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CHARACTERISTICS OF 17β-ESTRADIOL BINDING PROTEINS

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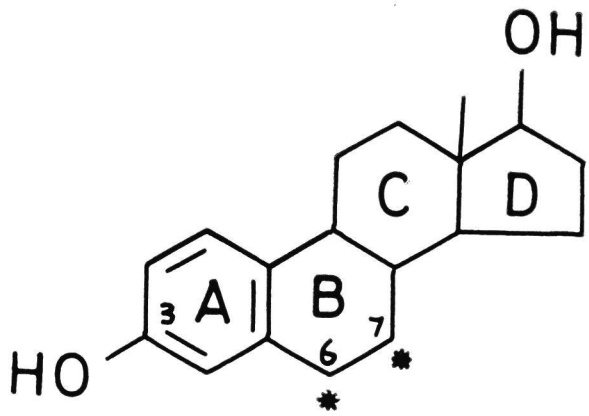
INTRODUCTION

THE PRESENCE OF a protein with high affinity and which binds specifically with 17β-estradiol has been demonstrated in the uterus (Toft and Gorski, 1966). This receptor protein molecule was thought to exist in a free form or is only very loosely bound to structural elements in the cytoplasm.

To date, one of the proposed mechanisms of steroid hormone action is that the hormone initially acts by binding to a specific cytosol receptor protein. The complex that is formed is then transported to the nucleus of the cell wherein it reacts with the nuclear chromatin. This combination in turn influences the synthesis of mRNA that will act as a template directing the synthesis in the endoplasmic reticulum of a specific enzyme protein.

The clinical importance of 17β-estradiol receptor assays has made inroads in the prediction of response to endocrine therapies in patients with metastatic breast cancer (McGuire *et al.*, 1975). Hence, several assay methods were developed for measuring estrogen receptors, e.g. sucrose density gradient, gel filtration, electrophoresis, tissue-slice and dextran-coated charcoal (DCC) techniques. The DCC assay was the most popular technique and was the method adopted in this study. The labelled hormone employed was 17β-estradiol tritiated at positions 6 and 7 of the steroid structure as shown below.

It is our aim in this study to isolate, purify and characterize these binding proteins from the rabbit tissue cytosol and thereby to study their characteristics in different tissue such as liver, uterus and kidney. These binding proteins have sometimes



been referred to as the hormone receptors. It is therefore realised that a study on 17β-estradiol binding proteins will throw more light in the understanding of the mechanism of estradiol action.

MATERIALS AND -ETHODS

Materials

17β-estradiol, Norit A and Dextran were purchased from the Sigma Chemical Co. (U.S.A.).

6,7-^{3}H estradiol (500 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England).

Experimental Animals

Adult female rabbits (New Zealand White) were obtained from the Central Animal House, Faculty of Medicine, University of Malaya.

Animals (7 days pregnant) were killed by cervical dislocation and the liver, uterus and kidney were quickly removed and placed on ice. All subsequent procedures were carried out at cold room temperature (4°C) unless indicated.

Preparation of 17β-Estradiol Binding Proteins

The fresh tissues of liver, kidney and uterus were homogenized (Sorvall-omnimixer) in six volumes of iced-cold 0.01 M Tris/HCl buffer (pH 7.4). The homogenate obtained was centrifuged (Sorvall, SS-3 automatic) at 755 xg for 20 min at 4°C and the resulting supernatant further centrifuged at 4°C for 20 min at 10,000 xg. The pellet was discarded and the supernatant recentrifuged at 10,000 xg for 20 min. Centrifugation at 100,000 xg (Beckman ultracentrifuge L2-65B in a 60 Ti rotor) was then carried out on the supernatant for 1 hr at 4°C. The resultant supernatant contained the 17β-estradiol binding proteins, also referred to as cytosol extract.

Precipitation of Binding Proteins with Ammonium Sulphate

The digest mixture containing 100 μl of labelled estradiol (37,000 cpm), 200 μl of cytosol extract of varying protein concentration and 0.01 M Tris/HCl buffer (pH 7.4) in a final volume of 500 μl was incubated at 37°C for 90 min. 500 μl of saturated ammonium sulphate solution was added at 0°C and the mixture allowed to stand in ice for 10 min. The mixture was then centrifuged at 6,000 xg for 15 min. The precipitate obtained was washed with 500 μl of saturated ammonium sulphate solution and re-centrifuged at 6,000 xg for 15 min. The resultant precipitate was transferred into a vial containing 5 ml of toluene-Triton X-100 scintillation cocktail. The vials were counted for 5 min each in a Beckman LS-100 Liquid Scintillation Counter.

For competitive binding studies, estradiol was added to the reaction mixture together with the labelled hormone.

Separation of Bound and Free Estradiol by Charcoal Adsorption

Labelled estradiol of varying volumes (10 to 200 μl, i.e. 2,000 to 40,000 cpm), 200 μl of cytosol extract, and buffer in a total volune of 500 μl were incubated at 37°C for 30 min and then cooled in ice. 1 ml of the suspension of dextran-coated charcoal in Tris/HCl/EDTA buffer (pH 7.4) was added. The contents were mixed and kept in ice for 10 min. The charcoal was spun down at 1,000 xg for 20 min and 500 μl of the supernatant collected into the counting vial containing 10 ml of Bray's scintillation mixture.

For competitive binding experiments, different amounts of the unlabelled estradiol were added to the reaction mixture together with the labelled hormone. In some cases, the cytosol extract was preincubated at 37°C for 30 min with the unlabelled 17β-estradiol prior to the addition of the labelled hormone.

The above standard procedure was modified with regard to incubation time, temperature, pH, ion and detergent concentrations.

RESULTS

Comparison of Separation Methods of 17β-Estradiol Binding Proteins

Ammonium sulphate precipitation appeared to yield slightly higher values of percentage binding than those obtained by dextran-coated charcoal (Fig. 1). The lower values in binding with the latter method could be due to the removal of some of the labelled estradiol from the bound hormone protein complex by the charcoal.

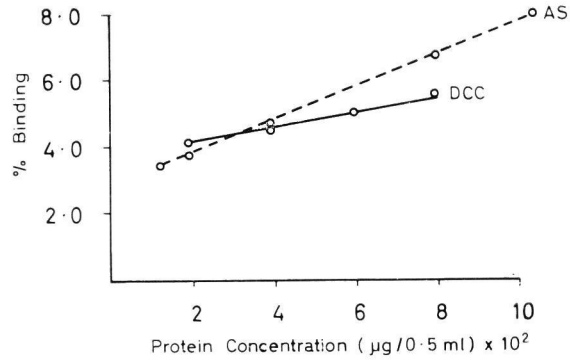


Fig. 1. Comparison of Dextran-coated Charcoal and Ammonium Sulphate Precipitation.
Increasing amounts of protein (200 μg – 800 μg/200 μl) were incubated with 100 μl of tritiated estradiol (37,000 cpm) at 37°C for 90 min. Separation of the bound from the free estradiol was effected by using (a) ammonium sulphate precipitation (AS) and (b) dextran-coated charcoal technique (DCC).

Influence of Incubation Time on the Binding

The rabbit uterine cytosol (700 μg/100 μl) was incubated with 100 μl of labelled 17β-estradiol (37,000 cpm) and 300 μl of Tris/HCl/EDTA buffer (pH 7.4) at 37°C for various time intervals ranging from 5 min to $2\frac{1}{2}$ hr.

The results showed a gradual decline in the binding of labelled hormone (Fig. 2). This decline in binding was seen in the first 30 min after which the percentage binding remained fairly constant. Anyway, there was no appreciable loss in binding

activity with time. The decrease in percentage binding within the first 30 min of incubation could be indicative of a denaturing process (Brecher *et al.*, 1967).

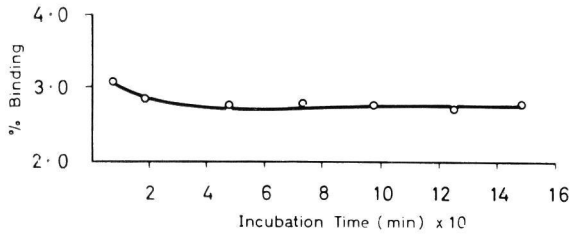


Fig. 2. Influence of Incubation Time on Tritiated Estradiol Binding.
The digest mixture consisted of rabbit uterine cytosol (100 μl), 100 μl of tritiated estradiol (37,000 cpm) and 300 μl of Tris/HCl/EDTA buffer. The digest was incubated at 37°C for various time intervals.

Binding as a Function of Cytosol Protein Concentration

Varying amounts of the tissue extracts were used for incubation with 100 μl of the labelled estradiol. In the liver and uterine cytosol, the binding of the labelled hormone increased with increasing protein concentration (Fig. 3). But the percentage binding in liver cytosol was higher than that in the uterine cytosol. The kidney cytosol did not change significantly in binding ability with varying protein concentration.

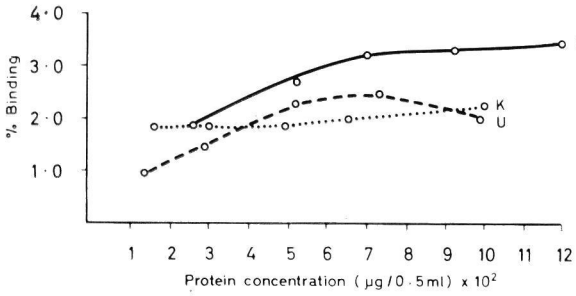


Fig. 3. Relationship between Protein Concentration and Percentage Radioactivity Bound
L = Liver cytosol, K = Kidney cytosol,
U = Uterine cytosol
Varying amounts (975–9,600 μg/200 μl) of tissue extracts, 100 μl tritiated estradiol (37,000 cpm) and buffer in total volume of 0.5 ml were incubated at 37°C for 30 min.

The binding of labelled estradiol to the cytosol proteins was a saturable process with respect to the labelled hormone concentration. The failure of kidney cytosol to show significant increase in binding despite increasing protein concentration indicated that the protein receptors had been saturated with the labelled hormone.

Displacement of Labelled 17β-Estradiol by the Unlabelled Hormone

Increasing amounts of cold estradiol (10 pg to 1,000 pg) were added to the incubation medium containing 500 μg of cytosol proteins and pre-incubated for 30 min at 37°C. 100 μl of labelled hormone (10,300 cpm) was then added and the mixture incubated for a further 30 min.

From the results obtained, the displacement effect by the unlabelled hormone appeared to be most significant in the 10–200 pg region for liver and uterine cytosol (Fig. 4).

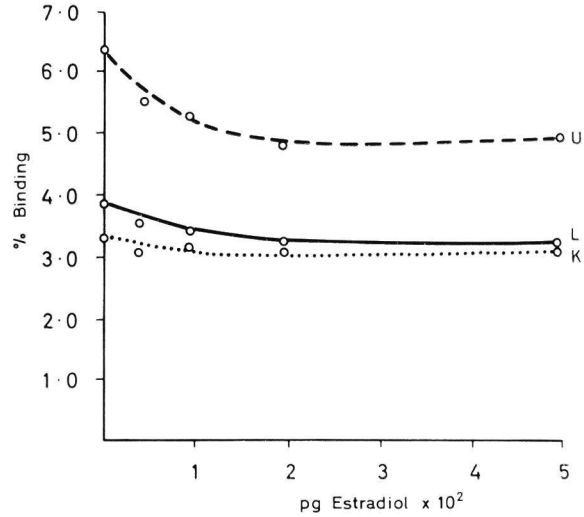


Fig. 4. Influence of Unlabelled Estradiol on Binding.
Protein concentration of the uterine, liver and kidney cytosol extract was 975 μg/200 μl, 9600 μg/200 μl and 3,300 μg/200 μl respectively.

Influence of Temperature on Binding

The digest containing a mixture of 50 μl of labelled estradiol (10,300 cpm), 100 μl (500 μg) of cytosol proteins and buffer in a final volume of 500 μl, was carried out at temperatures ranging from 4°C to 60°C for 30 min.

Temperature appeared to have a marked influence on the binding of labelled hormone (Fig. 5). For uterine cytosol, binding was maximum at 35°C – 40°C. Liver cytosol also showed a maximum binding at this range of temperature. However, kidney cytosol showed a maximum binding at a lower temperature of 25°C. In general, the uterine binding proteins were more sensitive to temperature changes.

Influence of pH on Binding

100 μl (500 μg) of cytosol proteins and 50 μl of labelled hormone (10,300 cpm) were incubated

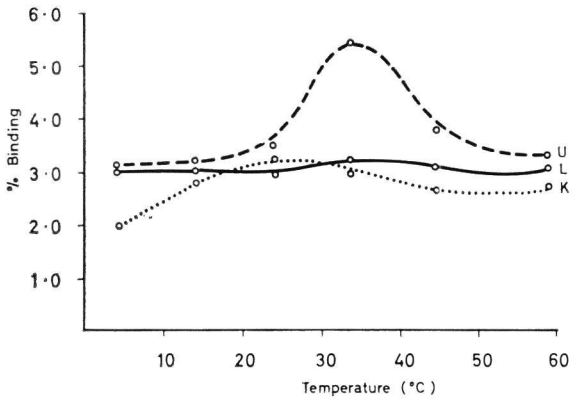


Fig. 5. Influence of Incubation Temperature on Binding.
U = Uterine cytosol, L = Liver cytosol,
K = Kidney cytosol

under hydrogen ions concentrations ranging from pH 3.0 to 9.0 for 30 min at 37°C. Bound estradiol was separated from excess free estradiol by charcoal adsorption.

A maximum binding was observed at pH 7.4 in the uterine cytosol (Fig. 6). Binding in the acidic medium was lower than that in a neutral or alkaline medium. In liver cytosol, maximum binding occurred in the range of pH 6.0 to 7.0. Kidney cytosol also showed a pH optimum of 7.0 for maximum binding.

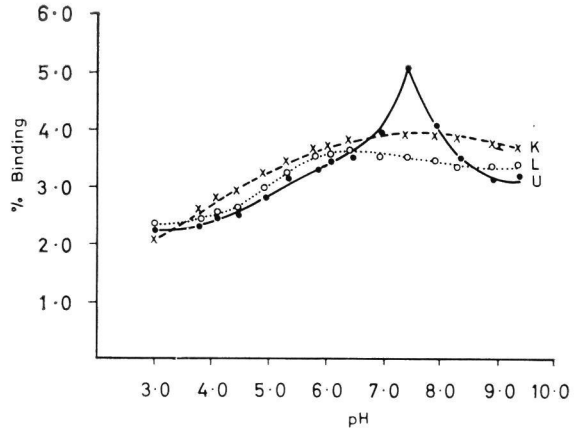


Fig. 6. Influence of pH on Binding.
K = Kidney cytosol, L = Liver cytosol,
U = Uterine cytosol

Effect of Magnesium Ions on Binding

100 μl (500 μg) of cytosol proteins, 50 μl of labelled hormone (10,300 cpm) were incubated at pH 7.4 in a medium containing magnesium ions ranging from 1 mM to 1,000 mM. The bound estradiol was separated from the free hormone by using a dextran-coated charcoal suspension in Tris/HCl buffer (pH 7.4) which did not contain EDTA.

In kidney cytosol, the binding appeared to decrease slightly in the presence of magnesium ions when present in a concentration of 5 mM to 10 mM (Fig. 7). Above this ions concentration, there was a slight increase in binding. The liver cytosol was not significantly affected by the magnesium ions. Uterine cytosol showed a marked sensitivity to magnesium ions in binding ability. Calcium ions gave similar binding pattern to that of the magnesium ions.

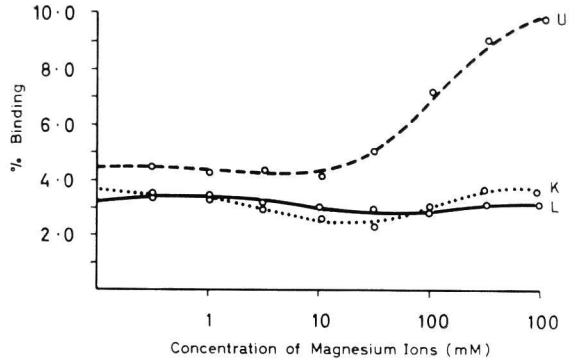


Fig. 7. Effect of Magnesium Ions
U = Uterine cytosol, L = Liver cytosol,
K = Kidney cytosol
100 μl (500 μg) of cytosol protein, 50 μl of labelled estradiol (10,300 cpm), 100 μl of buffer and 250 μl of magnesium ions of molar concentrations ranging from 1 mM to 1,000 mM were incubated at 37°C for 30 min.

Effect of Ionic and Non-Ionic Detergents on Binding

The ionic detergent used was sodium deoxycholate, "Triton X-100" and "Span" were the two non-ionic detergents employed. The incubation medium with a final volume of 500 μl contained 50 μl of labelled estradiol (10,300 cpm), 1 mg of cytosol protein and increasing amounts of the respective detergents ranging from 0.1% to 1.0% (v/v in the case of Triton X-100 and Span, w/v in the case of sodium deoxycholate). Incubation was carried out at 37°C for 30 min.

(i) *Rabbit Liver Cytosol*

Binding of labelled hormone increased with increasing concentration of the three detergents (Fig. 8). With Triton X-100 or sodium deoxycholate, a sigmoidal type relationship was observed.

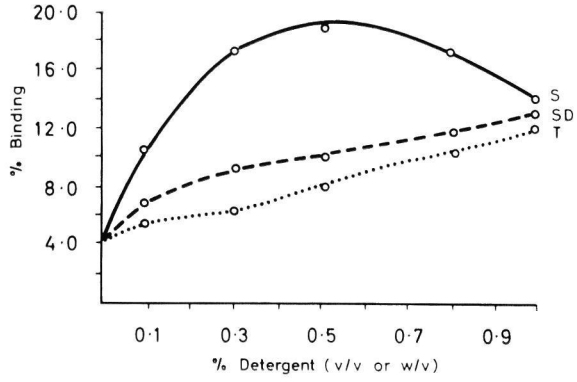


Fig. 8. Effect of Ionic and Non-Ionic Detergents on Binding in Rabbit Liver Cytosol.
T = Triton X-100, S = Span, SD = Sodium deoxycholate

(ii) *Rabbit Kidney Cytosol*

Generally, the detergents used enhanced the binding of labelled hormone (Fig. 9).

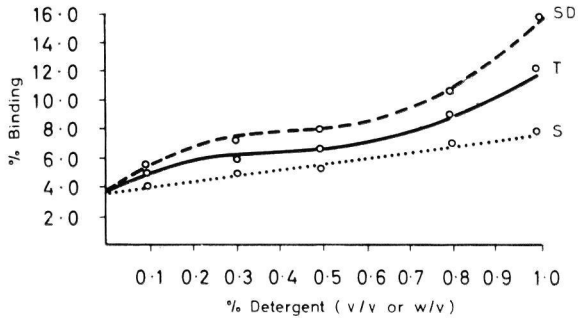


Fig. 9. Effect of Ionic and Non-Ionic Detergents on Binding in Rabbit Kidney Cytosol.
T = Triton X-100, S = Span, SD = Sodium deoxycholate
(Similar curves were obtained for uterine cytosol)

DISCUSSION

Although ammonium sulphate precipitation appeared to give a higher percentage binding than the dextran-coated charcoal method the latter was adopted because it was simpler and less time consuming. This method also gave better duplicate readings.

The binding of labelled estradiol to the cytosol proteins was a saturable process with respect to the labelled hormone concentration. The failure of kidney cytosol to show significant increase in binding despite increasing protein concentration indicated that the protein receptors had been saturated with the labelled hormone.

In the displacement experiment, both labelled and unlabelled estradiol competed for the same binding sites in the tissue cytosol extracts. Hence, a decrease in the binding of labelled hormone in the presence of cold estradiol was observed. In the target organs e.g. uterus, the amount of specific receptors of high affinity binding system present in the cytosol is larger in quantity than that of non-target tissues, such as liver or kidney. Since the binding of estradiol to this system is reversible, the addition of excess of cold estradiol will result in displacement of the labelled hormone. From the curves, it can be seen that the bound tracer could not be reduced to zero even with large excess of the competitor. The effect is clearly caused by the large excess of low affinity binding sites. The relatively high concentration of the low affinity sites rendered them less sensitive to displacement.

The measurement of the amount of estradiol bound by the tissue extract is one indication of the amount of specific receptors present. The charcoal method employed distinguishes the "specifically-bound" from the "non-specifically-bound" estradiol by removing most of the latter together with the excess of free estradiol. The suppressibility of the binding of labelled estradiol by unlabelled hormone which competes for the same specific receptors in the tissue extracts is a second and probably better indicator of the amount of specific receptors present.

The affinity of various steroids to the estrogen receptor has been investigated (Hahnel and Twaddle, 1974). Highest affinity to the estrogen receptor was found if the steroid structure had all three of the following features: (1) an aromatic ring A; (2) a phenolic hydroxyl group on carbon-3; and (3) an alcoholic hydroxyl group on carbon-17 in the β-configuration. The most important of the three structural requirements is the phenolic hydroxyl group, both with regard to position on the ring and its nature (i.e. phenolic or alcoholic).

The position and the nature of the oxygen function on ring D are not as critical. On the basis of these results it was postulated that binding of estradiol to the receptor requires a two-point attachment. Binding is initiated by attachment of the C-3 phenolic hydroxyl group to a highly specific centre. The strength of this attachment is influenced by steric factors (for instance, planar aromatic ring A versus chair or boat configuration in saturated ring A) and functional groups (for instance, weaker hydrogen bonding through C-3 alcoholic hydroxyl group versus C-3 phenolic hydroxyl group). Once the initial attachment between the C-3 phenolic hydroxyl group and the primary binding centre has been established, a specific change in configura-

tion of the receptor may be triggered which brings the second binding centre into closer proximity of the C-17β hydroxyl function of 17β-estradiol and facilitates binding.

Above an optimum temperature for maximal binding of the tissue cytosol, the percentage binding decreased rapidly. This was probably due to irreversible denaturation of the receptor protein. Temperature optima could be indicative of the involvement of hydrophobic interactions in binding (Talwar *et al.*, 1968).

The sensitivity of the binding to pH changes suggested stringent conformational requirement for binding (Ellis and Ringold, 1971). The receptors are irreversibly destroyed at low pH (Toft and Gorski, 1966).

Several reports have indicated that certain divalent ions either increase the extent of binding (Emanuel and Oakey, 1969) or stabilize the receptor in some way. It was found that a large excess of calcium ions causes aggregation of the receptor which is probably caused by charge neutralization. However certain other divalent ions e.g. Mn^{2+} and Zn^{2+} appear to cause a reduction in receptor binding.

The sigmoidal type relationship obtained in the experiment of detergents on binding suggested a a heterogeneity of the estradiol binding molecules in the tissue extract or possibly the involvement of cooperative interactions in the binding of the steroid to the receptor molecules in the presence of detergents. The increase in binding with labelled estradiol in the present of detergents was probably due to changes in conformation of the receptor molecule, exposing more of the binding sites to labelled hormone.

In conclusion, our studies indicate the presence of a protein which binds specifically with 17β-estradiol. This binding protein possesses certain characteristics which are different in various tissue cytosol as evidenced by the experiments on effect of temperature, pH, ion and detergent concentrations.

Further characterization of the binding proteins are being carried out using gel filtration and isoelectric focussing.

SUMMARY

17β-Estradiol binding proteins were isolated from female rabbits using differential centrifugation. Ammonium sulphate precipitation and charcoal adsorption techniques were used in the separation of the bound from the unbound hormone. The binding of estradiol was proportional to the amount of protein receptors present and excess of unlabelled estradiol caused displacement of the bound estradiol from the binding sites. The proportion of bound estradiol decreased with increasing tritiated estradiol in the incubation medium. Binding was dependent on the time of incubation and was also sensitive to temperature and pH changes. Calcium and magnesium ions affected binding only when high concentration of these were used and both ionic and non-ionic detergents increased the binding ability.

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