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ESTRADIOL BINDING SERUM PROTEINS IN NORMAL AND IMMUNIZED RABBITS AND THEIR PHYSIOLOGICAL SIGNIFICANCE

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SUMMARY

Characterization of serum proteins, which selectively bind estradiol for the regulation of its effective concentration, showed that the binding proteins differ in the normal and estradiol immunized rabbits. The estradiol binding protein in the control serum had a molecular weight of 65,000; Stokes radius 31 Å; frictional ratio 1·28 and sedimentation coefficient of 4·6 S while that in the immune serum had a molecular weight of 160,000; Stokes value 54 Å; frictional ratio 1·26 and sedimentation constant of 6·9 S. Binding affinity and binding capacity of the binding proteins in the sera of control and immunized rabbits showed that the former is a low affinity and high capacity protein with an association constant of $1{\cdot}67 \times 10^6$ M^{-1} and the number of binding sites $1{\cdot}125 \times 10^{-5}$ mol/ml serum, while the latter had an association constant of $1{\cdot}56 \times 10^9$ M^{-1} with 3×10^{-9} mol/ml serum binding sites. The difference in the nature of binding proteins was further authenticated by their relative mobilities on electrophoresis. The data suggested that estradiol binding serum proteins in the immunized rabbit are different from those in the control and this, in turn, has marked implications for the availability of hormone at the target site.

INTRODUCTION

Steroid hormone binding to serum proteins has been extensively studied in relation to the transport of these hormones, their availability and interaction with the receptors in the target organs, and their activity in the bound state. For many years, the concept of "vehicle function" of the plasma proteins [1] has prevailed and protein binding in blood has been considered just a mechanism of transport. Since the solubilization of plasma steroids at physiological concentration does not necessitate the presence of proteins, it seems that the steroid binding to serum proteins is not a pre-requisite for estrogen action *per se* [2]. It is also evident that unbound or free hormone in plasma is biologically active [3–5] and even under certain conditions the free mobility of the steroid is optimum in the absence of the binding proteins. Therefore, binding of steroid hormones to serum protein constitutes a regulatory function providing a 'buffer' system to control the chemical and biological activity *i.e.* the effective concentration of the free hormone. The presence of the specific sex steroid binding globulin [6] and corticosteroid binding globulin [7] may be cited as examples for such hormone regulatory systems in circulation. These proteins, although present in low concentrations in the plasma, have high affinity for the circulating steroids. Yet another uniquely adapted high affinity binding protein is the immuno-protein with very high ligand specificity. Even at steroid concentrations as low as or below 3×10^{-6} M these proteins show marked steroid specificity to the high affinity binding sites [8]. Since the relative strength of binding of steroid hormones to serum proteins has a regulatory influence on the availability of the hormone to the target tissues and since immunoproteins are better as models for carrier proteins of plasma, the as yet unknown effect of active immunization with estradiol on the transport mechanism and availability of hormone to the target site, required for its biological activity, has been thoroughly investigated.

MATERIALS AND METHODS

Animals. Adult female rabbits weighing 2·5 to 3·5 kg, were purchased from Haffkine Institute, Bombay and were maintained on Hind-Lever pellet diet supplemented with fresh greens and water *ad libitum*.

Chemicals. The antigen, estradiol-17β succinyl bovine serum albumin (E_2-BSA) was a gift from Cancer Chemotherapy National Service Centre, NIH, U.S.A. [6,7-^{3}H]-estradiol-17β (S.A. 0·0067 mg/mCi) was purchased from New England Nuclear, Boston and checked for its radiopurity by thin-layer chromatography in benzene:methanol (9:1) system before use. Sephadex G-200 and Dextran blue 2000 were obtained from Pharmacia Fine Chemicals, Sweden. Non-enzymatic protein markers were obtained from Mann Research Laboratories.

Immunization protocol

Rabbits were injected subcutaneously with 8 weekly injections of the antigen E_2-BSA (1 mg) emulsified in 2 ml of 1:1 mixture of saline and Freund's adjuvant. The animals were bled 10–12 days after the booster injection, (0·5 mg antigen in saline alone).

Counting of radioactivity

Radioactivity was measured in a Liquid Scintillation spectrometer Packard Tricarb Model 3314 at a tritium efficiency of about 35%. Samples were counted using 10 ml of toluene based scintillation liquid (2,5-diphenyloxazole (PPO), 4 g; 1,4 bis-2-(4-methyl-5-phenyloxazolyl)-benzene, (dimethyl POPOP), 100 mg; in 1 litre of distilled toluene). Aqueous samples were counted in 15 ml of diotol scintillation liquid (PPO, 3·2 g; dioxan, 250 ml; toluene 250 ml and methanol, 150 ml, POPOP, 65 mg; naphthalene 52 g).

In vivo *studies*

50 μCi tritiated estradiol in 2 ml of normal saline was injected to the control and immunized animals through the marginal ear vein. One hour after injection the blood was collected and processed.

In vitro *studies*

Serum samples (3 ml) from both the control and immunized animals were separately incubated with 1 μCi of [^{3}H]-estradiol for 1 h at 37°C in a Dubnoff metabolic incubator with constant shaking.

Sephadex G-200 chromatography of serum proteins

The columns (2·5 × 50 cm.) were equilibrated with 0·01 M Tris–HCl buffer pH 7·4 at 4°C. The void volume of the column was checked using Dextran blue-2000 and then calibrated with non-enzymatic protein markers—γ globulin, BSA, chymotrypsinogen, cytochrome-C and ovalbumin. The peak elution volumes of these proteins were determined spectrophotometrically in a Zeiss PMQ II Spectrophotometer. Incubated serum samples or serum from estradiol injected animals were applied on these calibrated columns. Fractions of equal volume were collected and the absorbance at 280 nm and radioactivity of each fraction were measured.

Calculation of molecular weight, Stokes radius and frictional ratio of estradiol binding proteins (EBP)

The respective elution volumes of the non-enzymatic protein markers used to calibrate the column were plotted against their respective molecular weights. From the standard curve thus obtained, the molecular weight of EBP was determined according to the method of Andrews[9]. Molecular Stokes radius and frictional ratio of the binding protein were calculated as described by Laurent and Killander[10].

Sucrose density gradient centrifugation analysis

Linear 5–20% sucrose gradients (4·8 ml) in 0·01 M Tris–HCl 0·001 M EDTA buffer, pH 7·4, were prepared in 5 ml cellulose nitrate tubes using a Beckman density gradient former and stored at 4°C for at least 12 h prior to use. Aliquots (0·2 ml) of 1:10 serum previously incubated with [^{3}H]-estradiol were layered on the surface of the gradient. Centrifugation was carried out at 48,000 rev./min for 12 h at 2–4°C using a Beckman Ultracentrifuge Model L with SW 50 rotor. At the end of the run, 2 drop fractions were collected directly into the counting vials by puncturing the bottom of the tube. Sedimentation coefficients of estradiol binding components were estimated according to the method of Martin and Ames[11] using BSA (4·6 S) and gamma globulin (7 S) as standards.

Paper electrophoresis

Control and immune sera (0·5 ml), preincubated at 37°C with [^{3}H]-estradiol, were subjected to electrophoresis on Whatman No. 1 filter paper as described by Rosenbaum, Christy and Kelly[15]. The mixture (200 μl) was streaked on the paper about 2 cm cathodal to the midline. Electrophoresis was carried out at 20°C for 17 h in 0·055 M glycine acetate buffer pH 8·6 with an applied voltage of 160 Volts. At the end of electrophoresis, the paper strips were removed, air dried and scanned in a Packard Radio chromatogram Scanner Model 7200. Based on the radioactivity scan and a strip stained with bromophenol blue, 1 cm wide strips were cut at right angles to the direction of migration of proteins and counted directly in toluene based scintillator.

Equilibrium dialysis

Serum samples diluted with Tris–HCl buffer (1:10) were dialysed in 2 cm wide dialysis tubing (Union Carbide Corp.) against 10 ml of the same buffer containing different amounts of radioinert estradiol and 20,000 c.p.m. of [^{3}H]-estradiol. Dialysis was carried out by continuous rotation of the bag for 48 h at 4°C. Duplicate aliquots of 0·5 ml were taken from inside and outside the bag for the determination of radioactivity. The percent binding of estradiol to protein was calculated by the method of Slaunwhite and Sandberg[12].

The data were expressed as a Scatchard plot by plotting the ratio of bound/unbound on the ordinate against the concentration of bound estradiol on the abscissa [13]. The association constant (Ka), the dissociation constant (Kd), and the number of binding sites (n) were calculated.

Ligand specificity

Competition of various neutral and phenolic steroids with estrogen binding sites in 10-fold diluted control and immune sera was studied by the method of equilibrium dialysis. Radioinert competitor (100 nM) was added to the tracer concentration of [6-7,^{3}H]-estradiol and the competition efficiency was determined [14].

Enzymatic studies

Samples of diluted control and immune sera (1:10 v/v), previously equilibrated with [6-7,^{3}H]-estradiol, were incubated with 650 μg/ml of either trypsin, deoxyribonuclease or ribonuclease for 0·5 h. An aliquot (0·2 ml) of each sample was then analyzed on a sucrose density gradient as described earlier.

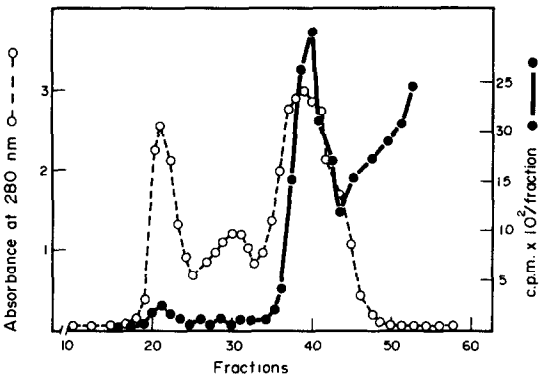


Fig. 1. Sephadex G-200 chromatography of control rabbit serum, obtained 1 h after the intravenous administration of [6,7-^{3}H]-estradiol. The sample was eluted from the column (2·5 × 50 cm.) with Tris–HCl buffer, pH 7·4 at 4°C. Radioactivity was determined in 0·5 ml aliquot of each fraction (3·4 ml) and then monitored for O.D. at 280 nm.

RESULTS

Analysis of estradiol binding serum proteins by Sephadex G-200 chromatography

Sephadex G-200 characterized by low degree of cross linking has been employed for the fractionation of serum proteins. On subjecting the sera of control and immunized animals, obtained 1 h after i.v. injection of [^{3}H]-estradiol, to Sephadex chromatography, it was seen that the proteins of each were resolved into three major 280 nm peaks (Figs. 1 and 2). However, estradiol and/or its metabolites were associated with the third protein peak of the control serum and with the second elution peak of the immune serum.

A similar chromatographic pattern of estradiol binding to serum proteins emerged after *in vitro* incubation with [6-7,^{3}H]-estradiol.

The amount of bound steroid calculated in each protein peak per unit optical density showed that the maximum steroid was associated with the second protein peak of the immune serum (0·5 pg) and with the third peak (0·33 pg) of the control serum.

Chemical identification of bound steroid

From 50 to 70% of the radioactivity present in the third and second protein peaks of the organic extracts

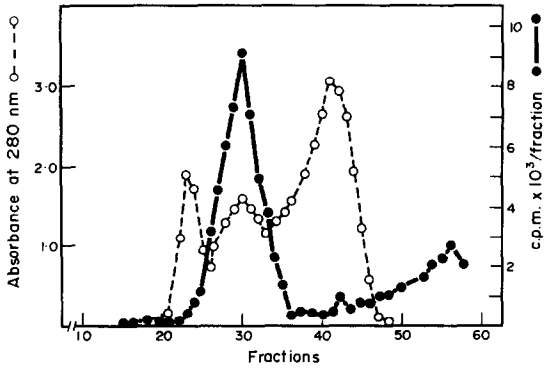


Fig. 2. Chromatographic behaviour of estradiol binding proteins of immune serum on Sephadex G-200 after injection of [^{3}H]-estradiol. Fractions of 3·1 ml each were collected and checked for absorbance at 280 nm and radioactivity.

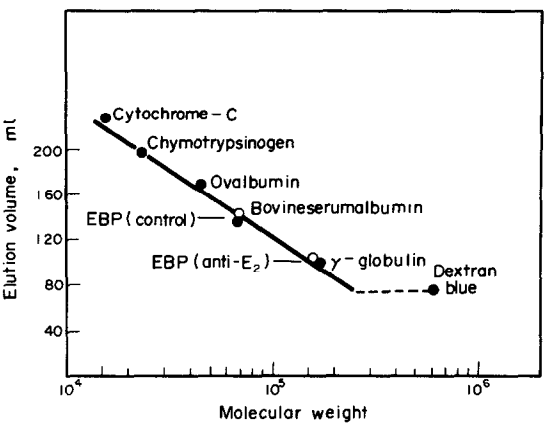


Fig. 3. Calibration curve of Sephadex G-200 column for measurement of molecular weights of control and immune EBPs. Blue dextran 2000 was chromatographed separately, while other protein markers, γ globulin, BSA, ovalbumin, chymotrypsinogen and cytochrome-C were chromatographed in a single run. Peak elution volumes were measured for each marker and plotted against their respective molecular weights.

of the control and immune sera respectively was identified as estradiol. The major part of the remaining radioactivity moved with authentic estrone on t.l.c. in benzene:methanol system. No estriol was detected.

Molecular parameters of control and immune EBPs

The molecular weights of the EBPs calculated from the calibration curve on the basis of their elution volumes gave values of 65,000 and 160,000 for the control and immune sera respectively (Fig. 3). Molecular Stokes radius was calculated from the distribution coefficients of these binding proteins. The distribution coefficients $(kd)^{1/3}$ of standard protein markers used to calibrate the column were plotted as a function of the Stokes radius. The Stokes values of EBPs, derived by interpolation of their respective $(kd)^{1/3}$, of

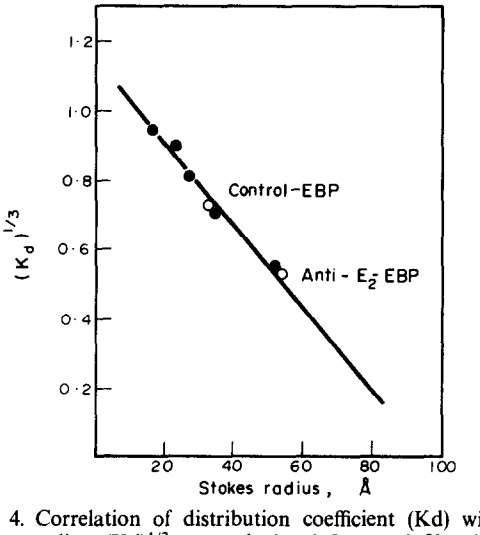


Fig. 4. Correlation of distribution coefficient (Kd) with Stokes radius. $(Kd)^{1/3}$ was calculated from gel filtration data resulting from chromatography as shown in Fig. 3. Stokes radius of respective proteins was taken from the literature. Molecular radius of control and immune EBPs were derived by interpolation of respective $(Kd)^{1/3}$.

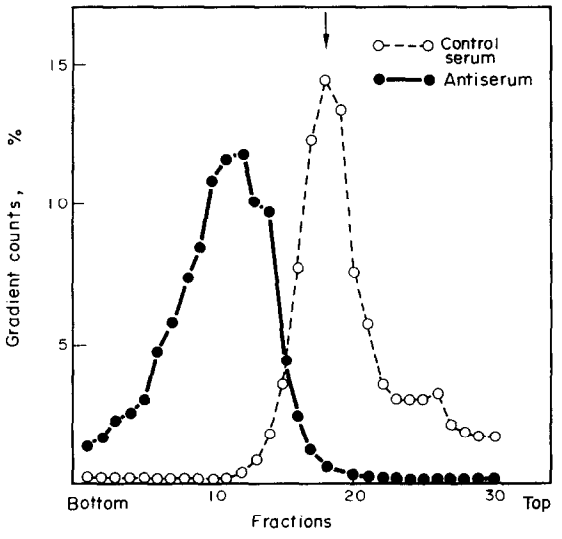


Fig. 5. Sucrose density gradient sedimentation profile of estradiol binding components, of control and immune sera, incubated with [6-7,^{3}H]-estradiol at 37°C for 1 h. The free radioactivity had been absorbed with Dextran-coated charcoal pellet prior to application. Standard reference in each run was a mixture of BSA and γ globulin.

control and immune sera were 31 Å and 54 Å respectively (Fig. 4). The frictional ratios of the EBPs were found to be 1·28 and 1·26 for control and immune sera respectively.

Sucrose gradient sedimentation of EBPs

The density gradient sedimentation profile of 0·2 ml aliquots of incubated control and immune sera is shown in Fig. 5. The sedimentation coefficients were calculated to be 4·6 ± 0·07 S (6 independent runs) for the control EBP and 6·9 ± 0·05 for the immune EBP.

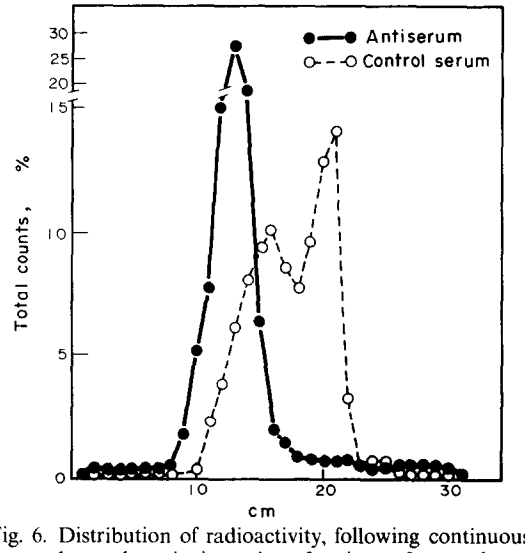


Fig. 6. Distribution of radioactivity, following continuous paper electrophoresis, in various fractions of control and immune sera. The amount of radioactivity in each fraction was expressed as a percent of the total counts eluted. The relative mobility (R_F) of the binding proteins was calculated in relation to the migration of bromophenol blue.

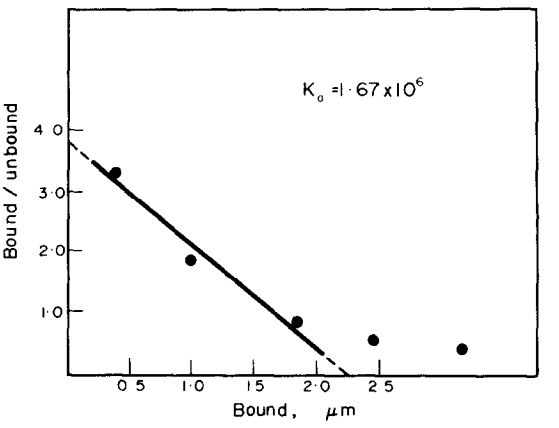


Fig. 7. Determination of the association constant (Ka), the dissociation constant (Kd) and the number of binding sites (n) of control serum. The data obtained by equilibrium dialysis of 1 : 10 diluted serum with increasing concentrations of estradiol were plotted according to Scatchard[13].

Paper electrophoresis

Figure 6 shows a comparison between the distribution of radioactivity following continuous paper electrophoresis of the sera of control and immunized rabbits incubated with the same amount of [6-7,^{3}H]-estradiol. The separation yielded two discernible radioactive peaks in the control—one moving fast with albumin and the other with relatively less mobility while there was only one peak in the immune serum corresponding to the mobility of gamma globulin in this system. The different estradiol binding proteins in the two sera had the relative mobilities of 0·70 (control serum II peak) and 0·43 (immune serum). However, the other estradiol bound protein peak in the control serum had a relative mobility of 0·53 between gamma globulin and albumin.

The binding affinity and the binding capacity of control and immune EBPs

Data on affinity for 17β-estradiol of 4·6 and 6·9 S proteins of control and immune sera calculated by

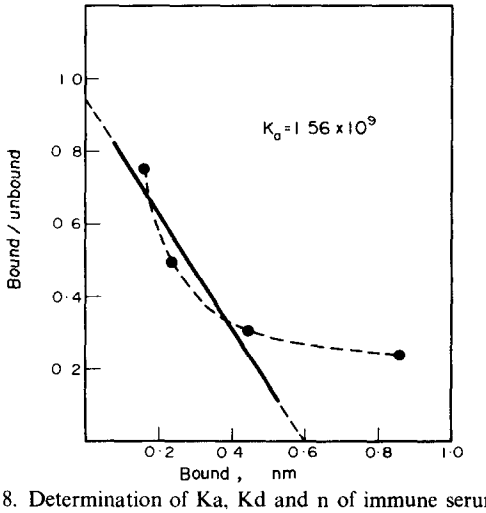


Fig. 8. Determination of Ka, Kd and n of immune serum EBP. Data were obtained by equilibrium dialysis at 4°C and plotted according to Scatchard. Ka was obtained by extrapolation of the straight intermediate segment to the coordinate axis.

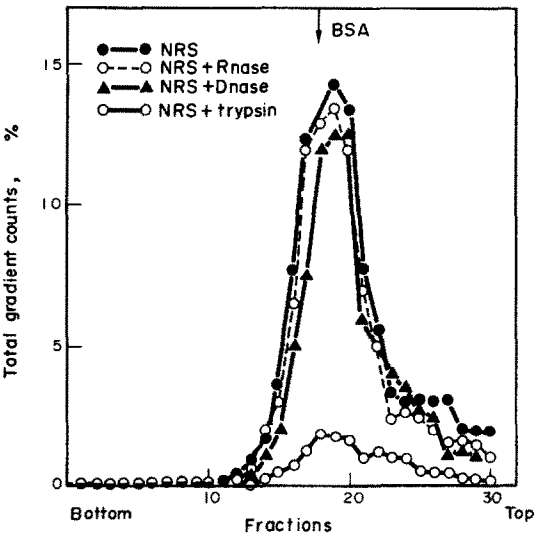
equilibrium dialysis method are presented according to Scatchard (Figs. 7 and 8). By extrapolation of the straight intermediate segments to the coordinate axis, a specific binding (high affinity) protein with association constant of $1{\cdot}56 \times 10^9$ M^{-1} for the immune serum and low affinity binding with association constant of $1{\cdot}67 \times 10^6$ M^{-1} for control serum were obtained. The number of binding sites in the specific binder of the immune serum was calculated from the plot and found to be 3×10^{-6} M while in the low affinity binder it was found to be $1{\cdot}125 \times 10^{-2}$ M serum. The dissociation constants as calculated from the Scatchard plot were found to be $0{\cdot}64 \times 10^{-9}$ M and $0{\cdot}59 \times 10^{-6}$ M for the immune and control sera respectively.

Ligand specificity of EBP in control and immune sera

Competition for EBP (control and immune) by neutral and phenolic steroids at a concentration of 100 nM, determined by the method of equilibrium dialysis, has been presented in Table 1. From the table, it appears that the immune EBP was highly specific for estradiol and estrone, while the other steroids showed negligible competition. In the case of control EBP, the steroids estradiol, estrone, testosterone, estriol, androstenedione and progesterone, compete in decreasing order. The synthetic estrogen, diethylstilbestrol, did not compete with either the control or the immune EBP. However, testosterone showed competition for the binding sites up to 47% in the control serum only.

Nature of the binder

Control and immune sera, equilibrated with [^{3}H]-estradiol and incubated with trypsin, showed displacement of radioactivity from 4·6 and 6·9 S regions, respectively, to the top of the gradient (Figs. 9 and 10). Comparable treatment of serum samples with either deoxyribonuclease or ribonuclease had no effect on the sedimentation pattern of EBPS, thus signifying the protein nature of the binders.



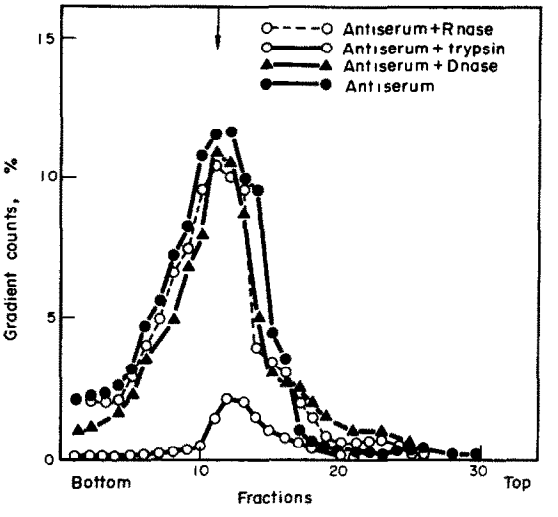


Fig. 9 and 10. Effect of enzyme treatment on sedimentation profiles of control and immune serum EBPs. Serum samples (1:10) were equilibrated with [6-7,^{3}H]-estradiol and then incubated with either trypsin, deoxyribonuclease (DNase) or ribonuclease (RNase), prior to centrifugation.

DISCUSSION

The results obtained in this investigation suggest that the facilitation and/or blockade of the estradiol-induced biological response in the target tissues is dependent on the availability of the free steroid in circulation. Further analysis of such a situation revealed that this is, in turn, dependent on the relationship between the concentration of the ligand, the concentration of the binding sites and the association constant. The presence of almost 100% of serum estradiol in the bound form and a very high association constant of the order of $1{\cdot}56 \times 10^9$ M^{-1} in estradiol-immunized rabbits strongly suggest that the unbound or in other words the effective concentration of estradiol is available in negligible amounts to the estradiol sensitive receptor cells in target tissues for the manifestation of biological response. That the concentration of the free steroid in circulation determines the biological responsiveness has further been confirmed in the control rabbit in which 15 to 20 per cent of estradiol in the free form has been detected with an association constant of $1{\cdot}68 \times 10^6$ M^{-1}.

Table 1. Ligand specificity of estradiol binding proteins in control and immune sera of rabbits

Unlabelled steroid (100 nM)	Control		Immune	
	Percent bound	Competition efficiency	Percent bound	Competition efficiency
—	80·8	—	98·2	—
Estradiol	38·5	100·0	23·4	100·0
Estrone	45·7	82·9	35·6	83·9
Estriol	65·3	36·6	84·2	18·7
Testosterone	60·8	47·0	96·3	2·4
Androstenedione	76·7	9·6	95·2	4·1
Progesterone	79·2	3·7	97·2	1·3
Diethylstilbestrol	80·2	1·4	98·0	0·3

The determination of physico-chemical characteristics of the high and low affinity EBPs facilitates the understanding of the physiological significance of these associations in the immune and control sera respectively. Characterization of control and immune binding components by enzymatic hydrolysis suggested that both the binders are protein in nature. However, other molecular parameters suggested that

although being protein in nature, the two binders differ physically. The control EBP was eluted with the third 280 nm peak of Sephadex G-200, representing mainly the bulk of serum albumin, with molecular weight of 65,000; molecular Stokes radius 31 Å; frictional ratio 1·28 and sedimentation coefficient of 4·6 S. On the other hand, immune EBP, eluted as the second peak, had a molecular weight of 160,000; Stokes value 54 Å; frictional ratio 1·26 and sedimentation coefficient of 6·9 S.

The ligand requirements for both the control and immune rabbit sera suggested that the structural integrity of the entire molecule of estradiol or estrone is essential for binding activity and that any modification in ring A structure of the phenolic steroid would prevent binding to the proteins. However, a competition efficiency of about 47% by testosterone suggested that common binding sites may be involved in the control serum.

The difference in the nature of the binding proteins has further been authenticated by their relative mobilities on electrophoresis (Fig. 6). While all other techniques employed to characterize the binding proteins showed the presence of a single binding component in the control serum, paper electrophoresis demonstrated two discernible radioactive peaks with relative mobilities of 0·73 and 0·53, although the bulk of radioactivity was associated with the protein having mobility similar to that of albumin. The binding protein with relative mobility of 0·53 could be due to the alpha and/or beta globulin of serum. Barlow, Maclaren and Pothier[16] demonstrated a similar binding of estradiol in this region on paper electrophoresis in the pregnant and non-pregnant human serum and characterized the peaks as alpha and beta globulins. Thus, this protein might also be contributing towards the relatively high association constant of control EBP (10^6 M^{-1}) than that of serum albumin alone ($K_a = 10^5$ M^{-1}) [17]. In the immune serum the EBP had a relative mobility of 0·43, which is similar to that of γ globulin.

Thus these molecular parameters are suggestive of the fact that the estrogen binding component in the immune serum has many of the characteristics of γ globulin and in the control of albumin. It, therefore, seems likely that the differences in the biological response in the immunized and non-immunized animals [18] may be due to the differences between the binding affinity and binding capacity of the binding proteins. The high dissociation constant, $0{\cdot}59 \times 10^{-6}$ M, and the manifestation of biological response in the target tissue of the control rabbit indicates that the complex dissociates easily in circulation and thus does not interfere with the biological activity of the steroid while binding to high affinity immunoproteins with low dissociation constant ($0{\cdot}64 \times 10^{-9}$ M) does not render the hormone free for its action at the target site.

Experimental support for this interpretation has been provided by the recent findings of Nieschlag *et al.*[19] and Sundaram *et al.*[20] who showed that the active immunization of male rabbits with testosterone and female rhesus monkeys with estrone abolished the biological effects of testosterone on Leydig cells and estrogen on ovarian function, respectively. These have been attributed to the increased levels of bound hormones in circulation due to the presence of high affinity immunoproteins, thereby decreasing the amount of hormone rendered available to the sensitive receptors. The increased levels of testosterone during pregnancy without androgenizing effects has also been explained on the basis of increased serum binding capacity thereby preventing the active androgenic steroid from acting on the normally sensitive genital tissues [21].

Thus our data on the inhibition of ovum implantation in actively immunized rats and rabbits [22] suggest that the manifestation of estrogen action is the result of an interplay of hormone production and its binding to serum proteins which in turn, regulates the effective concentration and the availability of the hormone to the target organ.

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REFERENCES

1. Bennhold H.: *Ergeb. inn. Med. U. Kinderheilk.* **42** (1932) 273 (cited from ref. 3 below).
2. Soloff M. S., Creange J. E. and Potts G. O.: *Endocrinology* **88** (1971) 427–432.
3. Westphal U.: In *Mechanism of Action of Steroid Hormones* (Edited by C. A. Villee and L. L. Engel). Pergamon Press (1961) pp. 33–89.
4. Slaunwhite W. R., Lockie G. N., Back N. and Sandberg A. A.: *Science* **135** (1962) 1062–1063.
5. Vermeulen A., Stoica T. and Verdonck L.: *J. clin. Endocr. Metab.* **33** (1971) 759–767.
6. Mercier-Bodard C., Alfsen A. and Baulieu E. E.: *Acta endocr., Copenh.* suppl. 147, **64** (1970) 204–224.
7. Hoffmann W., Forbes T. R. and Westphal U.: *Endocrinology* **85** (1969) 778–781.
8. Zimmering P. E., Lieberman S. and Erlanger B. F.: *Biochemistry* **6** (1967) 154–164.
9. Andrews P.: *Biochem. J.* **91** (1964) 222–233.
10. Laurent T. C. and Killander J.: *J. Chromatog.* **14** (1964) 317–330.
11. Martin R. G. and Ames B. N.: *J. biol. Chem.* **236** (1961) 1372–1379.
12. Slaunwhite W. R. and Sandberg A. A.: *J. clin. Invest.* **38** (1959) 384–391.
13. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
14. Milgrom E., Atger M. and Baulieu E. E.: *Steroids* **16** (1970) 741–754.
15. Rosenbaum W., Christy N. P. and Kelley W. C.: *J. clin. Endocr. Metab.* **26** (1966) 1399–1403.
16. Barlow J. J., MacLaren J. A. and Pothier L.: *J. clin. Endocr. Metab.* **29** (1969) 767–776.
17. Sandberg A. A. and Slaunwhite W. R.: *J. clin. Invest.* **36** (1957) 1266–1278.
18. Bhalla V. and Farooq A.: *Ind. J. physiol. Pharmac.* **15** (1971) 20.
19. Nieschlag E., Usadel K. H., Schwedes U., Kley H. K., Schoffling K. and Kruskember H. L.: *Endocrinology* **92** (1973) 1142–1147.
20. Sundaram K., Tsong Y. Y., Hood W. and Brinson A.: *Endocrinology* **93** (1973) 843–847.
21. Rivarola M. A., Forest M. G. and Migeon C. J.: *J. clin. Endocr. Metab.* **28** (1968) 34–40.
22. Bhalla V.: Ph.D. Thesis. All India Institute of Medical Sciences, New Delhi (1973).