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Nature and Characteristics of the Binding of Oestradiol-17β to a Uterine Macromolecular Fraction

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The binding of oestradiol-17β to two proteins, namely serum albumin and a uterus fraction, was studied *in vitro*. The former protein has a physiological function in the transport of the hormone and the latter is involved in the selective uptake of the steroid by the target organ. The uterus fraction shows a high degree of stereospecificity for the binding of the steroid. Cortisone, oestradiol-17α and testosterone are bound negligibly and progesterone to a much smaller extent than is oestradiol-17β. This property is in contrast with the wide variety of ligands bound by the serum albumin. The temperature and the presence of the steroid influence markedly the binding properties. Oestradiol binding to the uterus fraction is optimum at 37° and at pH 7–8·5. It is markedly decreased at pH values above or below this range, suggesting stringent conformational requirements. The tissue 'receptor' protein is a macromolecule with a minimum molecular weight of 100000. The protein moiety is essential for the binding function. The probable concentration of the total binding sites for oestradiol in the ovariectomized-rat uterus cytoplasmic fraction as determined *in vitro* is about 1 mμM at a steroid concentration of 50 mμM.

Among the endogenous compounds that regulate metabolic activities or growth of different tissues in multicellular organisms are a variety of steroid hormones. Oestradiol-17β, the natural oestrogen hormone secreted by mammalian ovaries, is transported in blood in association with serum albumin, an aspect that has been the subject of previous studies (Eik-Nes, Schellman, Lumry & Samuels, 1954; see review by Daughaday, 1959). It is taken up rapidly from the circulation and retained selectively by several organs, e.g. uterus, vagina and pituitary (Jensen & Jacobson, 1962; Stone, Baggett & Donnelly, 1963; Stone, 1963; Eisenfeld & Axelrod, 1965). These organs are also the sites of biological action of this hormone. Axiomatically speaking, the uptake of the steroid by the tissue is the earliest event of the action of the hormone on the tissue. The mechanisms involved in the uptake and retention of oestradiol by specific tissues therefore have an important bearing on the regulatory action of this steroid on the metabolic activities of these organs.

It was reported previously that [6,7-^{3}H]oestradiol-17β, when administered to ovariectomized rats, was found in the uterus mainly bound to a heavy particulate fraction and to a macromolecular component of the high-speed supernatant fraction (Talwar, Segal, Evans & Davidson, 1964; Talwar *et al.* 1965). Similar observations have been made by Noteboom & Gorski (1965), King & Gordon (1965), Toft & Gorski (1966) and Jensen, Desombre & Jungblut (1966). Results reported in the present paper show that the interaction between oestradiol and uterus extract is a 'tight' type of binding that withstands to a great extent the infinite dilution inherent in passage through Sephadex columns. The linkage is non-covalent in character, the bound steroid being completely extractable by organic solvents. The binding of the steroid to the uterus fraction is nevertheless highly specific, in contrast with the binding of the steroid to the carrier protein in the blood, serum albumin. Factors influencing oestradiol–protein interaction in the two cases have been studied, and it is shown that the binding of the steroid with the two types of proteins of physiological importance, though similar in some respects, also shows several differences.

MATERIALS AND METHODS

Materials

[6,7 - ^{3}H]Oestradiol - 17β (166 μc/μg. or 20·4 μc/μg.), [1,2-^{3}H]testosterone (125 μc/μg.) and [7-^{3}H]progesterone (90 μc/μg.) were products of New England Nuclear Corp.,

Boston, Mass., U.S.A. Diethylstilboesterol, progesterone, cortisone and oestradiol-17β, chromatographically homogeneous, were purchased from Calbiochem, Lucerne, Switzerland. Oestradiol-17α was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Sephadex G-100, G-50 and G-25 (bead form) were products of Pharmacia, Uppsala, Sweden. Crystalline bovine serum albumin was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Pronase was a Calbiochem product. Trypsin, ribonuclease, deoxyribonuclease and other enzymes were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Methods

Stock steroid solutions. These were prepared by dissolving a known weight of the steroid in ethanol, which ensured complete solubilization, and thereby quantitative transfers of small amounts. Appropriate samples of this solution were taken for experimental studies. The final concentration of ethanol in experimental studies was 1–2%.

Preparation of G-100–P-1 fraction. Uteri from calves and ovariectomized rats were used for the studies. The calf uteri were obtained from commercial sources wrapped in polyethylene bags kept in an ice bucket, and were processed within 4hr. of the removal of the tissue from the animals. The endometrial lining of the calf uteri was scraped off with a scalpel and homogenized in a glass homogenizer. The muscular tissue (myometrium) was passed through a meat mincer and homogenized vigorously with a Potter–Elvehjem type of homogenizer in TMK buffer (10mM-tris–HCl buffer, pH7·4, containing 1·5mM-$MgCl_2$ and 10mM-KCl). The homogenate was centrifuged at 15000g for 30min. The residue was discarded and the supernatant centrifuged at 105000g for 90min. In some cases, at this stage, solid $(NH_4)_2SO_4$ was gradually added to the supernatant with constant stirring to attain 85% salt saturation. The precipitate was dissolved in a small volume of TMK buffer and dialysed overnight against TMK buffer. The dialysed material (3ml.) or 105000g supernatant without prior concentration by $(NH_4)_2SO_4$ treatment was passed through a column (30cm. × 1cm.) of Sephadex G-100 pre-equilibrated and eluted with TMK buffer. The first protein peak (G-100–P-1) was used for the binding experiments. All experimental procedures were performed in the cold-room, unless otherwise stated.

Preparation of 0·25–G-100 fraction from calf uteri. Calf uteri dissected of extraneous tissue were passed through a meat mincer and homogenized in medium A [KCl (0·3M), EDTA (5mM), mercaptoethanol (7mM) and glycerol (20%, v/v)] in a VirTis homogenizer and subsequently in a Waring Blendor. The homogenate was centrifuged at 1500g for 15min. The supernatant was then centrifuged at 17000rev./min. in rotor 21 of a Spinco preparative ultracentrifuge for 45min. The pellet was rejected. To the supernatant, $(NH_4)_2SO_4$ was added gradually with stirring to bring the saturation to 25%. The pellet was dissolved in medium B [KCl (10mM), EDTA (5mM), mercaptoethanol (7mM) and glycerol (20%, v/v)]. The preparation was passed through a preparative column of Sephadex G-100. The first protein peak representing the macromolecules unretained by the gel was collected.

Binding studies. (i) Equilibrium dialysis. Rat uterus G-100–P-1 fraction from ovariectomized rats (3mg. of protein/ml.) was put in dialysis bags previously cleaned by

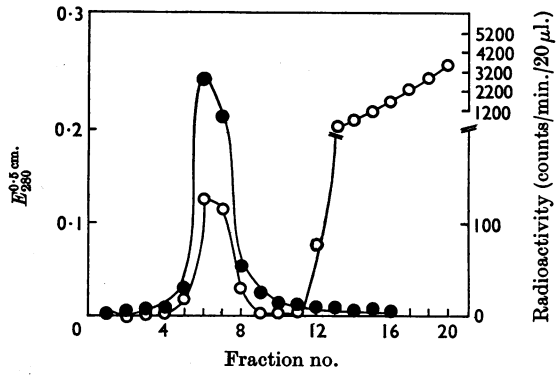

Fig. 1. Separation of protein-bound steroid from the free steroid. A 2·2mg. sample of rat uterus G-100–P-1 fraction in 2ml. of TMK buffer, pH7·5, was incubated with 92mμM-[6,7-^{3}H]oestradiol-17β for 15min. at 37° and passed through a column (0·8cm. × 22cm.) of Sephadex G-100. Fractions of volume 2ml. were collected. ●, $E_{280}^{0.5\,cm.}$; ○, radioactivity.

(*a*) boiling with 5% (w/v) Na_2CO_3 solution, (*b*) washing extensively in water containing 0·5M-EDTA and (*c*) washing in deionized water. The fraction was dialysed against 4ml. of TMK buffer containing a known amount of radioactive oestradiol of known specific radioactivity. The dialysis was continued for 36–40hr. with constant shaking in a cold-room (0–4°). At the end of this period the inside and outside volumes were noted and samples plated for determination of radioactivity. The concentration of bound steroid in mμmoles/l. was calculated as described by Sandberg, Rosenthal, Schneider & Slaunwhite (1966).

(ii) Binding studies with the use of Sephadex columns. The requisite concentration of radioactive steroid was incubated with known amount of uterus fraction in the cold (2–4°) or at 37°. The free steroid was separated from the protein-bound steroid by either of the following procedures.

(1) A 2ml. sample of the incubation mixture was passed through a pre-equilibrated column (1·2cm. × 35cm.) of Sephadex G-50 (medium grade) kept in a cold-room. Fractions of volume 3ml. were collected. The protein-bound steroid was usually recovered in two tubes. The specific binding capacity, i.e. counts/min. of oestradiol bound/mg. of protein, was very nearly the same in the two tubes. An average of the two was taken. As shown in Fig. 1, the free steroid is eluted from the column at a later stage.

(2) A 0·5ml. sample of the incubated mixture was passed through a column (27cm. × 0·9cm.) of Sephadex G-25 (coarse grade, bead form). Fractions of volume 1ml. were collected. The radioactivity and the extinction at 280mμ of each fraction were measured. The specific binding capacity in this case is represented by counts/min./E_{280} unit. In most cases the determinations were done in duplicate. The values were repeatable with a maximum deviation of about 10–15%.

(3) In some cases the free steroid was separated from the bound steroid on a Sephadex G-100 column (0·8cm. × 22cm.).

Measurement of radioactivity. A sample (usually 0·1ml.)

was taken in the radioactivity-counting vial and the aqueous phase was dried by evaporation. Then 10ml. of the scintillation mixture containing 4g. of 2,5-diphenyloxazole and 0·5g. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. of toluene was added. Radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter. The efficiency for ^3H counting was 30%.

Determination of protein content. The protein content was determined by the method of Warburg & Christian (1942).

Table 1. *Binding by rat uterus G-100–P-1 fraction of oestradiol on Sephadex columns*

Rat uterus G-100–P-1 fraction (4·2mg. of protein) was incubated with 0·1μM-[6,7-^{3}H]oestradiol-17β (specific activity 166μc/μg.) in 0·8ml. of TMK buffer, pH 7·5, for 60min. at 37°. The mixture was passed through a column (0·8cm. × 22cm.) of Sephadex G-50. The flow rate was 0·4ml./min. Fractions of volume 1ml. were collected. Fractions 6 and 7 were pooled and 0·8ml. of it was passed through a second Sephadex G-50 column. About 4% of the added steroid was bound in the first passage.

	Radioactivity (counts/min./ml.)	Protein (μg./ml.)	Specific binding of oestradiol (counts/min./mg. of protein)
First passage			
Tube no. 6	50800	1·46	34800
Tube no. 7	84850	2·4	35350
Second passage			
Tube no. 6	29200	0·95	29800
Tube no. 7	6800	0·24	28300

RESULTS

Use of Sephadex for studying binding. Most dialysis membranes adsorb oestradiol appreciably, which introduces an additional phase into the system. Oestradiol is relatively less polar than some other steroids (e.g. cortisone) and has lower solubility in aqueous medium. The concentrations employed for binding studies are in the range 1μM–0·1mμM. High adsorption on dialysis membranes presents a significant problem. These studies have also utilized Sephadex columns for separation of the protein-bound steroid from the free steroid. The binding of oestradiol to receptor protein in the uterus fraction is firm enough to resist total dissociation during passage through the column. The binding determined by this method for duplicate runs is repeatable with a maximum deviation of 10–15%. On successive passages through Sephadex columns there is a slow dissociation. The binding after two successive passages is shown in Table 1. Gel filtration as a technique for studying binding of steroids (cortisol) to specific plasma proteins (transcortin) has also been employed by several

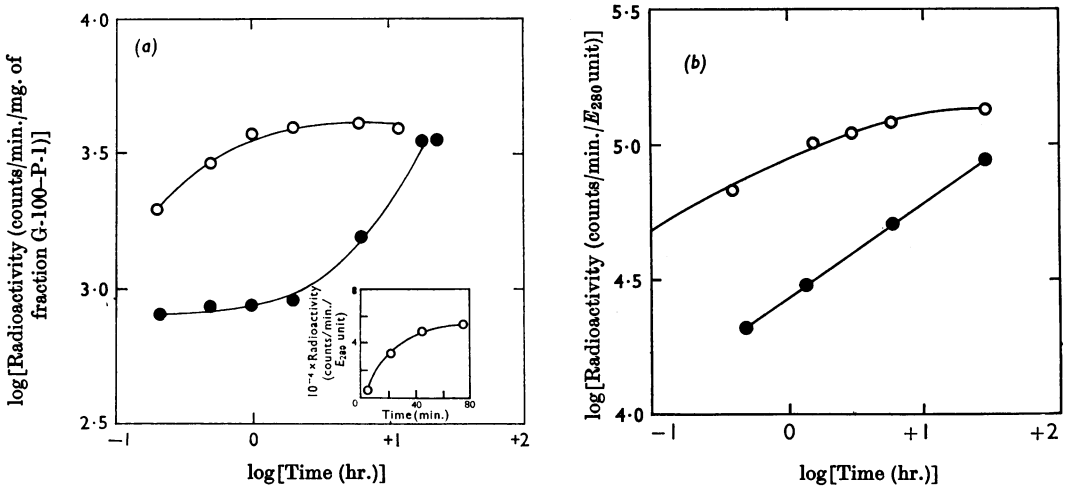

Fig. 2. Kinetics of the binding of oestradiol to rat uterus G-100–P-1 fraction and to bovine serum albumin at 37° and in the cold. In (*a*) 14ml. of G-100–P-1 fraction (21mg. of protein) was incubated at 37° (○) and at 2–4° (●) with 0·37μM-[6,7-^{3}H]oestradiol-17β (specific activity 20μc/μg.). At different time-intervals 2ml. fractions (3mg. of fraction G-100–P-1) were removed and passed through Sephadex G-25 columns. The inset in (*a*) indicates incubations carried out under identical conditions at 37° (○), but at various time-intervals 0·5ml. fractions were withdrawn and passed through Sephadex G-25 columns. In (*b*) 5ml. of bovine serum albumin (2mg./ml.) in TMK buffer was incubated with 0·36μM-[6,7-^{3}H]oestradiol-17β at 37° (○) and at 2–4° (●). Samples (0·5ml.) were withdrawn at different time-intervals and passed through Sephadex G-25 columns. Radioactivity associated with the protein peak was measured.

Table 2. *Influence of the presence of steroid and temperature on the oestradiol-binding capacity of rat uterus G-100–P-1 fraction*

A 1·5ml. sample of rat uterus G-100–P-1 fraction containing 2·58mg. of protein was incubated in the cold and at 37° with 0·31 μM-[6,7-^{3}H]oestradiol. The protein-bound steroid was separated from the free steroid by passage through a column (50cm. × 1·5cm.) of Sephadex G-25. To test whether maintenance of the uterus fraction at 37° is conducive to a better conformation of the protein for binding, fraction G-100–P-1 in Expt. 3 was incubated alone at 37° for 60min., after which oestradiol was added to it; the mixture was then chilled and passed through Sephadex G-25.

Description	Oestradiol bound (counts/min./mg. of G-100–P-1 protein)
Expt. 1	
Fraction G-100–P-1 + [6,7-^{3}H]-oestradiol incubated at 2–4° for 60min.	1219
Expt. 2	
Fraction G-100–P-1 + [6,7-^{3}H]-oestradiol incubated at 37° for 60min.	3589
Expt. 3	
Fraction G-100–P-1 incubated alone at 37° for 60min., and then [6,7-^{3}H]oestradiol added	1645

other workers, who have reported the advantages, precision and repeatability of this experimental method (Demoor, Heirwegh, Heremans & Declerck-Raskin, 1962; Murphy & Pattee, 1964; Doe, Fernandez & Seal, 1964). The method has been found by these authors to give values comparable with those obtained by equilibrium dialysis and ultrafiltration; however, others have found limitations (Quincey & Gray, 1963). This method has several operational advantages, but it measures essentially the 'tight' type of binding that is in a way the focus of study in these investigations.

Kinetics of the binding of oestradiol to the uterus fraction and bovine serum albumin at 37° and at 2–4°. Fig. 2 shows the extent to which oestradiol was bound to the uterus fraction and bovine serum albumin at 37° and in the cold as a function of time. The rate of binding of the steroid with the uterus fraction is fast and linear up to 20min. at 37°. Optimum binding is attained after 60–70min. The initial rate of binding of the steroid with the uterus protein is low at 2–4° and rises only after 6hr. of contact with the steroid. The kinetics of binding of oestradiol to bovine serum albumin are distinctly different from those of the binding of the steroid to the uterus protein fraction, especially at 2–4°.

The beneficial effect of a higher temperature and of the presence of steroid for binding is shown by

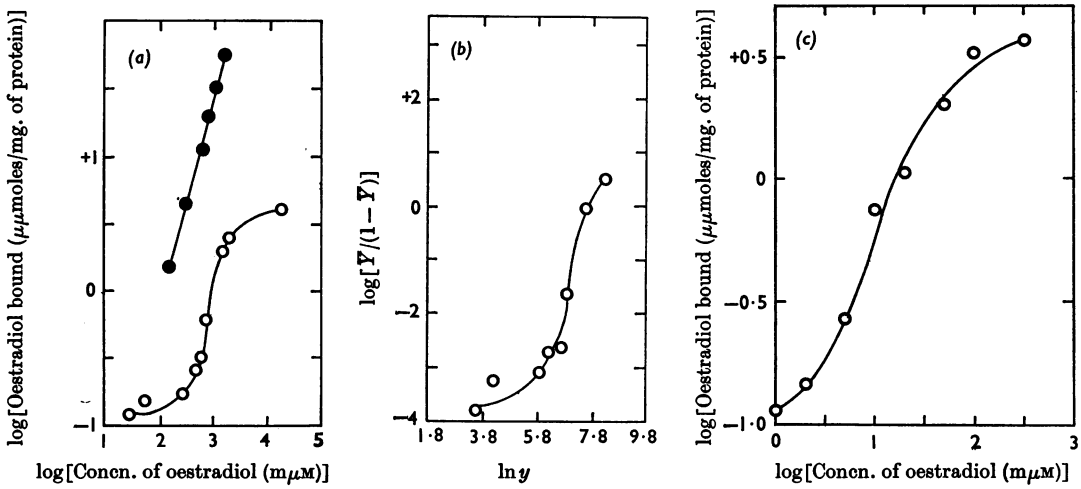

Fig. 3. Effect of oestradiol concentration on the binding of oestradiol. (*a*) Calf uterus 0·25–G-100 preparation (○) (1·85mg. of protein/ml.) and bovine serum albumin (●) (2mg./ml.) in TMK buffer were incubated with increasing concentrations of [6,7-^{3}H]oestradiol at 37° for 60min. The protein-bound oestradiol was separated from the free oestradiol by passage through a column (25cm. × 0·9cm.) of Sephadex G-25. (*b*) Hill plot of the data for 0·25–G-100 fraction (○) in (*a*). The activity, y, is taken to be equal to the concentration of oestradiol in the medium, and $\bar{Y}$ is the fractional saturation of the binding protein. The maximum binding of oestradiol to the uterine fraction was deduced from the reciprocal plot of concentration and the steroid bound. (*c*) Oestradiol bound to rat uterus G-100–P-1 fraction (○) at different steroid concentrations as determined by equilibrium dialysis. The experimental procedure is the same as described in Fig. 6.

the experiment reported in Table 2; 37° is a better temperature for binding, but the presence of the steroid during incubation is also necessary for optimum binding.

Effect of temperature on the binding of oestradiol. The rate of binding of oestradiol to the uterus fraction is influenced markedly by temperature (Fig. 4*b*). The rate of binding increases with the temperature and is optimum at 37°. The binding of testosterone to bovine serum albumin was found to be higher at 25° than at 8° (Schellman, Lumry & Samuels, 1954).

Relationship of the steroid concentration and steroid–protein interaction. Fig. 3 shows the amount of steroid bound to the uterus fraction and bovine serum albumin at various concentrations of the steroid. In these experiments calf uterus fraction and crystalline bovine serum albumin were incubated with different concentrations of radioactive oestradiol *in vitro* for 60 min. at 37°. Protein-bound steroid was separated from the free steroid on a column of Sephadex G-25 (coarse grade) as described in the Materials and Methods section. A number of differences between the binding of the uterus fraction and bovine serum albumin are apparent.

(*a*) The binding of the steroid to the uterus fraction exhibits a sigmoid curve, whereas the binding of the steroid to the serum albumin bears a linear relation under similar conditions. There is likelihood of the involvement of co-operative interactions in the binding of oestradiol to the uterus protein, though it may also be stated that this type of curve could also result from a heterogeneous population of oestradiol-binding proteins.

(*b*) The uterus protein has high affinity but a limited number of binding sites. The binding attains a saturation limit at 2μM-oestradiol concentration. No saturation is noticeable for albumin in the range of steroid concentrations investigated.

(*c*) Fig. 3(*a*) and the Hill plot (Fig. 3*b*) of the binding of oestradiol to the uterus fraction suggest the presence of two types of binding. At low concentrations of the steroid, a small amount of the steroid is bound tightly with high affinity and does not dissociate readily. The steroid bound in the second stage (to the same or a different protein) is 12 times that bound in the primary stage. The steroid bound in the second stage is also more 'labile' and is more readily available. The linear relation obtained for the binding of oestradiol to serum albumin, on the other hand, suggests that all binding sites for oestradiol in albumin are approximately of the same energy level.

Influence of pH *on binding.* The binding of

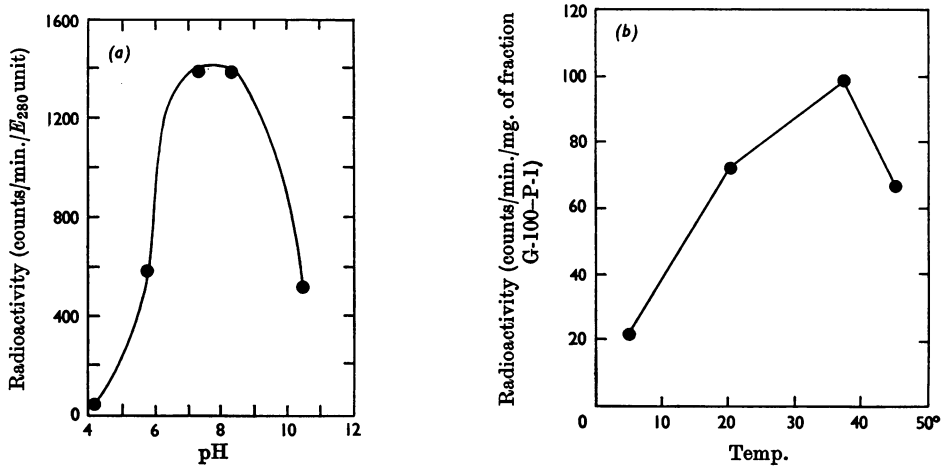

Fig. 4. (*a*) Effect of pH on the binding of oestradiol to rat uterus G-100–P-1 fraction. Ovariectomized rats were given 1·6 μg. (60 μc) of [6,7-^{3}H]oestradiol-17β intraperitoneally. The uteri were removed 1 hr. after the injection, homogenized in 0·9% NaCl and centrifuged at 30000*g* and 105000*g* successively to obtain the high-speed supernatant, which was passed through a column of Sephadex G-100. The first protein peak (G-100–P-1.E) had radioactive oestradiol associated with it. Samples of this fraction were adjusted to different pH values with the addition of dil. NaOH or dil. HCl, kept at the indicated pH for 20 min. at 37° and passed through a column (0·9 cm. × 25 cm.) of Sephadex G-25 pre-equilibrated with saline of the same pH. The specific radioactivities of the protein peaks at different pH values were determined. (*b*) Influence of temperature on the rate of binding of [6,7-^{3}H]-oestradiol to rat uterus G-100–P-1 fraction. A 0·4 ml. sample of fraction G-100–P-1 containing 2·27 mg. of protein was incubated with 9·2 μM-[6,7-^{3}H]oestradiol-17β for 15 min. at different temperatures in each case and passed through a column (1·2 cm. × 35 cm.) of Sephadex G-50 in the cold-room.

oestradiol to the uterus fraction varies considerably with the pH of the medium (Fig. 4*a*). The maximum binding takes place in the pH range 7–8·5. Acidification or alkalinization decreases the binding markedly. The binding is totally abolished at pH 4. This behaviour is in some respects different from earlier reports on the binding of various steroids to bovine serum albumin at different pH values. Levedahl & Perlmutter (1956) studied the influence of pH on the binding of testosterone to bovine serum albumin in the pH range 2·5–11; a progressive increase in binding was observed with pH. The binding of oestrone and progesterone with human serum albumin was also observed to increase progressively from pH 4 to 9 (Sandberg, Slaunwhite & Antoniades, 1957). Schellman *et al.* (1954) observed an increase in the binding of testosterone to bovine serum albumin in the pH range 6·5–8·5.

Extractability of oestradiol bound to uterus G-100–P-1 fraction. Oestradiol is bound to the uterus fraction in non-covalent linkage. It is readily extracted by organic solvents such as ethanol, acetone, chloroform and ether. A major part of the bound steroid is co-precipitated with the protein on treatment with cold trichloroacetic acid (Table 3).

The curve of binding of oestradiol to the uterus fraction at 37° resembles a typical enzyme reaction curve. The chemical nature of the protein-bound steroid was investigated with the objective of ascertaining whether oestradiol was converted into its derivatives during incubation with the binding protein. Radioactive oestradiol-17β was incubated alone and with the uterus fraction for 60 min. at 37° *in vitro*. The steroid was extracted in both cases with chloroform–ether (1:3, v/v) and analysed by

Table 3. *Effect of trichloroacetic acid and organic solvents on the* [6,7-^{3}H]*oestradiol bound to rat uterus G-100–P-1 fraction*

A 4 ml. sample of rat uterus G-100–P-1 fraction (6 mg. of protein) was preincubated with 0·32 μM-[6,7-^{3}H]oestradiol (specific activity 125 μc/μg.) at 37° for 15 min. and passed through a column (30 cm. × 1·5 cm.) of Sephadex G-50. The fraction containing protein-bound radioactive oestradiol was divided into three parts. In (1), 0·1 ml. was plated for counting. In (2), 1 ml. of cold 10% (w/v) trichloroacetic acid was added drop by drop to 1·5 ml. of the protein-bound oestradiol fraction in an ice bucket; the mixture was centrifuged and 0·1 ml. of the supernatant was plated for radioactivity. In (3), the fraction was extracted three times with 5 ml. of ether, and a sample of the extract was taken for counting.

Treatment	Radioactivity in supernatant (counts/min.)	Radioactivity extracted (%)
(1) None	13176	—
(2) Trichloroacetic acid	4860	37
(3) Ether	13425	100

Table 4. *Susceptibility of oestradiol-binding capacity of the calf and rat uterus fractions to various hydrolytic enzymes*

In Expt. 1, 0·25 ml. of calf endometrium G-100–P-1 fraction (E_{280} 0·64), with radioactive oestradiol previously bound *in vitro*, was incubated with the indicated enzyme in 0·1 ml. of TMK buffer for 1 hr. at 37°. The mixture was passed through a column (25 cm. × 1 cm.) of Sephadex G-25. The loss of bound oestradiol was evaluated. In Expt. 2, 1·0 ml. of rat uterus G-100–P-1 fraction (1 mg. of protein) in TMK buffer was incubated for 15 min. at 37° with the indicated amount of enzyme in 0·05 ml. of solution. Then 5 μl. of [6,7-^{3}H]oestradiol-17β (0·025 μg. ≡ 3 μc) was added and the incubation continued for 60 min. at 37°. A 0·05 ml. sample of the mixture was passed through a small column (7 cm. × 0·5 cm.) of Sephadex G-25 and the eluate collected dropwise directly into radioactivity-counting vials. The flow rate was so adjusted that the protein-bound oestradiol peak was well resolved from the free oestradiol peak. Parallel controls were run with radioactive oestradiol and enzymes.

Treatment	Protein-bound radioactivity (counts/min.): Enzyme + uterus fraction	Enzyme alone	Net bound	Loss of bound radioactivity (%)
Expt. 1				
None	435	—	—	0
Pronase (0·1 mg.)	0	—	—	100
Ribonuclease	435	—	—	0
Trypsin (0·5 mg.)	0	—	—	100
Expt. 2				
None	417	—	417	0
Trypsin (250 μg.)	121	22	99	76
Pronase (50 μg.)	88	5	83	80
Ribonuclease (0·5 unit)	464	40	424	0
Deoxyribonuclease (55 μg.)	477	43	434	0

thin-layer chromatography. The radioactivity in both cases migrated in a single fraction, which had the R_F value of oestradiol. No radioactivity was detectable at the R_F values of oestrone and oestriol. The chemical form of the steroid therefore seems to remain unchanged during its binding to the uterus fraction.

Chemical nature of the macromolecule binding oestradiol in the uterus. Oestradiol is bound in the uterus to a macromolecule that is not retained on Sephadex G-100. Its molecular weight is therefore likely to be above 100 000. The binding capacity is susceptible to the action of proteolytic enzymes (Table 4), whereas ribonuclease, deoxyribonuclease and several other enzymes do not affect the binding of oestradiol to the uterus fraction. Steroid-binding macromolecules in the uterus fraction may thus be proteins or at least the protein moiety is inherently involved in the 'receptor' function of the macromolecule for binding of oestradiol.

The isolation and probable chemical nature of receptor for serotonin has been reported (Wooley, 1958; Wooley & Gommi, 1964, 1966). It is believed to be a ganglioside or neuraminic acid-containing protein, as the response of the tissue to serotonin can be selectively destroyed by treatment with neuraminidase. With the courtesy of Dr D. W. Wooley and Mr E. Parr, some of this active enzyme preparation was made available to test whether the receptor for oestradiol is sensitive to neuraminidase.

Table 5. *Effect of neuraminidase on the binding of oestradiol to calf uterus G-100–P-1 fraction*

Purified *Clostridium perfringens* neuraminidase was a tested preparation obtained from Dr D. W. Wooley (courtesy of Mr E. Parr) and was active in abolishing the receptor activity for serotonin in the stomach.

Description	Oestradiol bound (counts/min./E_{280} unit)
Expt. 1: Binding of oestradiol in the presence of neuraminidase	
Fraction G-100–P-1 + [6,7-^{3}H]-oestradiol for 60 min. at 37°	1750
Fraction G-100–P-1 + [6,7-^{3}H]-oestradiol + 0·1 ml. of neuraminidase for 60 min. at 37°	1810
Expt. 2: Action of neuraminidase on the protein-bound oestradiol	
Oestradiol radioactivity bound to fraction G-100–P-1 in the absence of enzyme treatment	37000
Radioactivity bound to fraction G-100–P-1 after 1 hr. treatment with 0·05 ml. of neuraminidase at 37°	41400

The enzyme does not influence the rate of binding of the steroid to the uterus fraction, nor does it cause the release of bound steroid from the uterus fraction (Table 5). The receptor for oestradiol therefore does not seem to be similar to the receptor for serotonin. The neuraminic acid moiety may not be involved in the binding of the steroid.

Specificity for binding of the steroids. Two types of experiments were performed to test the specificity of ovariectomized-rat uterus G-100–P-1 fraction for the type of steroids bound by it. In the first series the uterus fraction was incubated separately with three different radioactive steroids at equivalent concentrations and the amount of steroid bound in each case was determined from the radioactivity bound/mg. of protein after passage through a Sephadex column. Table 6 shows that the amount of oestradiol-17β bound by the uterus fraction is about ten times that of progesterone under identical conditions. Under the same conditions testosterone binding was negligible.

In the second set of experiments the uterus fraction and the bovine serum albumin were incubated with 10 mμM - [6,7 - ^{3}H]oestradiol for 60 min. at 37° in the presence of increasing (0, 10, 50 and 100 times) concentrations of homologous and heterologous non-radioactive steroids. The extent of steroid bound in each case was determined in the protein peak after passage through Sephadex G-25 (coarse grade) columns. It is apparent that non-radioactive oestradiol-17β competes with radioactive oestradiol-17β for binding to the uterus fraction (Fig. 5). The increase in the amount of oestradiol bound at 0·1 μM and at 0·5 μM concentrations of non-radioactive oestradiol reflects the known increase in the binding capacity with increase of steroid concentration (Fig. 3). The stereoisomer, oestradiol-17α, and cortisone do not affect at all the amount of oestradiol-17β bound to the uterus fraction. The situation is different for bovine serum albumin. All steroids whether homologous or heterologous influence the amount of oestradiol-17β bound to the serum albumin in an equivalent manner.

Table 6. *Binding of various steroid hormones with rat uterus G-100–P-1 fraction*

A 2 mg. sample of rat uterus G-100–P-1 fraction in 1·5 ml. of TMK buffer, pH 7·5, was incubated with 50 mμg. of the steroid in each case for 15 min. at 37° and passed through Sephadex G-100.

Hormone	Steroid bound ($\mu\mu$g./mg. of protein)
Oestradiol-17β	9·2
Progesterone	0·89
Testosterone	0·0

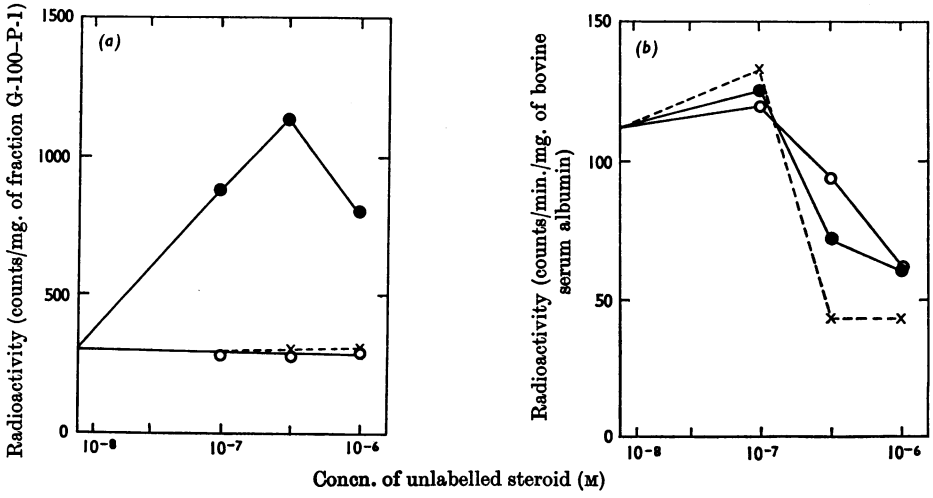

Fig. 5. Influence of other steroids on the binding of [6,7-^{3}H]oestradiol-17β to rat uterus G-100–P-1 fraction and to bovine serum albumin *in vitro*. A 0·5 ml. sample (0·8 mg. of protein) of G-100–P-1 fraction (*a*) or bovine serum albumin (*b*) in TMK buffer was incubated with 10 mμM-[6,7-^{3}H]oestradiol-17β in the absence or in the presence of the indicated concentrations of non-radioactive oestradiol-17β (●), oestradiol-17α (○) or cortisone (×) for 60 min. at 37°. Oestradiol-17β bound to the proteins in each case was determined after passage through a column (26 cm. × 1 cm.) of Sephadex G-25 (coarse grade).

Probable concentration of binding sites for oestradiol-17β in the uterus. To assess the total oestradiol bound by the uterine fraction (in contrast with the 'tightly' bound oestradiol), the binding of oestradiol-17β to the rat uterus G-100–P-1 fraction at different steroid concentrations was determined by equilibrium dialysis. The results are shown in Fig. 3(*c*). The type of the curve obtained is sigmoid, and is similar to the one obtained by gel filtration with the Sephadex columns (Fig. 3*a*). The two methods appear to give qualitatively similar results, though there are quantitative differences that, besides the nature of the method, may also be due to the different source of uterine fractions (Fig. 3*a* is for calf uterus and Fig. 3*c* for ovariectomized-rat uterus).

To determine the concentration of binding sites for oestradiol-17β in the uterus, an experimental approach used by Sandberg *et al.* (1966) for evaluation of the binding of cortisone to transcortin in the plasma was followed. The two systems have several common features. Transcortin is a specific binding protein for cortisone, its concentration in plasma is unknown and there are other non-specific binding proteins (such as albumin) also present in the plasma. These factors are similar to the situation encountered in the uterine fraction. To compensate for the non-specific binding, a parallel G-100–P-1 fraction was prepared from rat diaphragm, a tissue that does not concentrate oestradiol from blood. Fig. 6 shows a plot between b/u and b at different concentrations of oestradiol taken for dialysis, where b represents the bound oestradiol in mμmoles/l. and u represents the free oestradiol at each steroid concentration. Corrected values for the binding of steroid to the uterus fractions were obtained by subtracting the b/u values of diaphragm from the observed values for uterus along arbitrarily chosen lines of slopes $1/u_1$, $1/u_2$ etc. This curve does not show a linear relationship and thus may reflect the heterogeneity of binding sites operative at higher concentrations of the steroid.

The intercept on the abscissa of the first part of this curve (steroid concentration 50 mμM) gives a value of 2·05 mμM for nP_T, where n is the number of binding sites/molecule and P_T the total protein concentration. These values were obtained for 3 mg. of G-100–P-1 fraction, an amount obtained from about two uteri. The probable concentration of total cytoplasmic binding sites for oestradiol in each uterus of the ovariectomized rat would be about 1 mμM.

DISCUSSION

This paper describes some properties and characteristics of the binding of oestradiol-17β to two types of proteins. Both types have physiological functions to perform, one in the transport of the steroid in the blood and the other in the selective uptake of the steroid in a target organ. In both cases the linkage is of a non-covalent type and the steroid is readily extracted by organic solvents.

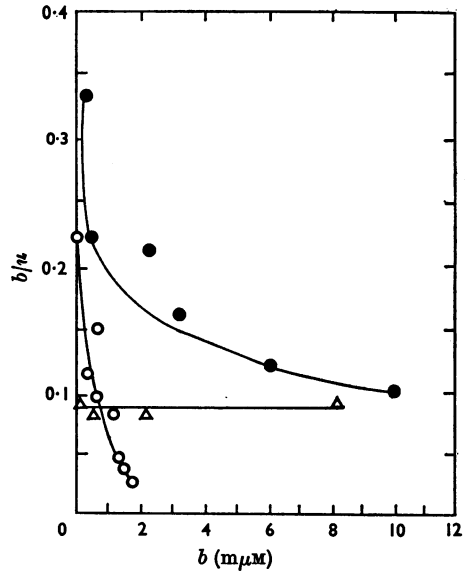

Fig. 6. Apparent and true binding of oestradiol-17β to rat uterus G-100–P-1 fraction. A sample (3mg. of protein) of G-100–P-1 fraction from ovariectomized-rat uteri (●) or rat diaphragm (△) in 1ml. of TMK buffer was dialysed against different concentrations of [6,7-^{3}H]oestradiol-17β in 4ml. of TMK buffer for 36hr. in the cold (0–4°). The inside (dialysis bag) and the outside volumes were noted and the radioactivity was determined in known samples. Controls were performed to assess the adsorption of the steroid by the dialysis bag. *b*, Steroid bound in mμmoles/l.; *u*, steroid unbound≡concentration of the steroid outside the dialysis bag. To compensate for the non-specific binding of the steroid, the corrected curve (○) for uterus was drawn by subtracting the *b*/*u* values for diaphragm from the apparent values for the uterus fraction along arbitrarily drawn lines having slopes of $1/u_1$, $1/u_2$ etc.

There is, however, one major difference. The serum albumin binds, not only oestradiol, but also other steroids as well as a variety of other substances such as azo-dyes, adenosine, sulphonamides, thiocyanates, metal cations, non-esterified fatty acids etc. The strength of binding of steroids to serum albumin seems to be inversely proportional to the number of polar groups in the steroid (Eik-Nes *et al.* 1954). According to Karush (1952) proteins like serum albumin possess a number of combining regions characterized by a high degree of configurational adaptability. This adaptability is associated with the ability of these regions to assume a large number of equilibrium configurations of equal energy.

The uterus fraction has a high degree of specificity for binding oestradiol-17β. Other steroids, e.g. cortisone (Talwar *et al.* 1964) or testosterone, are not bound by it. Progesterone is relatively poorly bound *in vitro*. There is even a discrimination for binding of the stereoisomer oestradiol-17α. The binding is therefore of a stereospecific character. The optimum binding occurs at 37°. Hydrophobic interactions may be important in the linkage of the steroid to the protein. The optimum binding is at pH7–8·5. There is a sharp fall in the binding at pH values above or below this range, which suggests the important influence that pH may have on the appropriate configuration of the protein for binding. The requirements for binding of steroids to serum albumin are not so stringent.

The rate of binding of the steroid to the uterus fraction increases on incubation of the fraction with oestradiol, suggesting the availability of more binding sites after the initial binding of the steroid to the macromolecules. This would indicate a type of co-operative interaction. The chemical form of the steroid remains unchanged on combination with the receptor, an observation that is consistent with earlier findings on the binding of this steroid in the uterus *in vivo* (Jensen & Jacobson, 1962).

The binding factors for oestradiol are present in the uterus in both the heavy particulate and the high-speed supernatant fraction (Talwar *et al.* 1964; Noteboom & Gorski, 1965; Toft & Gorski, 1966; Jensen *et al.* 1966). The heavy particulate fraction has also been subfractionated and purified. About 80% of the oestradiol radioactivity in the heavy particulate fraction is accounted for in the nuclear pellet (G. P. Talwar, unpublished work). The binding properties of the receptor in the cytoplasm and nuclear compartments of the uterus are identical in many respects. It is observed that, in uteri from ovariectomized animals, the protein fraction binding oestradiol is present initially only in the cytoplasm, and has a sedimentation coefficient of about 9·5s. When this fraction is incubated with oestradiol and uterus nuclei *in vitro* at 37° or when oestradiol is injected *in vivo* into animals, there is also the formation of another fraction binding oestradiol that has a sedimentation coefficient of about 5s and that is localized in the nuclei (Jensen *et al.* 1968). Recently the presence of a specific receptor for aldosterone in the nuclear fractions of the kidney has also been reported (Fanestil & Edelman, 1966). It is possible that this phenomenon may be of a general occurrence and that there may exist similar receptors for other steroid hormones in their respective target organs.

The uterine supernatant-fraction receptor is a macromolecule with a minimum molecular weight of 100000. It is protein in nature, or at least the protein moiety is inherently involved in the binding function. Ribonuclease, deoxyribonuclease and neuraminidase do not influence the ability of this fraction to bind oestradiol. The capacity for

binding oestradiol is of a finite magnitude and a plateau is reached both *in vivo* and *in vitro*. The probable concentration of the total binding sites in the ovariectomized-rat uterus at a steroid concentration of 50 mμM appears to be about 1 mμM as deduced from the experiments *in vitro*.

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