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THE BINDING OF ESTROGENS IN THE LIVER OF THE RAT: DEMONSTRATION AND ENDOCRINE INFLUENCES

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SUMMARY

The existence of an estrophilic macromolecule in the cytoplasm of rat liver is established, and some of its characteristics are examined by use of a Dextran-coated charcoal assay under conditions which minimize metabolism. The estrophile sediments at 4–5 *s* with aggregates of 8–9 *s* in either Tris–EDTA or Tris–magnesium buffer. The best resolution is achieved in magnesium buffers at high ionic strength. Estrogens bind to this protein with high association constants (K_a for estradiol $= 6.0 \times 10^9\ M^{-1}$) whereas androgens and glucocorticoids bind much less firmly. In experiments designed to examine the effect of various hormonal manipulations on the concentration of this estrophile in liver, the following observations were made: (1) There is no effect of 5 μg estradiol-benzoate in intact females; (2) Ovariectomy causes a 2–3 fold increase in capacity which decreases significantly, but not to control values, after the administration of 5 μg estradiol-benzoate; (3) Hypophysectomy causes a 5-fold decrease in capacity.

INTRODUCTION

The retention by the uterus and vagina of estradiol was first reported by Jensen *et al.* in 1962 [1]. After the administration of physiologic amounts of tritiated estradiol* to young rats, the retention of radioactivity by the liver was exceeded only by the uterus and vagina. That the liver responds to endogenous and exogenous estrogens has been documented [2]. There is a significant increase in liver wet-weight during pregnancy [3], an effect which is also seen after the administration of pharmacological doses of estrogens [4]. These observations coupled with the demonstrated lack of a specific binding protein for estrogens in adult rat plasma [5] have prompted a search for specific estrogen binding in rat liver. While this work was in progress, three reports appeared which were directed at the same question [6–8].

MATERIALS AND METHODS

Animals. Intact and hypophysectomized adult rats (CDF strain) were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. and maintained in a temperature-controlled environment with 12 h of light. Where indicated, animals were surgically altered two weeks prior to sacrifice.

Chemicals. [6,7-^{3}H] Estradiol-17β (44 Ci/mmol) was purchased from Amersham Searle and examined for purity bi-weekly with thin layer chromatography. Ribonuclease-free sucrose was purchased from Schwarz-Mann and the remaining chemicals from Fisher Scientific. Radioinert steroids were purchased from Steraloids or Research Plus and recrystallized when necessary.

Preparation of cytosol. Two buffers were used in this study, buffer A (50 mM Tris, 25 mM KCl, 2 mM Mg^{++}, pH 7.5) or buffer B (10 mM Tris, 1.5 mM EDTA, pH 7.5). Rats were killed by decapitation and the livers were blotted dry, weighed, and minced in the appropriate ice-cold buffer containing 0.5 M sucrose. After homogenization (Potter Elvehjem homogenizer with Teflon pestle) the crude preparation was centrifuged at 4000 *g* for 10 min and the supernate was stirred with dry prewashed Norite A (5 mg/ml) for 10 min at 4° in order to remove free steroids. After an additional centrifugation at 105,000 *g* for 45 min, the supernate was filtered quickly through glass filter disks (Whatman GF/C) under mild vacuum. A 0–40% ammonium sulfate fraction of the filtrate was prepared at 4° by the slow addition, with stirring, of solid ammonium sulfate. After 10–15 min, the precipitate, containing 46–49% of the estrogen binding protein activity and 18–24% of the cytosol protein, was collected by centrifugation at 12,000 *g* for 10 min, dissolved in a small vol. of buffer and dialysed for one h against four, 250 ml vol of the appropriate buffer. The small precipitate which formed during dialysis was removed by centrifugation at 12,000 *g* for 10 min and the supernate was filtered again and kept at 4°. The protein concentration of this final filtrate was determined using BSA as standard [9].

* The following trivial names are used in this paper: Estradiol refers to 17β-estradiol; estradiol-benzoate, 1, 3, 5(10)-estratrien-3, 17β-diol 3-benzoate; diethylstilbestrol, 3, 4-Bis (*p*-hydroxyphenyl)-3-hexene; BSA, bovine serum albumin.

Assay for estrogen binding. Estrogen binding capacity was determined in each sample using 2–4 × 10^{-8} M [^{3}H]-estradiol with the addition of unlabeled estradiol in four of the five incubation vials, one of which was used to determine non-specific binding. Bound steroid was separated from free using Dextran-coated Norite A (0.5% exhaustively washed Norite A suspended in the appropriate buffer containing 0.25 M sucrose and 0.1% Dextran at pH 7.5) in the following manner. Glass scintillation vials were placed on a platform shaker and 1.0 ml of the Norite suspension was added to each vial. One-quarter ml of the cytosol-steroid incubate was added in duplicate and the vials were shaken at 4° for 10 min (150 strokes/min). The entire charcoal–cytosol suspension from each vial was transferred to Pyrex test tubes (10 × 75 mm) and centrifuged at 4000 *g* for 3 min. One-half ml from each tube was counted in 10 ml of a xylene-based fluor (10.7 g butyl-PBD, Packard, dissolved in 2800 ml xylene containing 25% Triton X-114) at an efficiency of 45%.

Sucrose gradients. Cytosol, 0.5 ml, containing [^{3}H]-estradiol (3 × 10^{-8} M), plus or minus unlabeled estradiol, was incubated for two h at 4° after which the contents of each reaction vial were transferred to a pellet of Dextran-coated Norite A obtained by centrifugation of 1 ml of 0.5% Norite suspension. After 10 min, the Norite was removed by centrifugation and 0.25 ml of the clear supernate was transferred to 5–40% linear sucrose gradients prepared in the appropriate buffer. [^{14}C]-BSA and [^{14}C]-catalase, prepared by the method of Rice and Means [10], were added as markers and the gradients were centrifuged at 226,600 *g* for 16–18 h at 4° in a Beckman L5-50 ultracentrifuge. The gradients were collected in 5 drop fractions and counted in 10 ml of Xylene-based fluor.

Analysis of metabolic products. [^{3}H]-Estradiol, 2.4 × 10^{-8} M, (2.34 × 10^6 d.p.m./ml) was incubated with 1.0 ml cytosol for two or twenty h at 4° after which bound steroid was separated from free on 1.0 × 24 cm columns of Sephadex G-25. After addition of [^{14}C]-estradiol to determine recoveries, the bound fraction was extracted with five vol. of methanol and the precipitate which formed was washed twice with 5 ml of methanol. The methanol extracts were combined, evaporated and the residue was taken up in 10 ml of ether and washed three times with 5 ml of water. The ether was evaporated under a stream of nitrogen and chromatographed on Sephadex LH-20 columns, in benzene methanol: 85:15 v/v [11]. After addition of [^{14}C]-estradiol, the free fraction, obtained from Sephadex G-25 columns, was extracted twice with an equal vol. of ether. The ether extracts were combined, evaporated, and chromatographed on Sephadex LH-20, where a symmetrical peak of tritium, which co-chromatographed precisely with [^{14}C]-estradiol, was found. Estradiol, 14 mg, was added and the mixture was recrystallized from benzene methanol. The S.A. of the mixture was the same before and after crystallization. Recoveries of the [^{14}C]-estradiol were 92–100% for both the bound and free steroid.

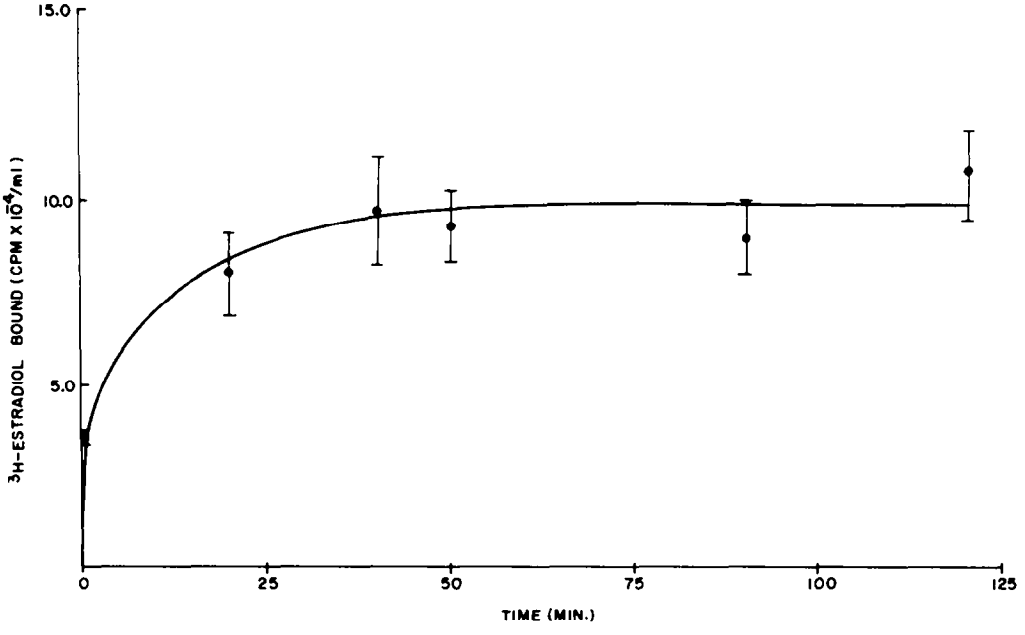

Fig. 1. Time course of specific estrogen binding in the cytosol of rat liver. The 0–40% ammonium sulfate fraction of liver cytosol, adjusted to a protein concentration of 10 mg/ml, was incubated at 4° with [^{3}H]-estradiol (10^{-8} M) in the presence and absence of diethylstilbestrol (10^{-5} M). Specific binding was determined as the difference between total binding and binding in the presence of DES. Each point is the mean (± S.E.M.) of triplicate measurements.

RESULTS

Assay conditions. The presence of enzymes in liver cytosol which metabolize estradiol complicates the problem of assaying hepatic proteins which bind this steroid. In order to minimize this complication, these studies were carried out at a relatively high concentration of estradiol thus minimizing the time for the binding reaction to reach equilibrium. The kinetics of binding of [^{3}H]-estradiol (10^{-8} M) at 4° are presented in Fig. 1. Under these conditions equilibrium was achieved in 1–2 h at 4°. In separate experiments, [^{3}H]-estradiol (10^{-8} M) was incubated with cytosol from intact female rats at 4° for 2 and 20 h. The bound and free fractions, separated using Sephadex G-25, were analyzed on Sephadex LH-20 as outlined in the Methods. Results of these experiments are presented in Table 1. One-half of the free estradiol was metabolized in about 2 h whereas it took about 20 h at 4° to metabolize 50% of the bound fraction.

Table 1. Metabolism of [^{3}H]-estradiol at 4° in the cytosol fraction of rat liver

Compound	G-25 Sephadex fraction	Percent of total radioactivity 2 h	20 h
17β-estradiol	Bound	81.7 ± 1.1	47.0 ± 6.4
	Free	53.8 ± 0.65	29.6 ± 1.2

[^{3}H]-estradiol, 2.4×10^{-8} M (2.34×10^6 d.p.m.), was incubated at 4° in 1.0 ml of a 0–40% ammonium sulfate fraction of cytosol protein from intact female rats (10 mg/ml) for 2 and 20 h. Bound radioactivity was separated from free on Sephadex G-25. Results are expressed as the mean percent ± S.E.M. relative to the radioactivity added to the ammonium sulfate precipitate of the cytosol.

Determination of the association constant for estradiol at 4°

A range of [^{3}H]-estradiol concentrations from 1×10^{-10} M to 1×10^{-8} M was used to obtain an association constant. In order to attain equilibrium at the lower concentrations, incubations were carried out for 20 h at 4° before separating bound from free. Both bound and free fractions were corrected for metabolism (see Table 1) and the association constants and capacities determined, Fig. 2.

Determination of binding specificity

These studies were conducted by adding, in separate tubes, fixed amounts of cytosol to a standard saturating concentration of [^{3}H]-estradiol and varying amounts of competitor. The association constants for the competitors were obtained from the relationship:

$$K_c = K_{E_2} \times (B/F)_c \times (F/B)_{E_2} \quad [12]$$

where: K = association constant, E_2 = estradiol, c = competitor. The concentrations of free and bound estradiol were determined by the Dextran-coated

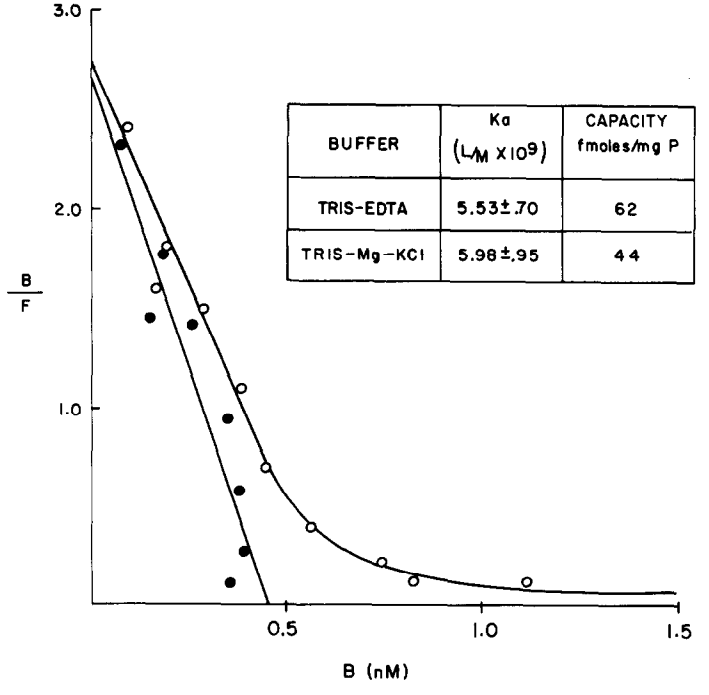

Fig. 2. Determination of the association constant and capacity of liver cytosol from 17β-estradiol using two different buffer systems. Nine concentrations of [^{3}H]-estradiol over a 100-fold range (0.3×10^{-9}–30×10^{-9} M) were incubated at 4° for 20 h. B/F values were calculated after correcting for metabolism in accordance with Table 1. The initial curvilinear relationship (O——O) between B/F and B was corrected according to Chamness and McGuire[22] (●——●) and the K_a and capacity were derived from the slope and intercept of the straight line. The slopes ($-K_a$) ± S.E.M. and capacities for both buffers are shown in the insert. The curves are the ones obtained using cytosol from intact animals prepared in buffer A (Tris–Mg).

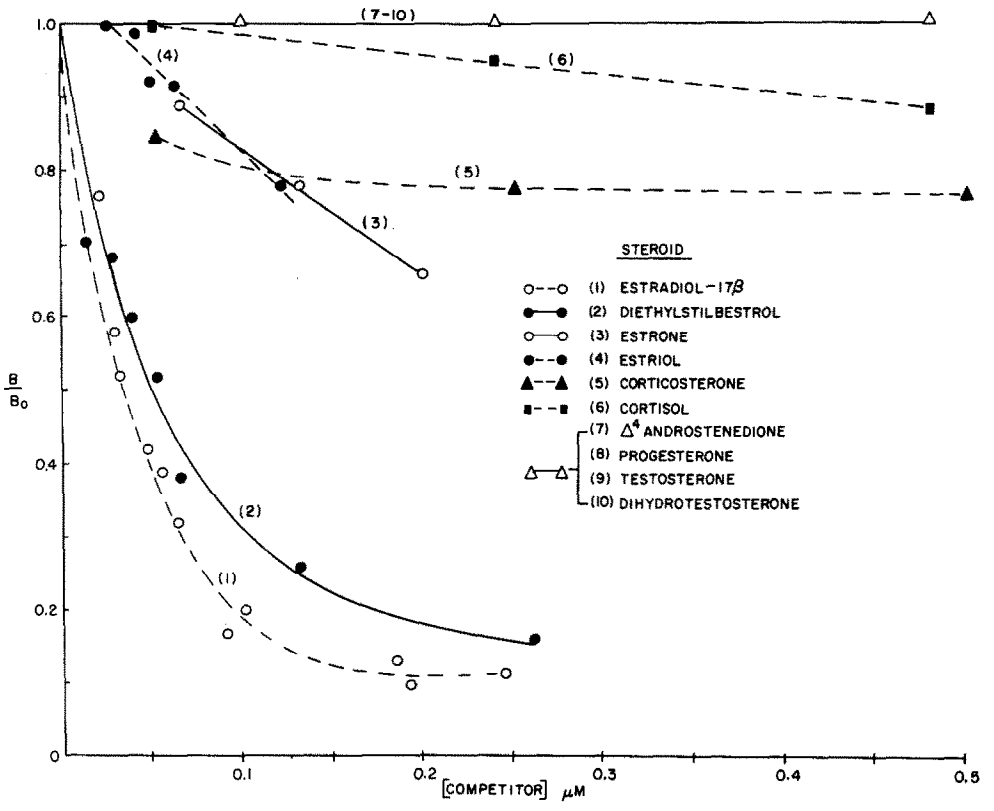

Fig. 3. Displacement curves of various steroids competing with [^{3}H]-estradiol for binding in liver cytosols. Aliquots (0.5 ml) of the 0–40% ammonium sulfate fraction of liver cytosol (10 mg/ml) were added to buffer A containing [^{3}H]-estradiol (10^{-8} M) alone or together with various concentrations of radioinert steroids. Binding was allowed to proceed at 4° for 2 h. Bound estradiol was separated from free using Dextran-coated charcoal as outlined in the methods. B/B_0 is the ratio of c.p.m./ml bound at each concentration of competitor divided by the c.p.m./ml bound with [^{3}H]-estradiol alone. B_0 values ranged from 4–6% of the total [^{3}H]-estradiol.

charcoal assay and the displacement curves are shown in Fig. 3. The concentration of bound competitor was assumed to be equal to the amount of [^{3}H]-estradiol displaced by a given concentration of competitor. The concentration of free competitor was obtained by subtraction. The association constant for estradiol used in the calculation is shown in the inset to Fig. 2. The association constants obtained at each concentration of competitor were averaged and are shown in Table 2.

Table 2. Association constants for the interaction of various steroids with the estrogen receptor protein in liver cytosol

Competitor	K_a	S.D.	Coefficient of variation (%)	Relative† K_a	Range of competitor concentration $M/L \times 10^{-9}$	(n)‡
17β-estradiol	5.98×10^9	9.48×10^8*	—	1.0	—	—
Diethylstilbestrol	2.01×10^9	2.18×10^8	11	0.34	65–262	3
Estrone	2.69×10^8	3.32×10^7	12	0.045	65–195	3
Estriol	1.12×10^8	3.79×10^7	34	0.019	49–610	4
Cortisol	1.82×10^7	6.68×10^6	37	0.0030	242–970	3
Androstenedione	2.09×10^7	7.37×10^6	35	0.0035	304–1220	3
Testosterone	Does not compete		—	—	61–1220	4
Dihydrotestosterone	Does not compete		—	—	60–1210	4
Progesterone	Does not compete		—		56–1120	4

* Standard error of the slope. †Relative $K_a = K_c/K_{E_2}$ ‡(n) is the number of values used to determine the association constant.

Aliquots (0.5 ml) of the 0–40% ammonium sulfate fraction of liver cytosol (10 mg/ml) were added to buffer A containing [^{3}H]-estradiol (10^{-8} M) alone or together with various concentrations of radioinert steroids. Binding was allowed to proceed at 4° for two h. Association constants for competitors were calculated as specified in the text.

Sedimentation characteristics of the estrogen binding proteins

Since some *in vivo* and *in vitro* studies require the isolation of intact nuclei, the effect of buffers containing cations required for nuclear isolation was examined. Ammonium sulfate fractions of liver cytosol, prepared in the presence and absence of magnesium, were examined on 5–40% linear sucrose gradients, Fig. 4. At low ionic strength, both buffers gave rise to sharp peaks in the 4–5 *s* and/or 7–8 *s* regions of the gradients. However, at high ionic strength there was marked aggregation in the magnesium-deficient buffer. These results were reproducible and independent of the amount of protein (1.4–2.0 mg) layered on the gradient (data not shown). Analysis of the radioactivity from the bound peak showed that 90% of it chromatographed with [^{14}C]-estradiol on Sephadex LH-20.

Effect of ovariectomy, hypophysectomy, and estradiol-benzoate on the binding capacity of the hepatic estrogen binding protein

Studies designed to examine the effect of some endocrine manipulations on the concentration of EBP were undertaken in a group of adult female rats. Experiments were begun 2 weeks after surgical removal of the appropriate gland. Completeness of ablation was confirmed in each case by visual inspection and by determination of uterine (saline injected, ovariectomized and hypophysectomized animals) and adrenal

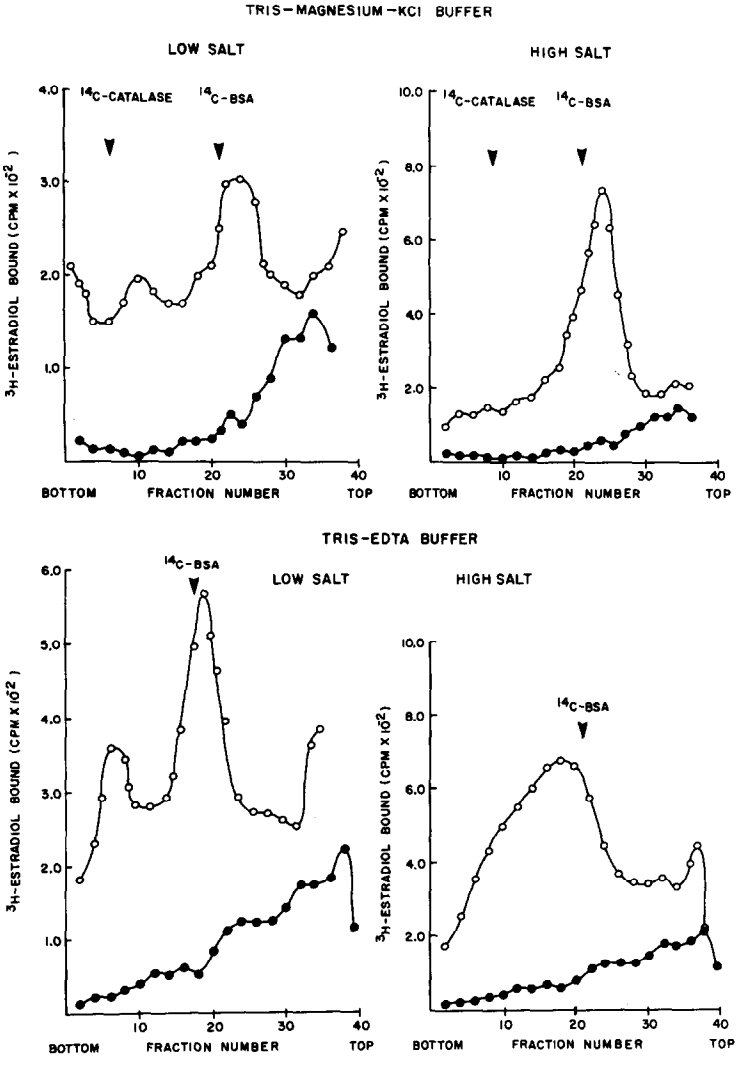

Fig. 4. Sucrose gradient centrifugation analysis of estrogen binding in rat liver cytosol. Ammonium sulfate fractions of liver cytosol (10 mg/ml) were prepared in each buffer and portions of each made 0.4 M in KCl (high salt). One-half ml of each preparation was incubated for 1–2 h with [^{3}H]-estradiol (10^{-8} M) with and without displacing amounts of radioinert estradiol. After unbound steroid was removed using DCC, aliquots, 0.2 ml containing 2 mg protein, were layered onto 5–40% linear sucrose gradients prepared in each buffer at low and high ionic strength (KCl, 0.4 M). After the addition of trace amounts of [^{14}C]-BSA (4.6 *s*) and [^{14}C]-catalase ~7.0 *s*), the gradients were centrifuged at 200,000 *g* for 16–18 h at 4° and analyzed as described in the Methods.

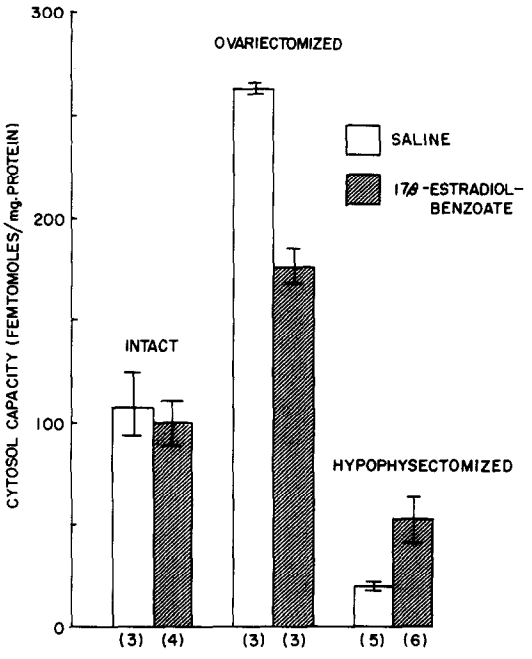

Fig. 5. Effects of estrogen administration on the concentration of estrogen binding sites in livers from intact, ovariectomized and hypophysectomized rats. Adult female rats were altered and injected as described in the text. Results are expressed per mg of protein in an ammonium sulfate precipitate of hepatic cytosol. Values shown are the mean ± S.E.M. The number of animals used to determine the values shown are in parentheses. The P values obtained using t-tests for these results are as follows. Animals given normal saline: intact (I) vs. ovariectomized (O) < 0.001; intact (I) vs. hypophysectomized (H) < 0.001; O vs. H < 0.001. Animals given 5 μg estradiol benzoate in saline (S.C.): I vs O < 0.001; I vs. H < 0.05; O vs. H < 0.001; O (saline) vs. O (estradiol) < 0.05; H (saline) vs. H (estradiol) $0.1 > P > 0.05$; I (saline) vs. I (estradiol) > 0.5.

weights (hypophysectomized animals). Daily subcutaneous injections, either 0.5 ml saline or 5 μg estradiol-benzoate in saline, were followed by sacrifice 18–20 h after the second injection. Ammonium sulfate fractions of hepatic cytosol were prepared in buffer A (Tris–Mg) as indicated in the text and legend to Fig. 4 but in this case incubation was with $2–4 \times 10^{-8}$ M [^{3}H]-estradiol to assure saturation of the binding sites. The results of these studies are presented in Fig. 5. There was no significant change in the receptor concentration of intact animals injected with estradiol. However, ovariectomy caused a two and one-half-fold increase in concentration which was partially reversed by administration of estrogen. Hypophysectomy caused the estrogen-binding concentration to decline to very low values. It is to be noted that these receptor concentrations were determined with cytosol which had undergone purification using ammonium sulfate. Although this caused a loss in total binding capacity, the specific binding (expressed as fmol/mg protein) increased approximately two-fold.

DISCUSSION

The data presented in this study show that the liver contains a protein which binds estradiol with specificity, binding affinity and sedimentation properties similar to the estrogen receptor identified in the uterus. For purposes of discussion, this protein will be called an estrogen receptor even though further experimentation is necessary in order to establish this unequivocally.

The use of low temperatures and short incubation times have kept metabolism to a minimum in this study. However, in spite of this, analysis of the free fraction revealed that nearly half of the [^{3}H]-estradiol had been metabolized in two h. In sharp contast to this observation, over 80% of the bound fraction was unaltered estradiol. It should be recalled that the bound tritium was obtained by gel filtration and that some of the non-estradiol tritium could have arisen from metabolites of estradiol binding to non-receptor protein. In addition, estrone binds fairly tightly to the receptor, $K_a = 2.7 \times 10^8\ M^{-1}$, and would be expected to be produced from estradiol and to compete for binding sites. These data suggest that in the liver, in addition to other functions, the receptor may serve to protect estradiol from enzymatic degradation.

It is apparent that in all the experiments reported in this paper there are two simultaneous reactions taking place; binding on the one hand and metabolism on the other. Therefore, the accurate determination of an association constant in liver presents some difficulties. At low concentrations of ligand, long incubation times are required for the binding reaction to come to equilibrium. We have used the metabolism data obtained at 10^{-8} M to correct for fractional metabolism at lower concentrations of added estradiol. It is certainly possible that we have underestimated the amount of metabolism taking place at the lower estradiol concentrations and to the extent that this is true there will be an error in both the association constant and capacity derived from this data. The linearity of the Scatchard plot speaks against this kind of error being excessive. However, it is because of this consideration that the capacities obtained using a two h incubation time are probably more accurate. All the data reported in this study, i.e., sucrose gradient and specificity experiments, were obtained using conditions in which the rate of binding greatly exceeded the rate of metabolism of the ligand.

When hepatic cytosol was prepared in buffers of low ionic strength and centrifuged in sucrose gradients of the same ionic strength, the predominant peak was 4–5 s with aggregates from 5–9 s. These patterns were independent of the presence or absence of magnesium and similar to those reported in uterus [13]. However, when these cytosols were adjusted to 0.4 M with respect to KCl and centrifuged on KCl containing gradients, the presence of magnesium prevented aggregation. Others have reported difficulty in obtaining satisfactory sedimentation patterns for estrogens in liver cytosol in the presence

of EDTA [7] and similar observations regarding the sedimentation behavior of receptors in KCl have been reported in the mammary gland [14]. However, in spite of this aggregation, the presence of magnesium did not affect the association constant (see inset to Fig. 2). The values for K_a reported here are in substantial agreement with those observed by others [6–8]. Magnesium has been retained in the buffers in this study in order to maintain nuclear integrity.

Data obtained from competition studies can be handled in many ways. In the past, this laboratory [15], along with others, has chosen to present competition data as suggested by Korenman[16]. His procedure involved experiments analogous to the ones used in this communication and the expression of relative binding affinities as the quotient: (mass of competitor required to displace 50% of the labeled steroid)/(mass of the unlabeled steroid required to displace 50% of the labeled steroid). Since the dose-response curves of competitors and the steroid to which they are compared are often not parallel (Fig. 3), the reader has to see the entire range of data in order to assess properly the significance of relative binding affinities calculated in this way. In order to communicate non-parallelism numerically, we have chosen to use the equation given in the text. Edsall and Wyman[12] have pointed out that, if the association constants obtained at widely different concentrations of competitor are the same, then there is substantial evidence that the competitor is binding at the same site as the ligand of interest. In impure systems, such as hepatic cytosol, lack of agreement could be attributed either to the competitor binding at a different site followed by an allosteric effect, or more probably, binding to other proteins in addition to the one being examined. This lack of agreement (or lack of parallelism in dose-response curves) is indicated in Table 2 by a large coefficient of variation of the K_a. For example, when diethylstilbestrol was used as the competitor, the coefficient of variation was only 11% indicating that diethylstilbestrol was binding at the same site as estradiol and that the relative K_a for this compound was an accurate one. Conversely, the coefficients of variation for estriol, cortisol and androstenedione are about three-fold higher, indicating that widely different association constants went into the calculations and that the K_a for these steroids must be regarded as only an approximate indication of their binding at the estradiol binding site. This comes as no surprise since liver is known to contain proteins which bind androgens and glucocorticoids [17–18]. Taken in its entirety, Table 2 suggests that the estrogen binding site(s) examined in this study is(are) reasonably specific for estrogens, a characteristic of steroid hormone receptors.

Interpretation of the results shown in Fig. 4 requires some comment because the assay used was not designed to be an exchange assay. It should be emphasized that the cytosol preparations were treated with charcoal prior to assay (see Methods) so that endogenous free estrogens were removed before the assay was begun. In addition, at the concentration of estradiol present in rat serum, approx. 10^{-10} M at proestrous, lower at other times [19], one would expect the receptors to be mostly empty. If one assumes that the receptor is uniformly distributed in hepatic water then the receptor concentration in the livers of intact animals is $1.2–1.6 \times 10^{-9}$ M. Katzenellenbogen and Ferguson[20] have shown, using an exchange assay for the receptor in rat uterine cytosol, that after the administration of 5 μg estradiol *in vivo*, there is a precipitious fall in the uterine cytoplasmic receptor concentration, and that the receptor concentration returns to basal values at 18–20 h. They have also shown (Katzenellenbogen, B., personal communication) that the difference between total cytoplasmic receptor and free receptor is negligible even at those times when the receptor concentration is changing rapidly. Hence, although an extrapolation from uterus to liver is necessary, we can, as a first approximation, accept the results in Fig. 5 at face value. Several effects are then apparent. The administration of 5 μg estradiol did not change the receptor concentration in intact animals. Ovariectomy resulted in a 2.4-fold increase in receptor concentration which was partially returned to that of the intact animal by the administration of estrogens. The implication of these experiments is that there are factors in the ovary which result in a lowering of the receptor concentration. One of these factors is estrogenic, but there must be an additional substance. In this connection, it is worth noting that Hsueh *et al.* have recently reported that progesterone treatment, in properly pretreated rats, leads to a fall in the concentration of the uterine estrogen receptor [21]. Finally, hypophysectomy led to a five-fold decrease in receptor concentration. This is consonant with the data of McGuire who found a 10-fold decrease after hypophysectomy [7]. He found that this loss of receptor could be partially restored by prolactin. We found that the administration of estradiol benzoate led to an apparent two-fold increase in hypophysectomized animals but this difference was not statistically significant ($0.1 > P > 0.05$). The possibility that this difference is real is being pursued using larger groups of animals.

The receptor capacities we have measured, 44 fmol/mg cytosol protein (corrected for loss due to ammonium sulfate purification) disagree somewhat with the results from Viladiu *et al.*[6], 13.4 fmol/mg, and Chamness *et al.*[7], 18.5 fmol/mg, but are in close agreement with those of Eisenfeld *et al.*[8], 58 fmol/mg. The reasons for the discrepancies are not immediately apparent. We also found a marked change after ovariectomy unlike Viladiu *et al.*[6], but this may have to do with the fact that those authors examined rats one day after ovariectomy while the animals in this study were sacrificed two weeks after surgery. Finally, although we found a significant decrease in receptor concentration after hypophysec-

tomy, the decrease is two-fold less than that observed by others [7]. The unexplained discrepancies noted above probably will not be resolved until a pure receptor is available as standard.

This study, along with reports of specific receptor-like proteins in the pancreas [23] and fetal brain and kidney [24–25], strengthen a statement made several years ago in reference to hormonal influences in the liver. "It is the liver which is probably affected in more ways and with more regularity and intensity by sex hormones than any other extra-genital organ; these effects encompass such a spectrum of sites and types of actions that they raise an important question concerning the significance of the distinction made between 'primary' target organs of those hormones and those which are 'secondary' in type" [26].

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