that binds also  $E_2$ . Our findings made clear that there are at least three DHT-binding materials in the rat prostate cytosol. One of them with the lowest  $R_f$  value ( $R_f=0.17$ ) bound DHT exclusively and, therefore, may be the DHT-receptor, beta protein. The result of Hansson et al. (1974) by PAGE that cytoplasmic androgen receptor in the rat testis slowly migrates may support our presumption. The other materials migrated

as two low peaks exhibited similar migration pattern to  $E_2$ -binding materials (to be called materials at peaks A and B). Considering steroid specificity, the material at peak B, at least, distinguished from alpha protein, although whether material at peak A is identified with the alpha protein is remained unsolved.

We found two  $E_2$ -binding materials in the rat ventral prostate cytosol. One of them at peak B ( $R_f=0.65$ ) exhibits specific binding to the estrogen with a few binding sites and high affinity toward the hormone. These are characteristics of the receptor for steroids. But, it was not made clear if this material is a receptor that enters the nucleus with the "two-step" mechanism as proposed in the first place by Jensen et al. (1968) and Gorski et al. (1968) for the estrogen receptor in the uterus and affects the cellular function of the tissue. At the present time we can merely offer a few minor evidences for the problem as follows. The material at peak B was observed in the nucleus as well as in the cytosol. Although the endoplasmic reticulum adhering to the nuclear membrane cannot absolutely be removed by the method of Blobel-Potter (Blobel et al., 1966) used in this study and this fact, therefore, raised the problem of contamination of the component of the cytosol to the nuclear fraction, the difference of composition of steroids between in the cytosol and in the nucleus denies the possibility of contamination. It was found from tissue incubation studies that  $E_2$  was converted to the  $E_1$  in the prostate cytosol as reported by Unhjem (1970). On the contrary to the cytosol,  $E_2$  was predominant in the nucleus with a virtual amount of unknown steroid. In addition to these facts, the material at peak B preferentially binds  $E_2$ . These findings suggest that the material at peak B binds  $E_2$  and transports it into the nucleus. Further study is necessary to clear this point.

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(1978年3月28日受付迅速掲載)



## 和文抄録

## ラット前立腺におけるエストロゲン結合物質の性状

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前立腺腫瘍の治療としてエストロゲン投与がおこなわれるが、その作用機序を明らかにする手始めとして、去勢ラット腹側前立腺の胞体可溶分画および核におけるエストロゲン結合物質の性状について検討を加えた。その結果、次の諸点が明らかとなった。(1) 各臓器の胞体可溶分画を得た後、それに対する放射性エストラジオール-17 $\beta$  の結合を炭末吸着法によって検討すると、前立腺において最もよく結合し、続いて肝臓、腎臓、精巣、精囊の順となり、骨格筋にはわずかしか結合しなかった。(2) 前立腺可溶分画を放射性エストラジオール-17 $\beta$  のみ、あるいはそれに非放射性の各種ステロイドを加えてインキュベーションした後、ポリアクリルアミドゲル電気泳動法によって分析を加えると、エストロゲンに特異的に結合するものと、ステロイド特異性のないものの2種の物質が認められた。ともに血清タンパクの混入によるものではな

い。また炭末吸着法を用いたスキッチャード・プロット分析によって、結合部位数および解離定数を求めると、それぞれ  $9.3 \times 10^{-16}$  モル/mg タンパク、 $K_d = 2.2 \times 10^{-11}$  M と計算された。(3) 前立腺組織細片を放射性エストラジオール-17 $\beta$  を含む培養液でインキュベーションした場合でも、核抽出液および胞体可溶分画中に2種の結合物質が認められた。(4) 放射性エストラジオール-17 $\beta$  とインキュベーションした組織から得た核と胞体可溶分画に対して、薄層クロマトグラフィーによってステロイド分析をおこなうと、胞体可溶分画においては、エストラジオール-17 $\beta$  の多くがエストロンに変換されているが、核においては多くのエストラジオール-17 $\beta$  のままで存在することが明らかになった。これらの結果は、去勢ラットの腹側前立腺に、結合部位数が少なくエストロゲンに親和性の高い高分子物質の存在を強く示唆している。