ESTROGEN RECEPTORS OF RAINBOW TROUT LIVER

CHARACTERIZATION OF ESTROGEN RECEPTORS IN THE LIVER CYTOSOL OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

BY

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SUMMARY

Two types of estrogen binding sites were found in the rainbow trout liver cytosol, using the equilibrium binding assay. The higher affinity, lower capacity type I site had a Kd range of 0.53-5.9 nM and a concentration range of 14-95 pmoles/g protein. The lower affinity, higher capacity type II sites had a Kd range of 65-265 nM and a concentration range of 20-180 pmoles/g protein. These estrogen binding sites are both bound by ^{3}H-moxestrol. The estrogen binding sites in the serum were also examined and two components were found with the higher affinity component having a Kd of 2.63 nM and a concentration of 25 pmoles/g protein while the lower affinity component had a Kd of 79 nM and a concentration of 200 pmoles/g protein. However, these estrogen binding components are unable to bind the synthetic estrogen, DES, and therefore cannot account for either of the binding components found in the liver cytosol which bind DES as readily as 17β-estradiol.

The cytosol estrogen binding sites bind non-estrogens in addition to estrogens. Sucrose gradient centrifugation of the cytosol yielded two estrogen binding peaks, one at 4.4S, the other at 3.7S. The heavier peak contained binding sites able to bind progesterone as well. Gel filtration of the cytosol also resulted in two peaks, one at

43,000 daltons, the other at 33,000 daltons. Again the heavier peak could be partially competed out by progesterone. The half life of these binding components was 60 minutes at 37°C, while no decrease in binding was observed after 4 hours at either 0 or 12°C. After 17β-estradiol treatment in vivo type I sites (relative to type II sites) were 74% depleted after 8.5 hours and 40% depleted after 24 hours. Finally, o,p'-DDT and p,p'-DDT, components of technical grade preparations of the insecticide DDT were both able to compete for estrogen binding sites in the rainbow trout liver cytosol and therefore may be able to affect the expression of estrogen inducible genes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

"Gene expression is a change in the nature of, or rate at which, different genes are transcribed" (Tata and Smith, 1979). During development of an embryo from zygote into a highly organized multicellular organism, cells undergo differentiation into functionally more specialized forms, and this involves the preferential synthesis of specific proteins, for example, hemoglobin in erythrocytes, antibodies in lymphocytes, and ovalbumin in the tubular gland cells of chick oviduct (for review, see Browder, 1980). It is generally believed that cellular differentiation could arise by the selective expression of specific genes, and there are two major investigations which support this possibility. First, transplantation of nuclei from several varieties of adult tissue (skin cells, interstitial cells, and lymphocytes) into enucleated eggs has resulted in the complete development of normal frogs, indicating that the nuclei of these specialized cells are totipotent, that is, that all genes needed in all specialized cells are present and are not lost or inactivated permanently (Gurdon, 1962; Gurdon, Reeves and Laskey, 1975; Wabl, Brun, Dupasquier, 1975). Second, nucleic acid hybridization experiments have ruled out the possibility of selective gene amplification as a mechanism for differential gene activity (David-

son, 1976). The genetic information in somatic cells was shown to be identical to that of embryonic cells and therefore the differences between specialized cells must be due to differential expression of genes.

Control of differential gene expression can occur at the level of transcription or post-transcription (RNA synthesis, splicing, stabilization, or transport) or at the level of translation or post-translation (protein synthesis or processing). However, it appears that in many systems, regulation is at the transcriptional level (Dawid and Wahli, 1979).

A useful model in which to study differential gene expression is steroid hormone-induced activation of well-defined gene products, in particular, the chick oviduct system. The evidence to date shows that steroid hormones act at the level of transcription (Dawid and Wahli, 1979). The oviduct tubular gland cells are induced by estrogen and progesterone to synthesize large quantities of egg white proteins, especially ovalbumin (Palmiter, Moore, Mulvihill, and Emtage, 1976; Cox, 1977; Hynes, Groner, Sippel, Chinguygen-Hau, and Schutz, 1977; Garapin, Lepennec, Roskam, Perrin, Cami, Krust, Breathnach, Chambon, and Kourilsky, 1978; Mandel, Breathnach, Gerlinger, LeMuer, Gannon, and Chambon, 1979; O'Malley, Roop, Lai, Norstrom, Catterall, Swaneck, Colbert, Tsai, Dugaiczyk, and Woo, 1979; Oka and Schimke, 1969). A major drawback to this system is the sub-

stantial cell division and cytodifferentiation accompanying primary hormone stimulation (Palmiter, 1975), making difficult the analysis of early molecular events leading to gene regulation. Many researchers have therefore turned to an alternate system, the egg yolk precursor protein, produced in oviparous animals. In the egg-laying vertebrates, vitellogenin (Vg) is a multicomponent glycoprotein that is synthesized in the liver, secreted into the blood and then taken up by developing oocytes (Deeley and Goldberger, 1979). This system has many advantages: 1) Vg is a unique and stable protein; 2) its synthesis is regulated by estrogens, in particular, 17β-estradiol; 3) the hormonal induction is completely reversible, that is, the synthesis of Vg ceases upon withdrawal of the hormone, and can be re-induced; 4) both primary and secondary inductions occur in fully differentiated cells, cytodifferentiation is not a prerequisite; 5) induction is faithfully reproducible <u>in vitro</u> in hepatocyte culture; 6) study of precursor-product processing is facilitated by the multicomponent nature of Vg and its cleavage in cells (oocytes) different from those in which it is synthesized (hepatocytes); 7) post-translational modification and processing can be studied easily due to the unequal distribution of amino acids, phosphate, sugars and lipids on the final product molecule; 8) finally, the induction of Vg in males where it is not normally produced provides an

effective control (for review, see Tata and Smith, 1979).

I Vitellogenin Synthesis

The estrogen-induced synthesis of Vg in livers of oviparous vertebrates is a valuable model system for the investigation of the molecular events involved in the regulation of gene activity. Induction in males makes a good control system as they do not normally synthesize Vg, and therefore a primary response is ensured (Tata and Smith, 1979).

In the serum of 17β-estradiol-treated male or female rainbow trout, a polypeptide of 170,000 daltons is observed which is identified as vitellogenin since it is immunologically identical to the major protein of the egg yolk, lipovitellin (Chen, 1983). Pulse-labelling of estradiol-treated liver cubes in vitro showed preferential incorporation of ^{35}S-methionine into two polypeptides of 170,000 and 160,000 daltons which are immunologically identical to serum vitellogenins. These two polypeptides are shown to share a common primary structure (Chen, 1983). Total RNA isolated from the liver of estradiol-treated fish directed synthesis of vitellogenin polypeptide with the molecular weight of 160,000 in the cell-free protein synthesizing system derived from rabbit reticulocytes. Hence, the primary translation product of the rainbow trout vitellogenin gene is a polypeptide of 160,000 daltons which is

post-translationally modified (eg. glycosylation, lipidation and/or phosphorylation) into a polypeptide of 170,000 daltons before it is released into the blood (Chen, 1983).

By Northern blot analysis it is revealed that administration of 17β-estradiol in vivo resulted in rapid accumulation of at least two species of mRNA sequences in male or female rainbow trout adults of any stage. One of the estradiol-inducible mRNA molecules has a size of about 6300 bases and codes for vitellogenin, while the other has a size of about 1800 bases but the protein product remains to be identified (Chen, 1983). The cDNA and genomic genes of these two estradiol-inducible mRNAs have been cloned, and are used as molecular probes for further analysis of their expression controlled by estrogens both in vivo and in vitro (Chen, 1983).

In Xenopus, Vg mRNA can be detected 4.5 hours after estradiol administration and its accumulation is linear (300 molecules/cell/day) until the twelfth day when it peaks and then begins to decline (Baker and Shapiro, 1977). Sixty days after induction the level of Vg mRNA is undetectable, that is, there is no significant translation of the Vg gene (Baker and Shapiro, 1977). If the animals are then restimulated with 17β-estradiol, Vg mRNA can be detected in some animals as early as 1 hour later (Baker and Shapiro, 1978). Therefore, expression of Vg is reversible and

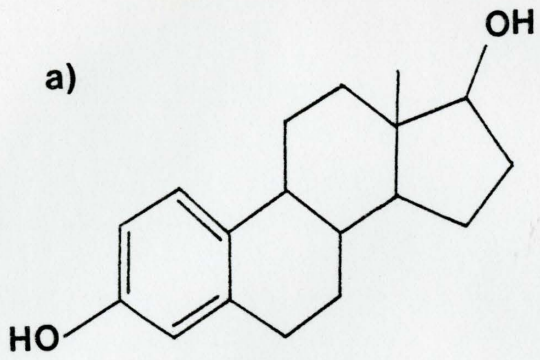
appearance of Vg mRNA occurs sooner in secondary stimulation than in primary stimulation where there is a lag period. Accumulation of Vg mRNA is also about ten times more rapid in early secondary stimulation (19,000 molecules/cell/day) than in early primary stimulation, and the peak is reached by the sixth day and is two times as large as the peak level after primary stimulation (Baker and Shapiro, 1977). That is, secondary stimulation is more effective than primary, the liver cell has changed in some way so that the Vg gene is more efficiently transcribed. Similar results are observed in the chicken (Deeley, Udell, Burns, Gordon and Goldberger, 1977; Burns, Deeley, Gordon, Udell, Mullinix and Goldberger, 1978) and in the rainbow trout (Chen and Howard, private communication). This change is probably not due to synthesis of additional, new estrogen receptor molecules (Baker and Shapiro, 1977), rather it may be due to decreased metabolism of 17β-estradiol.

II Estrogens and their Metabolism

Estrogen is a group of steroid hormones characterized by the presence of an aromatic A ring containing an hydroxyl group on carbon 3 and the absence of a methyl group on carbon 10 (see Figure 1) (Turner and Bagnara, 1971). Diethylstilbestrol (DES) is an active analog of 17β-estradiol and is used in some experiments because of its greater

Figure 1 Chemical structures of the three major estrogens; a) 17β-estradiol, b) estrone, and c) estriol, and two analogues; d) DES, and e) moxestrol. (Dorfman and Unger, 1965).

a)



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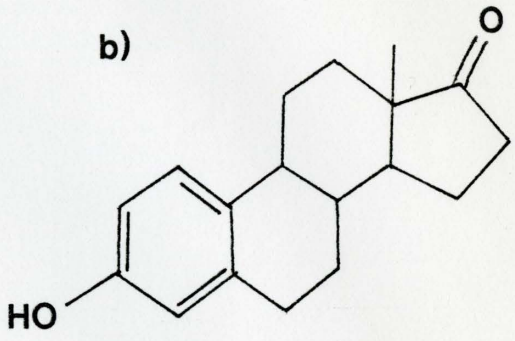

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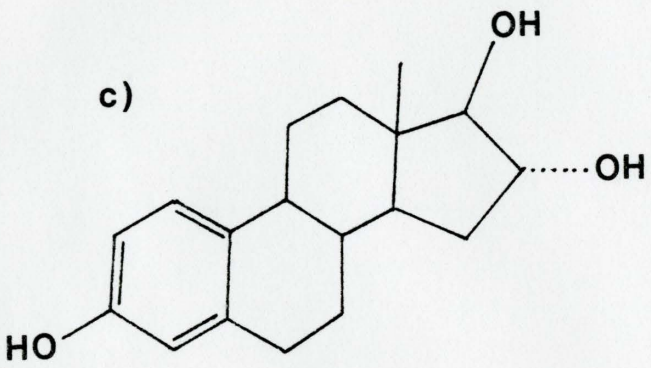

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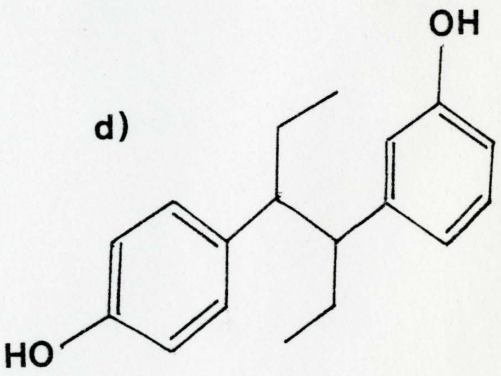

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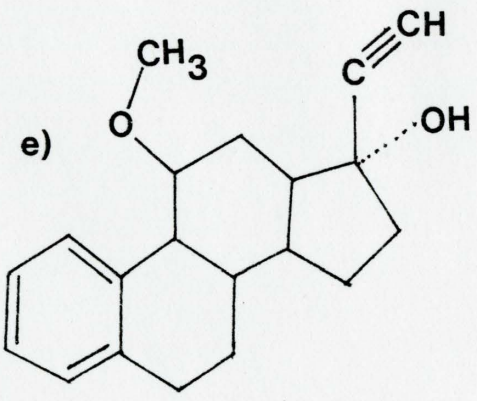
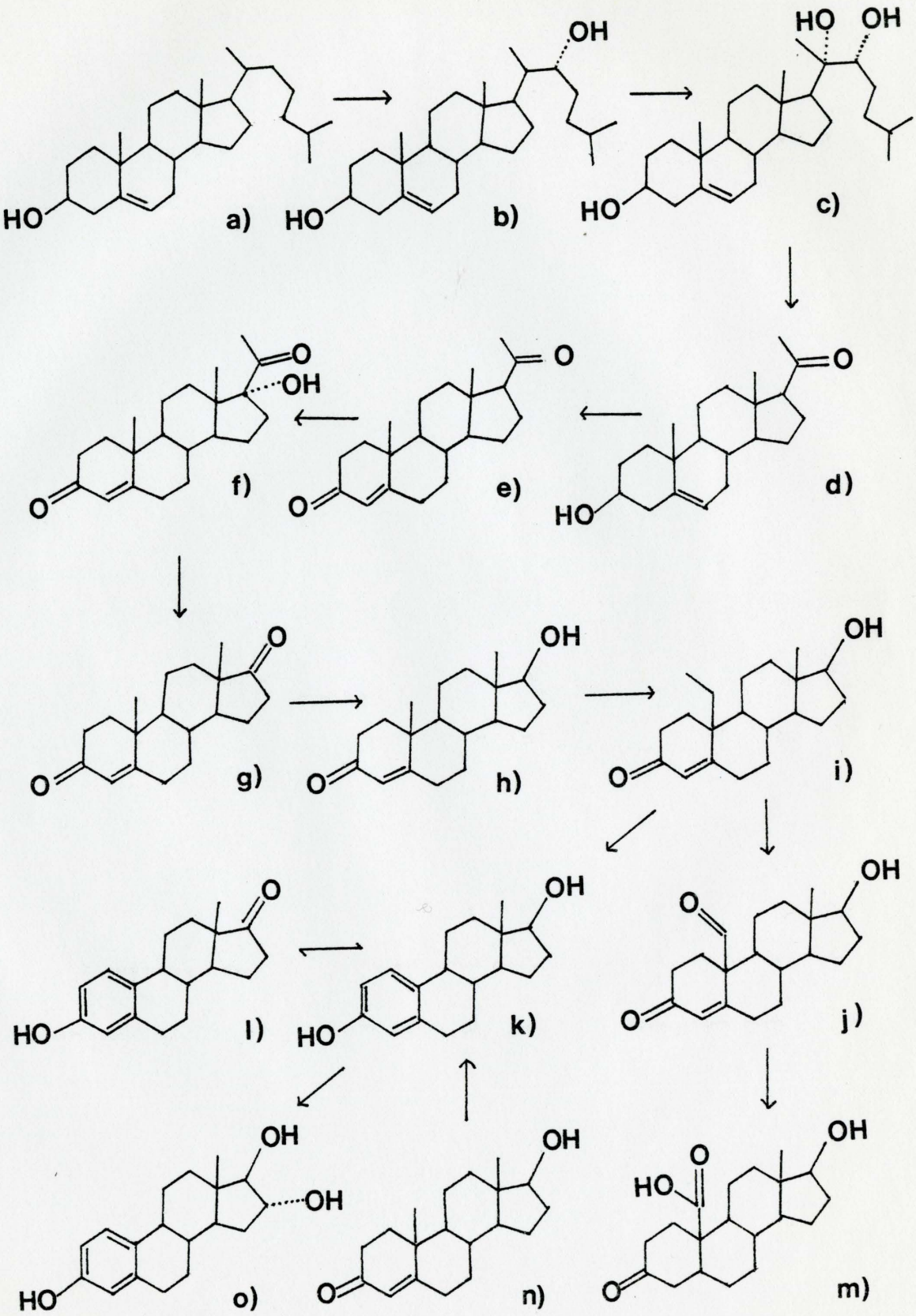


stability (Turner and Bagnara, 1971). In the rainbow trout, the three major estrogens, 17β-estradiol, estrone and estriol, are produced in the ovary in response to gonadotropin (Schreck, 1973). A possible pathway of synthesis is shown in Figure 2, although this is composed from in vivo and in vitro studies done in mammals (Dorfman and Unger, 1965). It is not known whether fish estrogen synthesis also follows the same or a similar pathway. After synthesis the estrogens travel via the blood to one of the target tissues, the liver (Foster and Breton, 1975). There are two steroid binding systems found in female rainbow trout plasma, a 'sex binding protein' with a high affinity for 17β-estradiol and testosterone, and a 'transcortin' type system which binds 17β-estradiol, testosterone, progesterone and corticosteroids (Foster and Breton, 1975). These proteins are possibly responsible for transporting 17β-estradiol to the target tissues.

There are four physiological periods in the annual reproductive cycle of the female rainbow trout; pre-vitellogenic (March to April); endogenous vitellogenesis (May to June); exogenous vitellogenesis (August to December) and a period of ovulation and spawning (January to February) (van Bohemen and Lambert, 1981). Vitellogenin synthesis occurs in response to estrogen, mainly 17β-estradiol, although estrone seems to act in a cooperative manner (van Bohemen, 1982). Estrogen synthesis increases from a neg-

Figure 2 Proposed scheme for the biosynthesis of the three major estrogens, 17β-estradiol, estrone, and estriol; a) cholesterol, b) 22-hydroxycholesterol, c) 20,22-dihydroxycholesterol, d) pregnenolone, e) progesterone, f) 17-hydroxyprogesterone, g) 4-androstenedione, h) testosterone, k) 17β-estradiol, l) estrone, m) 10β-carboxy-17β-hydroxestr-4-en-3-one, n) 19-nortestosterone, o) estriol (Dorfman and Unger, 1965).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ligible amount in February to a maximum of 2.6 nmol/ovary in October, and then the levels of the two estrogens decreased (van Bohemen and lambert, 1981; Scott, 1983). These increases are parallelled by Vg levels (van Bohemen and Lambert, 1981).

Few studies have been done on the metabolism of 17β-estradiol or other estrogens in teleosts, in spite of the interest in their reproductive role in female fish. However, the metabolism of 17β-estradiol by liver microsomes from juvenile rainbow trout was studied and the following metabolites were identified: estrone, estra-1,3,5(10)-triene-3,16β,17β-triol, and estra-1,3,5(10)-triene-3,7β, 17β-triol, as well as other polar metabolites hydroxylated at positions 6 and 6 (Hansson, 1980; Hansson and Refter, 1980). As the formation of hydroxylated metabolites was inhibited by CO, the participation of cytochrome P450 was indicated. Also found were sex-related differences in steroid metabolism. Higher levels of cytochrome P450 and 17-hydroxy-steroid oxidoreductase were observed in mature male rainbow trout as compared to female (Hansson, 1980). 6β-hydroxylase has a low activity during maturation of female trout and then increases in activity before spawning when the plasma level of 17β-estradiol is decreasing (Hansson; Hansson and Refter, 1980). It too is higher in male rainbow trout (Hansson, 1980). It is suggested that perhaps Vg synthesis interferes with estradiol metabolism as both

take place in the endoplasmic reticulum (Hansson, 1980). In culture, male Xenopus hepatocytes metabolize 17β-estradiol three times faster than do female hepatocytes but accumulate Vg mRNA only 1/5 to 1/15 as fast (Tenniswood, Searle, Wolffe, and Tata, 1983). However, if male Xenopus are treated with 17β-estradiol 16 weeks prior to culturing, the rate of estrogen metabolism in the hepatocytes is much lower, comparable to female hepatocytes (Tenniswood, Searle, Wolffe, and Tata, 1983). In summary, some metabolites of 17β-estradiol have been isolated and seem to be products of cytochrome P450-catalyzed metabolism and products of an oxidoreductase and a hydrolase. Other enzymes are probably also involved. Higher levels of estradiol in maturing females seem to be due to a lower rate of metabolism.

III Estrogen Specific Receptors

The classic theory of steroid hormone action is the two-step model where specific cytoplasmic hormone receptor molecules mediate the interaction between steroid and nuclear components to alter gene expression (Jensen, Suzuki, Kawashima, Stumpf, Jungblut, DeSombre, 1968). There is, however evidence that steroid hormones may, in some tissues act on the plasma membrane to change gene activity via adenylate cyclase (Baulieu, 1983). Specific binding sites for estrogen have been found on rat endometrial and liver cell membranes (Pietras and Szego, 1977), and progesterone has

been demonstrated to interact with Xenopus oocyte cell membranes, decreasing adenylate cyclase activity, and therefore affecting reinitiation of meiosis (Baulieu, 1983).

The two-step model by Jensen, (Jensen, Suzuki, Kawashima, Stumpf, Jungblut, and DeSombre, 1968), states that the hormone after entering the target cell, interacts with a specific high affinity receptor. This hormone receptor complex then undergoes a conformational change or activation, which results in its translocation to the nucleus. There it interacts with one or nore nuclear components, which results in activation of specific genes. Therefore the receptor must be present in the target tissue in limited amounts, it must be specific and have a high affinity for the hormone.

a) Cytosol Receptors

Many different systems have been examined for estrogen receptors. In the chicken, a protein of high specificity, high affinity (Kd=4-26 x 10^{-10}M), and low capacity (n=3.3 x 10^{-11}moles/g protein) has been purified from cockerel liver cytosol (Lazier and Haggarty, 1979). This protein was shown to become depleted from the cytosol after in vivo injection of estradiol, probably due to its translocation to the nucleus (Snow, Clark, Eriksson, Hardin, Chan, Jackson, and Means, 1978). Another group however, looked at the same system but used a protease inhibitor and mox-

estrol, an estradiol analog with a higher specificity and stability, and found a higher affinity (Kd=1.4-1.8 x 10^{-10} M) estrogen specific receptor with comparable binding capacity (n=4.1 x 10^{-11}moles/g protein) (DeBoer, Shippe, Ab, and Gruber, 1982). They also found a hormone and temperature dependent transformation from a 4S form to a 5S form of the protein and suggest that this transformation occurs as a result of activation of the complex.

Two estrogen receptors have been characterized in rat liver cytosol. The first is the classic, high affinity (Kd=1 x 10^{-10}M,n=1 x 10^{-11}moles/g protein) receptor which sediments at 8-9S on a sucrose gradient, and translocates to the nucleus after treatment with estrogen (Chamness, Costlow, and McGuire, 1975). The second estrogen binding component (known as the unusual estrogen binding protein, UEBP), has a higher capacity (n=1 x 10^{-7}moles/g protein) and lower affinity (Kd=1 x 10^{-8}M), sediments at 4S and binds androgens as well as estrogens. However it does not bind other steroids or non-steroidal estrogens (eg. DES) (Miroshichenko, Smirnova and Rozen, 1981). Several functions have been postulated for this component; it may serve to uptake and concentrate secreted androgens, estrogens and their metabolites in the liver cells, or it may be important for the regulation of the intracellular distribution of sex steroids between their receptors and enzymes of their metabolism (Miroshichenko, Smirnova and Rozen, 1981). Also, unlike the classic receptor

UEBP is not found in the 30% $(NH_4)_2SO_4$ precipitated cytosol. Whole cytosol is required for its isolation.

b) Nuclear Receptors

As theorized by Jensen (Jensen, Suzuki, Kawashima, Stumpf, Jungblut, and DeSombre, 1968), the cytosolic steroid receptor is found to translocate to the nucleus in many systems. There it presumably interacts with nuclear components to modify gene expression. In the chicken, receptor translocation is demonstrated after 17β-estradiol injection in vivo. There is a decrease in cytosol receptor (Lazier and Haggarty, 1979) and a corresponding increase in nuclear binding (Lazier, 1975). This nuclear binding component was shown to have a Kd (=3.4 x 10^{-10}M) similar to the nuclear receptor found in the rat liver (Chamness, Costlow, and McGuire, 1975). The rat uterus is an interesting system. The cytosol contains two types of estrogen receptors, similar to the situation found in the rat liver (Chamness, Costlow, and McGuire, 1975). In the nucleus, two receptors are also observed, one is the cytosol type I site after translocation but the nuclear type II site has no apparent relationship to the cytosol type II site (Clark, Markaverich, Upchurch, Eriksson, Hardin, and Peck, 1980). These type II sites may be involved in gene activation by binding the estrogen receptor complex or they could be precursors of the cytosol type I sites or they perhaps are

metabolic enzymes specific for estrogens (Clark, Markaverich, Upchurch, Eriksson, Hardin, and Peck, 1980).

IV Nuclear Events

The next step in understanding hormone-induced gene expression is to look at the nuclear events. A system that has been particularly well characterized with respect to nuclear interactions is the progesterone-induced expression of egg white proteins in the chick oviduct. The first question that arises is 'what is the identity of the nuclear acceptor sites?'. Many nuclear components have been considered for this function; the nuclear envelope, histones, non-histone basic proteins, histone and non-histone-DNA complexes, pure DNA, ribonucleoproteins, and the nuclear matrix (Bonner, 1980). Several groups have reported specific binding of the chick oviduct progesterone receptor complex to specific DNA sequences (Mulvihill, 1982; Dean, 1983; Compton, Schrader and O'Malley, 1983), and determined a consensus sequence of the binding site (Mulvihill, 1982). However, this sequence is A-T rich and it has been shown that such stretches of DNA are less stable than G-C rich sequences and hence are subject to non-specific, non-saturable, enhanced binding of steroid receptors due to destabilization of the DNA double helix (Bonner, 1980). If a minimum of DNA damage is permitted it is found that chromatin is a much stronger and more specific binder of

the progesterone-receptor complex than pure DNA, and chromatin is saturable whereas pure DNA is not (Bonner, 1980). Chromatin also displays a seasonal change in binding capability that correlates with the in vivo response of the oviduct to progesterone (Bonner, 1980). Therefore it seems likely that chromatin is the location of the nuclear acceptor sites for binding of the progesterone-receptor complexes.

The chromatin can be dissected into three protein fractions (Bonner, 1980). The first, called CP-1, containing histone proteins, is obtained by treatment of chromatin with 3M NaCl at pH 6.0. Following this treatment, the nuclear acceptor proteins for estrogen are still present in the chromatin. The next treatment, 4M guanidine hydrochloride (pH 6.0) removes the CP-2 fraction, and also unmasks all the nuclear acceptor sites. Therefore,this fraction contains the masking proteins responsible for seasonal changes in acceptor sites, developmental changes and changes following induction. Finally, treatment with 7M guanidine hydrochloride (pH 6.0) removes all acceptor activity and this fraction is called CP-3. This fraction was shown to be protein in nature (treatment with pronase before reconstitution lead to loss of receptor activity). Further fractionation and analysis of this fraction showed that the acceptor proteins are hydrophobic, slightly acidic and simple (non-conjugated) proteins of MW 13,000 to 18,000 daltons,

and contain at least two molecular species (Bonner, 1980).

The acceptor proteins could act directly by binding progesterone receptors at each responsive gene or they could act indirectly by binding the progesterone receptor at regulatory genes which in turn switch on the structural genes. Three models for the mechanism of action of steroid receptor complexes on transcription have been proposed. Model 1 has the progesterone receptor binding directly to the acceptor protein and thus initiating transcription (Bonner, 1980). Model 2 has the receptor binding DNA next to the acceptor protein (the DNA could be in a conformation altered by the acceptor protein to facilitate progesterone receptor binding, eg. hairpin loops, left-handed helices, or single-stranded regions). And Model 3 utilizes the subunit nature of the progesterone receptor, and has subunit B binding the acceptor protein followed by dissociation from subunit A which binds at adjacent DNA sequences. The latter two models are not inconsistent with DNA binding by the progesterone receptor complex, however, the specificity of the DNA binding is determined by the acceptor protein, not by the DNA sequence directly.

Although the Vg system has not been examined in this detail, it is likely that a similar situation exists. However, another aspect of Vg induction has been examined; changes in the DNA and chromatin structures during gene activation. That is, DNase I sensitivity of the chromatin,

and DNA methylation were examined. Active chromatin is preferentially digested by DNase I, an endonuclease with little sequence specificity (Elgin, 1982). In particular, some specific sites in chromatin are hypersensitive to DNase I, and these sites may indicate positions where the DNA is more accessible to macromolecular probes, or may be origins of replication, or positions where DNA rearrangement takes place (Elgin. 1982). In addition, DNA hypomethylation in many systems appears to be correlated with gene expression.

In Xenopus, the A1 and A2 Vg genes are twice as sensitive to DNase I digestion in estradiol-treated males as compared to control animals, or animals withdrawn from estradiol (Dickson, Aten, and Eisenfeld, 1978). There was no change in the DNase I sensitivity of the Vg gene in erythrocytes after the same treatment (Gerber-Huber, 1981). This indicates that estradiol treatment results in an increase in DNase I sensitivity of Vg genes in hepatocytes.

In the chicken, Gallus gallus, estradiol induces the appearance of three DNase I hypersensitive sites at the 5' end of the major Vg gene (Vg II) in addition to the internal and 3' hypersensitive sites present prior to treatment (Burch and Weintraub, 1983). Two of these sites are stable and apparently are transmitted to daughter cells after hormone withdrawal. However, the third site appears only transiently, perhaps due to binding of the hormone re-

ceptor (Burch and Weintraub, 1983). The sites do not appear in brain cells, erythrocytes, or fibroblasts, while a different set appears in oviduct cells that respond to estradiol but do not express Vg II (Burch and Weintraub, 1983).

It appears that in Xenopus, the Vg genes do not become hypomethylated when induced by estrogen, at least not at the sequences GCGC (Hha I) or CCGG (Msp I/Hpa II) (Folger, Anderson, Hayward, Shapiro, 1983). However, in the chicken Vg II gene region, a single Msp/Hpa II recognition site (CCGG) becomes demethylated in livers after estradiol treatment (Wilks, 1982; Geister, Mattaj, Wilks, Geldran, and Jost, 1983). This site is upstream from the gene and demethylation occurs after initiation of transcription and after appearance of the 5' flanking hypersensitive sites (Wilks, 1983; Geister, Mattaj, Wilks, Geldran, and Jost, 1983). However, one chicken was found to be minus this site but still able to express Vg II (Wilks, 1982). Thus, Vg gene activation and demethylation are not clearly correlated, although there is a correlation between Vg gene induction and DNase I sensitivity in both Xenopus and the chicken.

V The Estrogenic Activity of a DDT Analog, o,p'-DDT

An analog of the insecticide DDT, o,p'-DDT, which is found contaminating technical grade preparations of DDT

(p,p'-DDT), was shown to have estrogenic activity (Burlington and Lindeman, 1950). In ovariectomized rats, o,p'-DDT administration increased uterine weight, glycogen content and the activities of various enzymes involved in glycolysis and the hexose monophosphate shunt pathway, while treatment with the isomer p,p'-DDT enhanced to a lesser extent most of the uterine enzyme activities but had no uterotropic or glycogenic effect (Bitman, Cecil, Harris, and Fries, 1968). These phenomena may be explained in several ways. Steroid metabolism may be altered resulting in an increased sensitivity to estrogen, o,p'-DDT may bind to estrogen receptors, or it may bind to other molecules that can elicit estrogenic effects (Bulger and Kupfer, 1983).

It was found that o,p'-DDT inhibited ^{3}H-17β-estradiol binding to rat uterus cytosolic estrogen receptors (Nelson, 1974). Furthermore, sucrose density gradient sedimentation analysis showed that o,p'-DDT binds to the 8-9S cytosolic estrogen receptor of rat uterus (Forster, Wilder, Heinrichs, 1975; Kupfer, 1975; Kupfer and Bulger, 1976). This binding inhibition was shown to be non-destructive (there are the same number of binding sites before and after inhibition) and competitive (inhibition occurs at the same binding site as estradiol). The DDT derivative was also capable of translocating the estrogen receptor to the nucleus in a manner similar to that of estradiol (Kupfer and

Bulger, 1976). Its isomers p,p'-DDT and o,p'-DDE were not capable of estrogen binding (Bulger and Kupfer, 1983).

In view of the significant amounts of both o,p'-DDT and p,p'-DDT found in Great Lakes fish (Sonstegard, Leatherland, Mukhter and Bend, submitted for publication) it would be interesting to discover whether or not the two insecticides could bind to the rainbow trout liver estrogen receptors. If so, these chemicals could have an estrogenic effect and therefore could interfere with the natural breeding processes.

VI Rationale and Objectives

As a consequence of the above literature survey, it is apparent that our understanding of the molecular mechanism of estrogen controlled specific activation of the Vg gene in *Salmo gairdneri* is incomplete.

The main objectives of this work are:

a) to partially purify the rainbow trout estrogen receptors present in the liver cytosol, to determine dissociation constants and concentrations of sites,

b) to determine the ability of various steroids to compete for the estrogen binding sites of the partially purified protein fraction,

c) to study the depletion of cytoplasmic sites from cytosol after hormone treatment and finally,

d) to examine the abilities of o,p'-DDT and p,p'-DDT, two components of technical grade DDT, to bind to the rainbow trout liver estrogen receptors.

MATERIALS AND METHODS

Animals

Rainbow trout (Salmo gairdneri), ranging in size from 20 to 30 cms, were obtained from Goossens Trout Farm, Otterville, Ont. They were kept in large tanks in a constant flow of 12°C dechlorinated water. Commercial fish pellets (Martins Feed Mills, Elmira, Ont.,) were supplied every three to four days.

In depletion studies, fish were injected intraperitoneally with 10 ug 17β-estradiol/100 g fish (dissolved in cod liver oil) and sacrificed after 8.5 or 24 hours. Sacrifice was performed by excision of the liver through a ventral midline cut. If serum was required, the fish was first anaesthetized with methanesulfonic acid salt (Sigma Chemical Co., St. Louis, MO) and then the tail is amputated and the blood collected.

Chemicals

(2,4,6,7-^{3}H)-17β-estradiol (91.5-112 Ci/mmole), (11β-methoxy-^{3}H)-moxestrol (87.5 Ci/mmole) were from New England Nuclear, Boston, MA. They were supplied in toluene-ethanol (9:1) and kept at -20°C. Before use they were evaporated to dryness and redissolved in propylene glycol which was then brought up to the appropriate conc-

entration by dilution in Buffer B (0.5M KCl, 1.5M EDTA and 10mM Tris/HCl pH 8.4). Unlabelled steroids and BSA were obtained from Sigma Chemical Co., St. Louis, MO. Liquid scintillation fluid was purchased from Amersham Chemical Co., Arlington Heights. Charcoal-dextran suspensions were prepared from Norit A (Fisher Chemical Co., Fairlawn, NJ) and Dextran T70 (Pharmacia Fine Chemicals, Dorval, Que.). Sephacryl G-200 was also obtained from Pharmacia and DDT was from Aldrich Chemical Co., Milwaukee, WI. Iodoacetic acid and benzamidine were acquired from Sigma and were dissolved in ethanol to make 500 mM and 100 mM solutions respectively. DTT (dithiothreitol) was also purchased from Sigma and dissolved in Buffer A (0.33M sucrose, 3mM MgCl, and 20mM Tris/HCl pH 8.4) to make a 100mM solution.

Cytosol Preparation

All preparations were carried out at 0-4^{o}C. The excised livers were thoroughly perfused with 0.6% NaCl. After weighing, the tissue was sliced into large pieces and rinsed in saline two or three times (the saline should be clear after the final rinse). Then the liver was chopped into very fine pieces and homogenized in Buffer A (2.5 ml/g tissue). This homogenate was centrifuged at 2500 rpm in a JA 20 rotor, and the supernatant was transferred to a Beckman Type 65 rotor in the Beckman L3-50

ultracentrifuge and spun at 34,000 rpm for 90 minutes. For large scale preparations a Type 30 rotor was used at 27,000 rpm for 140 minutes and the resulting supernatant (fat layer removed) frozen in liquid nitrogen until required. Otherwise, the supernatant was subjected to a 0-35% $(NH_4)_2SO_4$ precipitation. The precipitate, obtained by centrifugation in a JA 20 rotor for 20 minutes at 10,000 rpm, was drained thoroughly and dissolved in Buffer B. Endogenous steroids were removed by incubation of the cytosol preparation with 0.1 volumes of charcoal/dextran suspension A (made by mixing equal volumes of a solution of 10% Norit A and a solution of 1% Dextran T70, both dissolved in 3mM EDTA and 20mM Tris/HCl pH 8.4). The charcoal/dextran was removed by centrifugation in a JA 20 rotor at 11,000 rpm for 10 minutes. The supernatant was used in the binding assays.

Equilibrium Binding Assay

Aliquots of 20 ul of the cytosol preparations were added to Buffer B (to make 300 ul) which contained final concentrations of 0.1 to 80 nM of ^{3}H-17β-estradiol with and without 100 times excess of unlabelled DES. Tubes were incubated for 16 hours and then 0.5 volumes of charcoal/dextran suspension B (made by mixing equal volumes of a solution of 2% Norit A and a solution of 0.2% Dextran T70, both dissolved in 3mM EDTA and 20mM Tris/HCl pH 8.4)

were added to the tube and mixed gently for 30 minutes. The charcoal/dextran was removed by centrifugation at 15,000 rpm for 1 minute, transferred to new tubes and recentrifuged to ensure that all charcoal/dextran was removed. Aliquots of 300 ul were taken and added to liquid scintillation fluid and the radioactivity in each sample was determined in Beckman scintillation counters, at efficiencies of 20-25%. The results of the assay are converted from cpm to nM before being plotted on graphs.

Competition Assay

In the competition assay, various steroids, both estrogens and non-estrogens, are compared to 17β-estradiol in their ability to displace binding of ^{3}H-17β-estradiol to estrogen receptors under the same conditions described in the equilibrium binding assay. One hundred fold excess of the competitors and cold 17β-estradiol are incubated with a constant concentration of ^{3}H-17β-estradiol overnight, and then the relative binding of the tracer is determined. The better the competitor, the greater the amount of ^{3}H-17β-estradiol it will displace. 17β-estradiol is used as the standard, that is, it displaces 100% of specific isotope binding.

Sucrose Gradient Centrifugation

Continuous linear gradients were made with a grad-

ient maker and varied from 5-20% sucrose (in Buffer B). Samples of cytosol incubated with and without DES or progesterone were applied to the top of the gradients and spun at 40,000 rpm for 21 hours in the SW 50.1 rotor. The gradients were then fractionated and added to scintillation fluid and then counted in the scintillation counter. BSA was used as a molecular weight marker (its peak was determined by reading absorbance at 280 nm in a UV spectrophotometer).

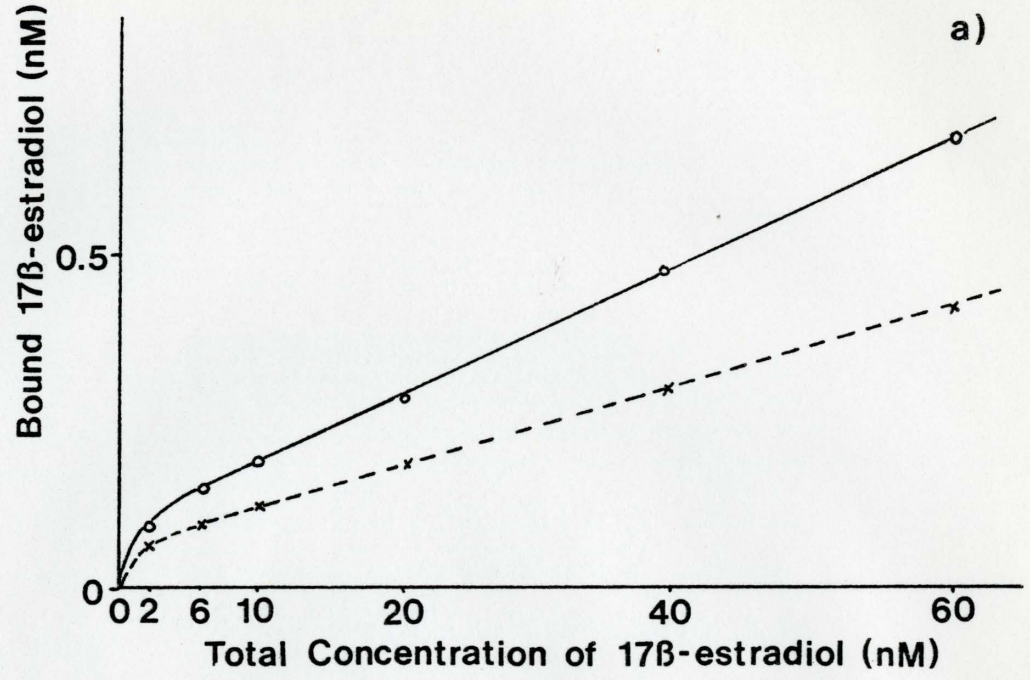
RESULTS

A) Partial Purification and Characterization

Using the equilibrium binding assay, one can determine the dissociation constants and concentrations of binding sites for 17β-estradiol in the rainbow trout liver cytosol. Two classes of binding sites were observed. The first class has a high affinity for the steroid but a low number of binding sites, whereas the second class is lower in binding affinity but has a higher number of binding sites. Figure 3 shows the results of a representative assay and the Scatchard plot of this assay. The Kd and n values calculated from this assay were 3.44 nM and 2.7 x 10^{-11}moles/g protein respectively for the type I binding component, and those for the type II binding component were 117 nM and 2.67 x 10^{-10}moles/g protein respectively. The assay was performed on 10 different preparations of cytosol and the Kd of the high affinity component had a range of 0.53 to 5.9 nM while the concentration of binding sites in this class ranged from 1.4 to 9.5 x 10^{-11}moles/g protein. The lower affinity binding component had a Kd ranging from 65 to 265 nM and concentration of sites ranging from 0.2 to 1.8 x 10^{-10}moles/g protein (Table 1). There is, on average, a 50 fold differ-

Figure 3 a) Binding of ^{3}H-17β-estradiol by rainbow trout liver cytosol, both in the presence (x---x) and absence (o——o) of 100 fold excess of DES. The cytosol was prepared in batch from 150 livers. Each point represents the average of three determinations.

b) Scatchard plot of specific binding of 17β-estradiol by rainbow trout liver cytosol. The dissociation constant (Kd) for the type I (high affinity) component is 3.44 nM and the concentration of binding sites is 27 pmoles/g protein. The Kd of the lower affinity, type II component is 117 nM while the concentration of binding sites is 267 pmoles/g protein.


a)
Bound 17β-estradiol (nM)
0.5
0
0 2 6 10 20 40 60
Total Concentration of 17β-estradiol (nM)

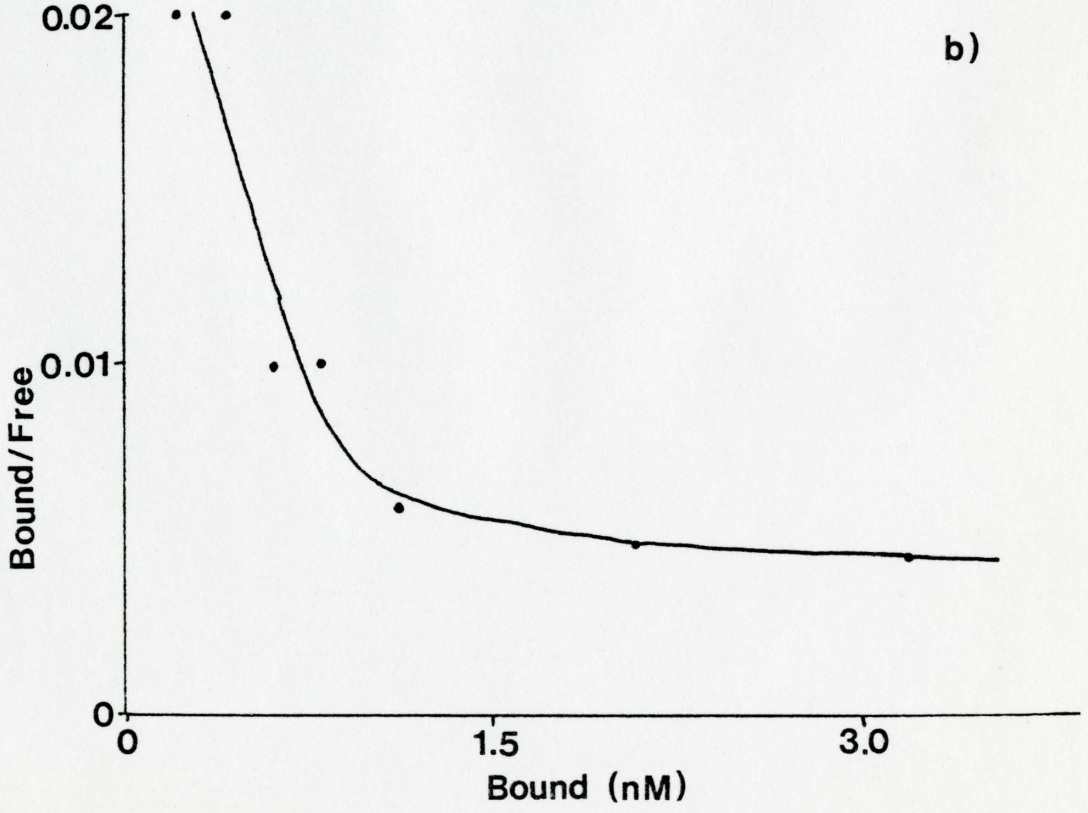

b)
0.02
0.01
0
Bound/Free
0 1.5 3.0
Bound (nM)

Table 1 Dissociation constants (Kds) and concentrations of binding sites for type I and type II classes of receptors in the rainbow trout liver cytosol.

Kd for type I (nM)	n value for type I (moles/g protein)	Kd for type II (nM)	n value for type II (moles/g protein)
2.14	9.3×10^{-11}	100	1.8×10^{-9}
0.53	5.4×10^{-11}	108	6.0×10^{-10}
2.51	9.5×10^{-11}	113	1.5×10^{-9}
1.90	8.6×10^{-11}	65	5.4×10^{-10}
1.80	1.4×10^{-11}	265	1.1×10^{-9}
1.65	1.5×10^{-11}	110	9.0×10^{-10}
2.80	3.4×10^{-11}	140	1.2×10^{-9}
2.50	3.0×10^{-11}	115	8.1×10^{-10}
5.90	4.0×10^{-11}	123	9.1×10^{-10}
3.44	2.3×10^{-11}	117	2.0×10^{-10}

Each assay was performed on the cytosol of at least two rainbow trout and at least eight different concentraions of ^{3}H-17β-estradiol were used, each concentration being done in duplicate, triplicate, or quadruplicate.

ence between the dissociation constants of the type I and type II sites and a 20 fold difference between their respective binding site concentrations. The concentration of K^+ in Buffer B in the range of 0.05 to 1 M did not affect the binding of the ligand to either class of receptors (data not shown). Furthermore, both binding components were still evident when ^{3}H-moxestrol was used as ligand instead of ^{3}H-17β-estradiol in the equilibrium binding assay (Table 2).

The possibility that serum contamination is the source of one or both classes of the cytosol binding components was examined. When the equilibrium binding assay was performed with the serum in the presence of 100 fold excess of DES, no specific binding could be discerned (that is, DES did not compete for any ^{3}H-17β-estradiol bound sites). However, when an excess amount of cold 17β-estradiol was used intead of DES, two classes of specific binding activities were detected (Fig. 4). The Kd and n values calculated for the higher affinity component were 2.63 nM and 2.5 x 10^{-11}moles/g protein respectively and those for the lower affinity component were 79 nM and 2.0 x 10^{-10}moles/g protein. Although these Kd and n values are in the same order of magnitude as those observed in the cytosol, they probably represent different binding components since the former cannot be competed out by DES. This point is further illustrated by sucrose gradient

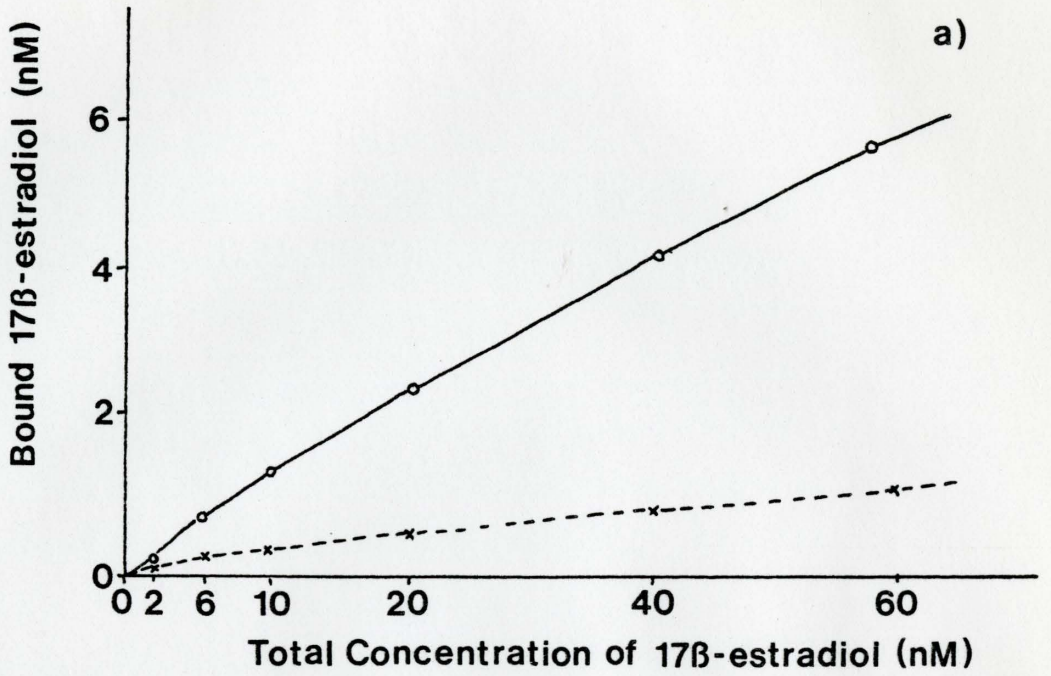
Table 2 Summary of Kd and n values using the tracers ^{3}H-17β-estradiol and ^{3}H-moxestrol.

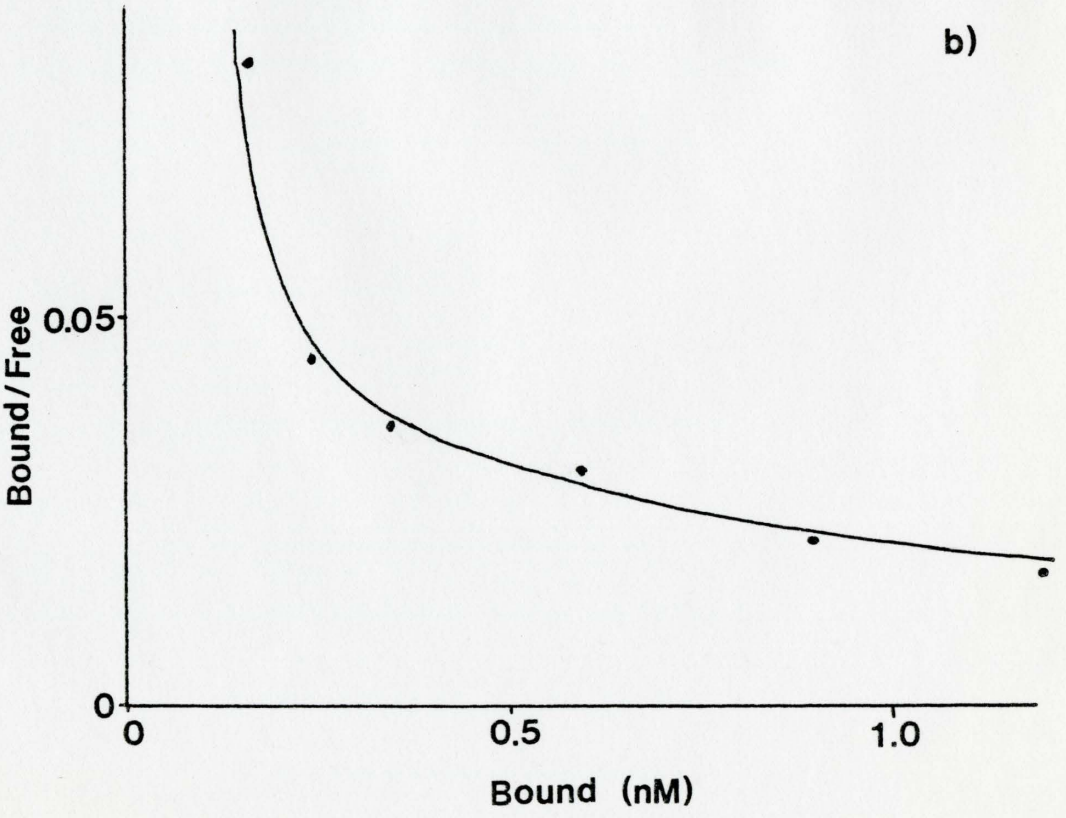
Ligand	Binding Component I		Binding Component II	
	Kd (nM)	n (nM)	Kd (nM)	n (nM)
^{3}H-17β-estradiol	2.31	0.23	287	9.60
^{3}H-moxestrol	2.01	0.08	253	1.34

Three different liver cytosol preparations were made (each from two 8-10" rainbow trout) and two binding assays were done on each preparation: one using ^{3}H-17β-estradiol as ligand, the other using ^{3}H-moxestrol. Each point in the binding assays was done in triplicate.

Figure 4 a) Binding assay performed on rainbow trout serum using ^{3}H-17β-estradiol with (x---x) and without (o——o) the presence of 100 fold excess of cold 17β-estradiol. The serum was extracted from three fish, and each point is the average of four determinations.

b) Scatchard plot of specific binding of ^{3}H-17β-estradiol by rainbow trout serum. The dissociation constants calculated are 2.63 nM for the higher affinity component and 79 nM for the lower affinity component. The concentrations of binding sites were determined to be 25 pmoles/g protein for the higher affinity component and 200 pmoles/g protein for the lower affinity component.


a)
Bound 17β-estradiol (nM)
6
4
2
0
0 2 6 10 20 40 60
Total Concentration of 17β-estradiol (nM)


b)
Bound/Free
0.05
0
0
0.5
1.0
Bound (nM)

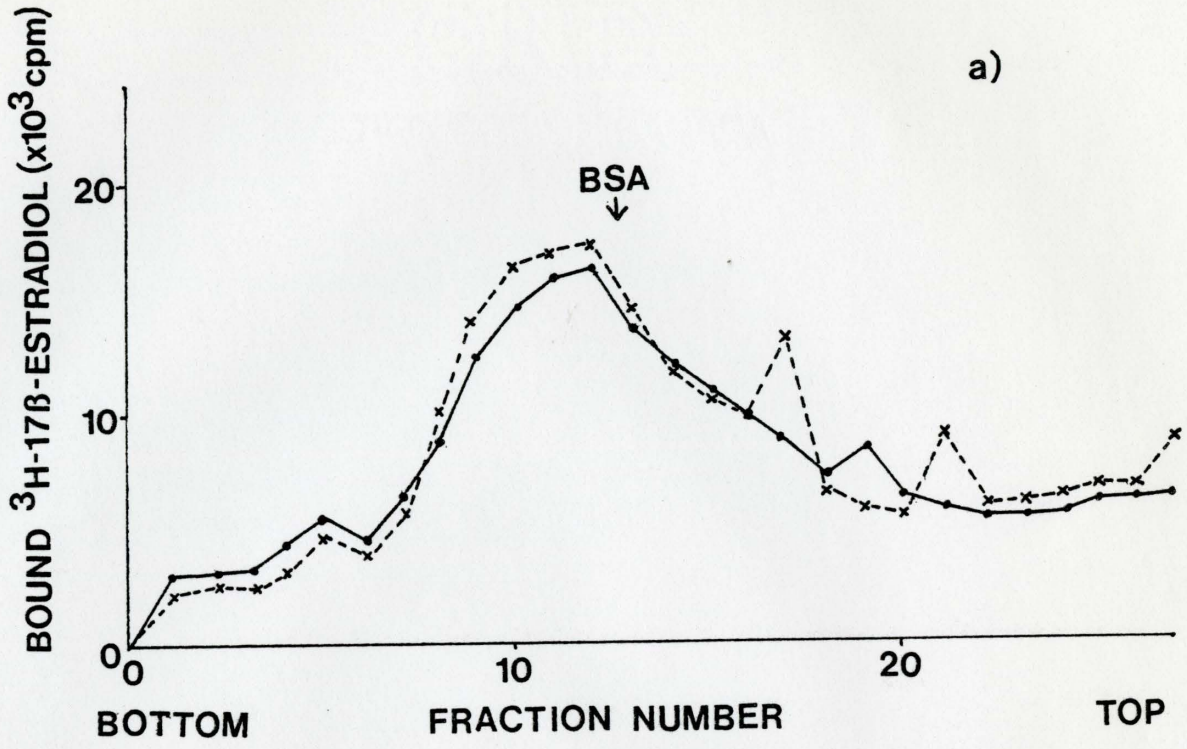
centrifugation of serum incubated with ^{3}H-17β-estradiol in the presence and absence of 100 fold excess of either DES (Fig. 5a) or cold 17β-estradiol (Fig. 5b). A large peak of ^{3}H-17β-estradiol binding was also observed, however, only 17β-estradiol, not DES, is able to compete for these binding sites.

The stability of specific estrogen binding activities in the cytosol was studied. From three independent determinations, the half life of the binding activities at 37^{o}C was found to be 60 minutes (Fig. 6). Furthermore, it was also found that there was no decrease in binding activity over the period of the assay (4 hours) at either 0 or 12^{o}C (data not shown).

The cytosolic estrogen binding sites were further characterized by sucrose gradient centrifugation. Following incubation of ^{3}H-17β-estradiol with the cytosol in the presence and absence of 100 fold excess of DES, the preparations were run through 5-20% linear sucrose gradients (Fig. 7). Two specific binding peaks were observed in the gradients. The S values for these two peaks were determined to be 4.4S and 3.7S using BSA (4.6S) as a marker. When progesterone was added to the cytosol instead of DES, a reduction in binding of 79% occurred in the 4.4S peak (Fig. 7). When the cytosol was passed through a column of Sephacryl S-200, two specific 17β-estradiol binding peaks were observed (Fig. 8). The molecular weights of these two

Figure 5 a) Sucrose gradient centrifugation of serum labelled with 17β-estradiol both in the presence (x---x) and absence (o——o) of 100 fold excess of DES. Serum was extracted from two fish, labelled overnight, and then layered over a 5-20% linear sucrose gradient and spun at 40,000 rpm for 21 hours. Fractions of 5 drops (170 ul) each were collected and counted in scintillation fluid.

b) Sucrose gradient centrifugation was carried out on serum as described above except that 100 fold excess of cold 17β-estradiol (x--- was used in place of DES.


a)
BSA
BOUND ^{3}H-17ß-ESTRADIOL (x10^{3}cpm)
20
10
0
0
10
20
BOTTOM
FRACTION NUMBER
TOP

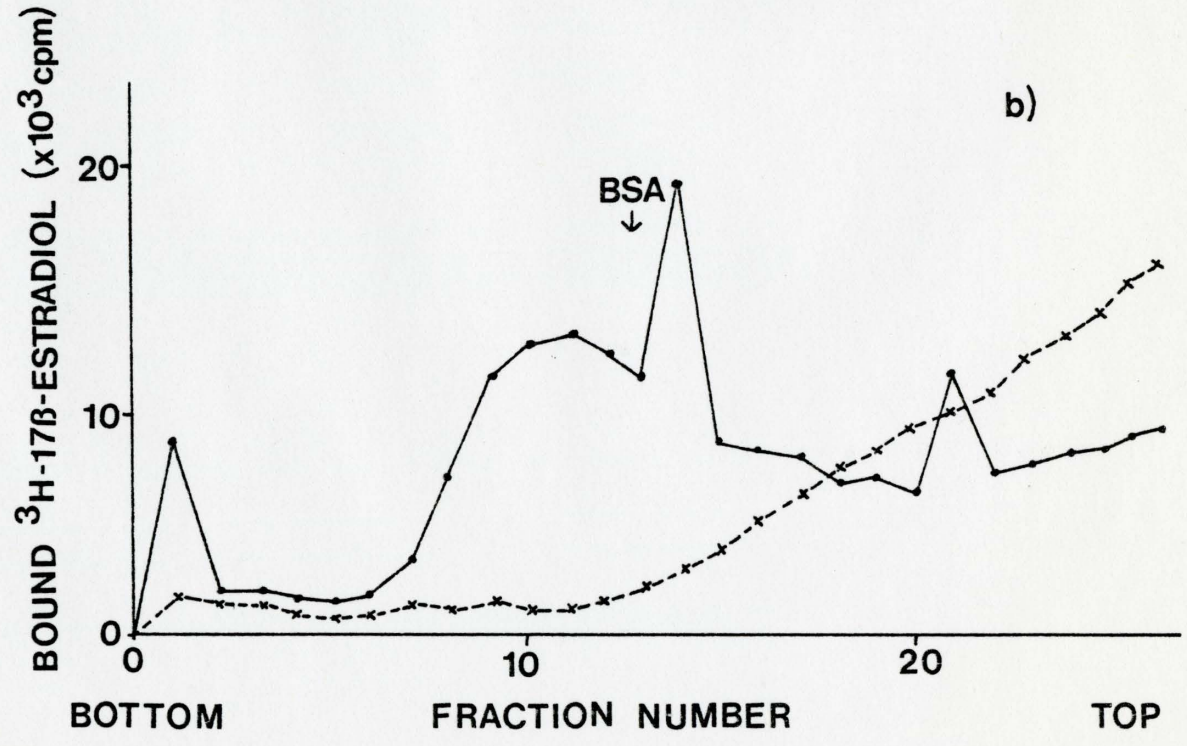

b)
BSA
BOUND ^{3}H-17ß-ESTRADIOL (x10^{3}cpm)
20
10
0
0
10
20
BOTTOM
FRACTION NUMBER
TOP

Figure 6 Degradation of 17β-estradiol binding sites in rainbow trout liver cytosol at 37°C. This experiment was performed by incubating cytosol at 37°C for various times prior to incubating overnight with ^{3}H-17β-estradiol, with and without the presence of 100 fold excess of DES, at 0°C. The specific binding is plotted against the time of incubation at 37°C. The controls were done at 0 and 12°C instead of 37°C and no decrease was observed during the time period used (4 hours).

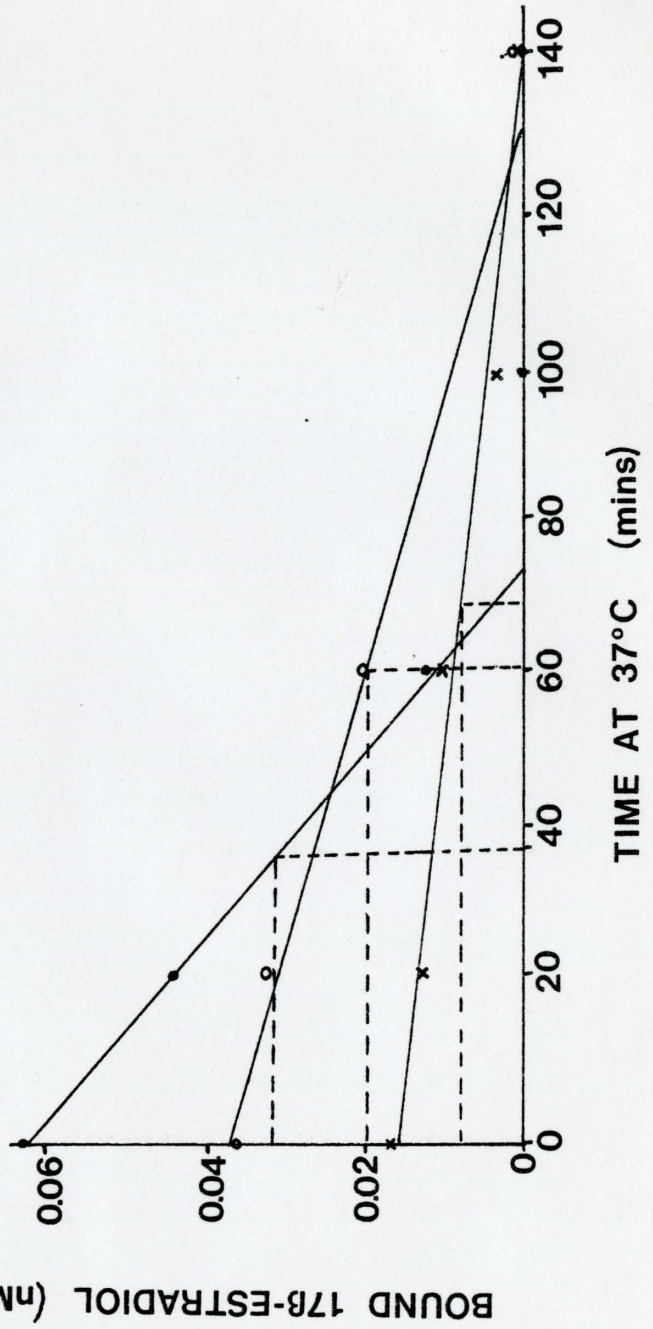

BOUND 17β-ESTRADIOL (nM)
0.06
0.04
0.02
0
0
20
40
60
80
100
120
140
TIME AT 37°C (mins)

Figure 7 Sucrose gradient centrifugation of rainbow trout liver cytosol. This experiment was done by incubating cytosol (prepared from the livers of three 8-10" rainbow trout) overnight in 20 nM ^{3}H-17β-estradiol with (x---x) and without (o——o) the presence of 100 fold excess of DES, and also in the presence of 100 fold excess of progesterone (●——●). These cytosol preparations were then layered over 5-20% sucrose gradients and spun at 40,000 rpm for 21 hours before fractionation. BSA was also run through the sucrose gradient as described, except the fractions were assayed for protein content using ultraviolet absorbance spectroscopy at 280 nm.

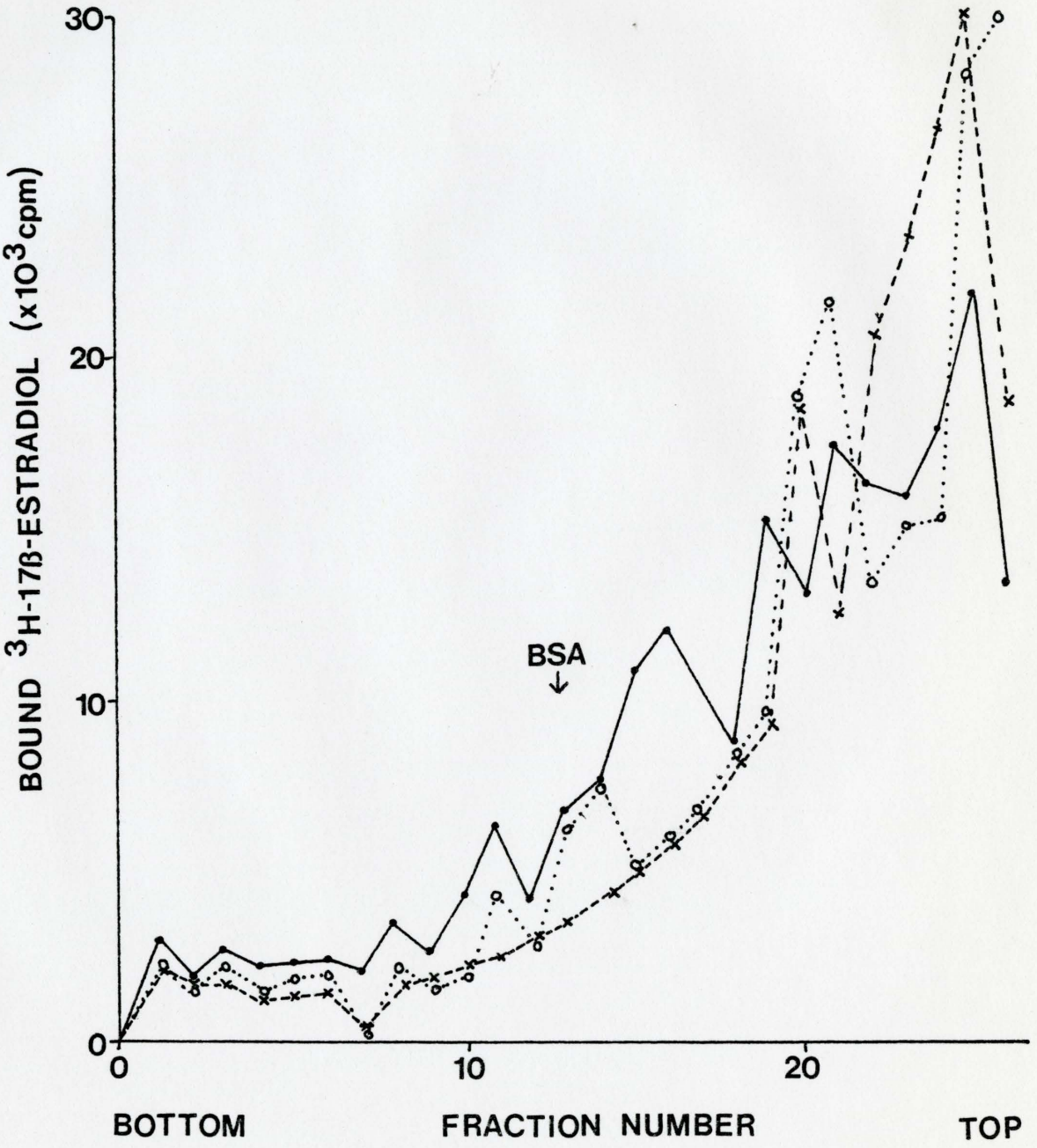
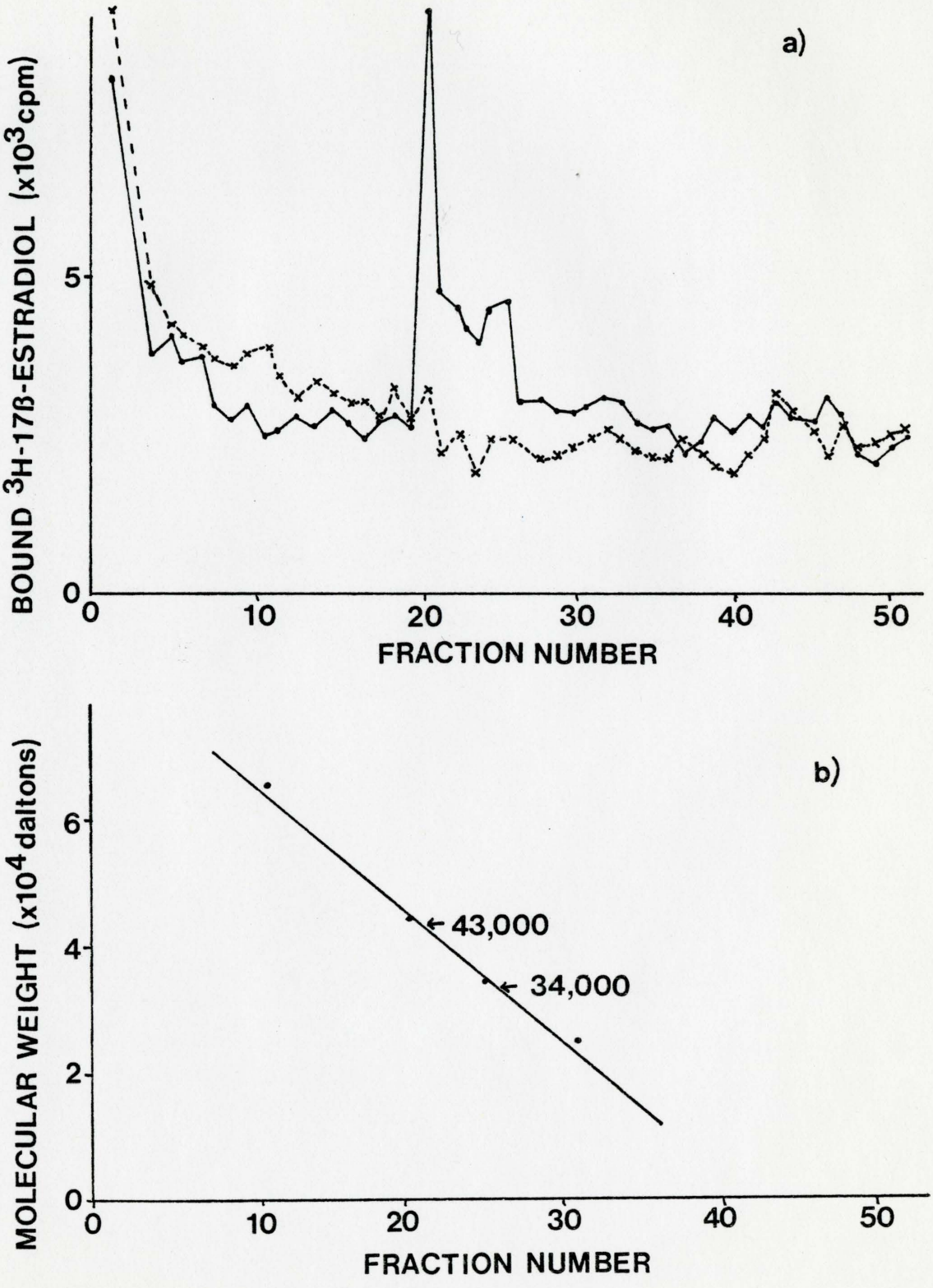

30
20
10
0
BOUND ^{3}H-17β-ESTRADIOL (x10^{3}cpm)
BSA
0
10
20
BOTTOM
FRACTION NUMBER
TOP

Figure 8 Separation of 17β-estradiol binding components of rainbow trout liver cytosol by Sephacryl S-200. a) Cytosol was extracted from the livers of two, 8-10" rainbow trout. It was incubated overnight with 20 nM ^{3}H-17β-estradiol both in the presence (x---x) and absence (o——o) of DES in 100 fold excess. The preparations were then applied to the top of a 1.5 x 35 cm column of Sephacryl S-200 and eluted with Buffer B at a rate of 50 mls/hr. Fractions of 1 ml were collected and 300 ul aliquots were counted in scintillation fluid. b) The molecular weight standards used were albumin (A) at 66,000 daltons, ovalbumin (O) at 45,000 daltons, pepsin (P) at 34,700 daltons and trypsinogen (T) at 24,000 daltons. The two estradiol-binding components were calculated to have molecular weights of 43,000 daltons and 33,000 daltons by order of elution.


a)
BOUND ^{3}H-17β-ESTRADIOL (x10^{3}cpm)
5
0
0
10
20
30
40
50
FRACTION NUMBER
b)
MOLECULAR WEIGHT (x10^4 daltons)
6
4
2
0
← 43,000
← 34,000
0
10
20
30
40
50
FRACTION NUMBER

binding components were calculated to be 43,000 and 33,000 daltons using the following molecular weight standards: albumin (66,000 daltons), ovalbumin (45,000 daltons), pepsin (34,7000 daltons), and trypsinogen (24,000 daltons). When 100 fold excess of progesterone was incubated with the cytosol before running through the column, a reduction occurred in binding to the higher molecular weight peak (data not shown).

B) Interaction with Other Steroids

The competition of various steroids for the estrogen specific binding sites in the cytosol was examined and the results are presented in Table 3. Each hormone was compared to 17β-estradiol in its ability to displace ^{3}H-17β-estradiol binding. DES was found to compete for 98% of the bound ^{3}H-17β-estradiol. Estrone was able to compete for even more sites than 17β-estradiol (121%). On the other hand, estriol and 17α-estradiol demonstrated somewhat lower competition, 54% and 69% respectively. The non-estrogenic steroids were also found to compete for ^{3}H-17β-estradiol bound sites. Progesterone and testosterone competed for 83% and 84% of sites respectively as compared to 17β-estradiol. Hydrocortisone could replace 64% of the 17β-estradiol bound sites while androstanolone could replace only 38% of these sites. These results demonstrate that some of the 17β-estradiol binding sites are also

Table 3 Relative binding affinities of steroids to the liver cytosol receptors of estrogen.

Competing unlabelled ligands	Amount ^{3}H-17β-estradiol bound (nM)	Percent Competition[a]
Control (no ligand)	0.368	-
17β-estradiol	0.106	100
DES	0.111	98
Estrone	0.051	121
Estriol	0.227	54
17α-estradiol	0.187	69
Progesterone	0.151	83
Hydrocortisone	0.200	64
Androstanolone	0.268	38
Testosterone	0.279	84

This assay was performed using a batch preparation of cytosol (150 livers). Each sample was incubated overnight with 20 nM ^{3}H-17β-estradiol plus 100 fold excess of one of the cold competitors. Each competitor is compared to 17β-estradiol in its ability to replace ^{3}H-17β-estradiol from specific binding sites.

[a]The percent competition is calculated using the following formula: $\frac{\text{Binding by competitor-Binding by 17}\beta\text{-estradiol}}{\text{Binding by control-binding by 17}\beta\text{-estradiol}} \times 100.$

capable of binding non-estrogenic steroids.

C) Depletion After Estrogen Interaction

Cytosol receptors specific to 17β-estradiol should, according to Jensen's two step model (Jensen, Susuki, Kawashima, Stumpf, Jungblutt and DeSombre, 1968), translocate to the nucleus after interacting with the hormone. This can be shown by examining the depletion of cytosol receptor sites after hormone treatment in vivo. The depletion of estrogen binding sites from the liver cytosol after 17β-estradiol treatment in vivo (10 ug/100 g fish) was studied. Livers were extracted from the treated fish at 8.5 and 24 hours post-treatment, and cytosol was prepared from these livers. These cytosol preparations were subjected to equilibrium binding assays from which the Kd and n values were calculated. The treated fish preparations showed a reduction of 74% in the number of type I sites relative to type II sites after 8.5 hours (as compared to untreated fish) and a reduction of 40% after 24 hours (Table 4). However, these results could also be explained by an increase in type II sites relative to type I sites.

D) Interaction with DDT

Studies in the rat have shown that ,p'-DDT, a component of technical grade DDT, has estrogenic effects.

Table 4 Depletion of 17β-estradiol binding sites from the rainbow trout liver cytosol.

Incubation time (hrs)	number type I sites/100 type II sites			% reduction in type I sites after treatment
	control	17β-estradiol	mock	
8.5	27 (10)	7 (10)	18 (10)	74
24	10 (30)	6 (10)	-	40

Animals of 6-8" in length were injected with 10 ug/100 gms 17β-estradiol dissolved in cod liver oil. Control fish were not injected and mock injected fish were given cod liver oil alone. The fish were sacrificed 8.5 and 24 hours later, their livers removed, and cytosol was prepared. The cytosol preparations were subjected to binding assays and Scatchard plots were constructed to determine binding site concentrations (n_1 and n_2 for type I and type II sites respectively). The number of fish used in each assay is shown in brackets. Each point in the binding assay was done in triplicate. The percent reduction in type I sites after 17β-estradiol treatment was determined by the following formula: $\frac{c-e}{c} \times 100 = \%$ reduction

where c is the number of type I sites/100 type II sites in the control and e is the number of type I sites/type II sites in the treated animals.

A competition assay was performed to discover whether this chemical could interact with rainbow trout liver cytosol estrogen receptors. It was found that o,p'-DDT was able to compete for 30% of the specific 17β-estradiol binding sites (Table 5). Furthermore, it is interesting to note that p,p'-DDT, the major component of technical grade DDT, is not only able to compete for these specific receptors (Table 5), but also to a much greater extent.

Table 5 Effects of o,p'-DDT and p,p'-DDT on 17β-estradiol binding sites in the Rainbow Trout liver cytosol.

Concentration	a) Concentration of bound ligand (nM)				b) % Competition			
of ligand	Control	DES	o,p'-DDT	p,p'-DDT	Control	DES	o,p'-DDT	p,p'-DDT
1 nM	0.028(3)	0.019(9)	0.026(9)	0.021(9)	0	100	29	71
5 nM	0.117(3)	0.063(9)	0.103(9)	0.100(9)	0	100	26	31
20 nM	0.331(3)	0.311(9)	0.324(9)	0.296(7)	0	100	30	175

This assay was carried out using the same procedure as the competition assay. a) This shows the concentration of bound ^{3}H-17β-estradiol in the presence of the three cold competitors, DES, o,p'-DDT, and p,p'-DDT. The cytosol was incubated with three different concentrations of ^{3}H-17β-estradiol. The number of repeats is shown in brackets. b) The % competition is shown in this table. o,p'-DDT and p,p'-DDT are compared to DES in their ability to reduce ^{3}H-17β-estradiol binding.

DISCUSSION

A) Partial Purification and Characterization

A biphasic curve was obtained when rainbow trout liver cytosol was used in equilibrium binding assays, with ^{3}H-17β-estradiol as ligand. The Scatchard plots of these assays demonstrated the presence of two types of estrogen binding sites; type I with a high affinity, low capacity (Kd=0.53-5.9 nM, n=14-95 pmoles/g protein) and type II with a lower affinity, higher capacity (Kd=65-265 nM, n= 20-180 pmoles/g protein). The variations found in the Kd and n values are possibly due to several factors; individual differences from fish to fish, differences in the recovery of binding sites from preparation to preparation, and different times of year in which the fish were sacrificed.

In the chicken, only one type of estrogen binding protein was apparent (Lazier and Haggarty, 1979), however, the dissociation constant of that receptor (0.4-2.6 nM) was similar to that of the rainbow trout type I binding sites. The concentration of the chicken liver cytosol binding sites (16-30 pmoles/g protein) was also within the range for rainbow trout type I binding sites. Therefore, the higher affinity, lower capacity type I binding sites

of the rainbow trout liver cytosol may be analogous to the estrogen receptor found in the chicken.

Perhaps one of the two estrogen binding components of rainbow trout liver cytosol is an artifact caused by binding to metabolites of ^{3}H-17β-estradiol. Alternately, one or both components may be metabolic enzymes or other non-receptor molecules. It had been shown that the synthetic estrogen, moxestrol, is useful in receptor studies because it showed less binding to non-receptor protein (Okret, Wrange, Nordenskjold, Silversward and Gustofssen, 1978). It formed tighter complexes with estrogen receptors (Bouton and Raynaud, 1979), and it was metabolized much more slowly (Raynaud, Bouton, Gallet-Bourquin, Philibert, Tournemine and Azadian-Boulanger, 1972). DeBoer, Shippe, Ab and Gruber, (1982), used ^{3}H-moxestrol as the ligand in equilibrium binding assays in cockerel liver cytosol. These workers obtained a higher binding affinity (Kd=0.14-0.18 nM) than did Lazier and Haggarty, (1979), who used ^{3}H-17β-estradiol as ligand. This difference is probably a result of the tighter complexes formed by moxestrol with the receptor molecules. ^{3}H-moxestrol was used in place of ^{3}H-17β-estradiol in the equilibrium binding assay on rainbow trout liver cytosol. Both classes of binding sites were still apparent, with Kd values similar to those found using ^{3}H-17β-estradiol as ligand (Table 2). The only difference was that much lower concentrations of binding

sites were found with the synthetic estrogen. Assuming that moxestrol is not readily metabolized in rainbow trout liver cytosol, then it may be concluded that neither of the two classes of binding sites are artifacts due to binding of non-receptor proteins by labelled metabolites of the ligand or binding of the intact ligand to metabolic enzymes. The lower n values obtained with ^{3}H-moxestrol may be a result of a more selective binding of the ligand to receptors (that is, the synthetic estrogen may not readily bind damaged or conformationally altered binding sites).

Contamination of the rainbow trout liver cytosol by serum estrogen binding components may account for one or both classes of binding activities. Serum binding of estrogen in rainbow trout was studied by Foster and Breton, (1975), and two binding classes were detected. One class demonstrated a high affinity and low capacity for 17β-estradiol (Kd=5.6 nM, n=11.6 uM). The binding affinities of these two classes were similar to those found in the liver cytosol. In this study equilibrium binding assays carried out on the serum using DES as the cold competitor showed no specific binding. However, when cold 17β-estradiol was used instead of DES, two classes of specific binding sites were observed (Fig. 2) and their binding affinities were similar to those found by Foster and Breton, (1975). Therefore, unlike the cytosol binding classes, the serum does

not contain sites capable of binding the non-steroidal estrogen, DES. The point is further illustrated by sucrose gradient centrifugation of the rainbow trout serum. A single 17β-estradiol binding peak is observed which sediments in the region of 3-6S (Fig.5). Foster and Breton, (1975), found a somewhat narrower peak in the region of 5-6S. This peak of ^{3}H-17β-estradiol binding is competed out only by an excess of the same non-radioactive steroid, but not only by an excess of DES (Fig.5). Therefore, it is unlikely that rainbow trout liver cytosol estrogen binding sites can be accounted for by serum contaminants because the cytosolic receptors, unlike those found in the serum, can be bound by the non-steroidal estrogen DES.

Next, the size of the estrogen binding components in the cytosol was examined by sucrose gradient centrifugation and by gel filtration. Sucrose gradient centrifugation demonstrated the presence of two specific estrogen binding peaks; one at 3.7S, the other at 4.4S (Fig. 7). In the chicken, two specific estrogen binding peaks were seen, one at 4.9S, and the other, a result of dimer formation, at 9.6S (DeBoer, Shippe, Ab, and Gruber, 1982). This dimer formation is not seen in the rainbow trout liver cytosol. In the rat liver cytosol, the high affinity component sediments at 8-9S while the lower affinity binding class sediments at 4S (Chamness, Costlow, and McGuire, 1975).

Two estrogen binding peaks were also obtained after passing the ^{3}H-17β-estradiol bound rainbow trout liver cytosol through a column of Sephacryl S-200. The molecular weights estimated for these peaks were 33,000 daltons and 43,000 daltons (Fig. 6). In the chicken, the estrogen receptor was estimated to be 40,000 daltons by gel filtration (Lazier and Haggarty, 1979). This value falls between the molecular weights estimated for the rainbow trout estrogen binding sites.

Therefore the sizes of the two estrogen binding components of the rainbow trout liver cytosol were shown to be 3.7S (MW 33,000) and 4.4S (MW 43,000). To demonstrate differences in the binding specificities between these two binding peaks, progesterone was added with ^{3}H-17β-estradiol to the cytosol. In the sucrose gradient, progesterone was able to compete out 79% of the radioactive ligand from the 4.4S peak, but not from the 3.7S peak (Fig. 7). That is, the 4.4S binding sites are able to bind progesterone as well as 17β-estradiol. In gel filtration of the cytosol, again progesterone was able to compete for specific 17β-estradiol binding sites in the higher molecular weight peak (43,000 daltons) but no in the lighter peak (33,000 daltons). Therefore, it is possible that the heavy binding component of the rainbow trout liver cytosol is a general steroid binding protein (and therefore accounts for the binding of non-estrogens in the competition

assay) while the lighter component is specific for estrogens.

The rainbow trout liver cytosol receptors were further characterized. Their stability at 37°C was examined, and their half life was determined to be 60 minutes (Fig. 6). There was no decrease in receptor binding after 4 hours of incubation at either 0 or 12°C. This finding was not surprizing because the rainbow trout thrive at temperatures between 8°C and 12°C and will die if kept at temperatures above 20°C for very long. In the serum, 17β-estradiol binding dropped to 5% of its initial value after 30 minutes at 60°C (Foster and Breton, 1975). Although these serum proteins are not the same as the cytosol binding proteins, this does give another example of the thermolability of rainbow trout estrogen receptors.

B) Interaction with other Steroids

The rainbow trout liver cytosol receptors were further characterized by studying their hormone specificity. Both estrogens (17β-estradiol, 17α-estradiol, estrone and estriol) and non-estrogens (progesterone, testosterone, hydrocortisone and androstanolone) were able to compete for ^{3}H-17β-estradiol bound sites to various degrees in the rainbow trout liver cytosol (Table 3). This is unlike the situation found in the cockerel liver cytosol where only estrogens are able to compete for the estrogen receptor binding (Lazier and Haggarty, 1979). Perhaps this diff-

erence can be accounted for by the presence of one of the two binding classes in rainbow trout liver cytosol. In the rat liver cytosol, Chamness, Costlow and McGuire (1975) reported the presence of two estrogen binding sites, one with a high affinity and low capacity (Kd=0.1 nM, n=10 pmoles/g protein) that was specific for estrogen, and the other with a lower affinity and higher capacity (Kd=10 nM, n=100 nmoles/g protein) that was found in mature males and was specific for estrogens and androgens. In an analogous way, one of the two classes of binding sites in the rainbow trout liver cytosol may be estrogen specific, while the other is a general steroid binding protein.

C) Depletion After Estrogen Interaction

Jensen, Suzuki, Kawashima, Stumpf, Jungblutt, and DeSombre, (1968), proposed that receptors interact specifically with estrogen and then translocate to the nucleus to change the expression of certain genes. In the rainbow trout liver cytosol, it would seem logical that only an estrogen specific binding component would translocate to the nucleus after 17β-estradiol treatment. The hypothesis is that the type I binding sites are estrogen specific and translocate to the nucleus after hormone interaction, while the type II sites are general steroid binding sites that do not directly affect gene expression. The reasoning is that since the type I sites have a higher affinity for 17β-

estradiol, and a lower capacity, they would be able to bind to low amounts of estrogens and still alter gene expression. The type II sites may function to accumulate excess steroids in the cytosol, or perhaps they are precursors of type I sites. To be consistent with size analysis data, the type I sites would correspond to the 3.7S, 33,000 dalton component while the type II sites would correspond to the 4.4S and 43,000 dalton component which binds progesterone as well as 17β-estradiol.

The depletion of cytosolic estrogen binding sites was studied in the cockerel liver after 17β-estradiol treatment in vivo (Lazier and Haggarty, 1979). This depletion reached a maximum (80%) at 1.5 hours after treatment, and then slowly increased to control values. At the same time analysis of the concentration of salt soluble nuclear receptors demonstrated a corresponding increase in binding sites. In the rainbow trout, type I sites were depleted relative to type II sites (Table 4). It was necessary to describe the depletion in this manner because the concentration of binding sites in different preparations of cytosol cannot be directly compared, and therefore it is necessary to assume that the concentration of type II sites does not change (this may not be a valid assumption as these sites may increase in concentration). In any case, given the assumption, the type I sites are 74% depleted after 8.5 hours and only 40% depleted after 24 hours.

This implies that maximum depletion occurs prior to 24 hours when binding sites are already partially replenished.

D) Interaction with Steroids

The estrogenic activity of DDT was first noticed when Leghorn cockerels treated with DDT had decreased testis growth and diminished development of secondary characteristics (Burlington and Lindeman, 1950). It was determined that o,p'-DDT, which constitutes from 11% to 28% of technical grade DDT, was estrogenic and increased weight, water content, glycogen and RNA in uteri and oviducts of rat, chicken and quail (Bitman, Cecil, Harris, and Fries, 1968). Studies on the effects of DDT on fertility range from no effect in rats (Ottoboni, 1969; Wren, Weyant, Fries, and Bitman, 1971) to a decreased frequency of ova implantation after mating in mice (Lundberg, 1973). However, it has been reported that o,p'-DDT given to neonatal female rats causes permanent sterility (Heinrichs and Gellert, 1971). In the gull, o,p'-DDT causes feminization in male embryos which possibly accounts for skewed sex ratios and poor breeding success noted in some gull populations (Fry and Toone, 1981). It appears that o,p'-DDT acts via estrogen receptor binding in humans, rats and quail (Nelson, 1974; Kupfer and Bulger, 1977; Turner and Eliel, 1978). The mechanism is not o,p'-DDT mediated destruction of estrogen receptors (Forster, Wilder, and

Heinrichs, 1975) and o,p'-DDT does translocate the estrogen receptor to the nucleus of the rat uterus (Kupfer and Bulger, 1980).

The effect of DDT in the rainbow trout has not been studied. However, significant amounts of both p,p'-DDT and o,p'-DDT have been found in the related species, Coho Salmon, in the Great Lakes (Sonstegard, Leatherland, Mukhtar, and Bend, 1984). Therefore it is likely that the rainbow trout as well, is contaminated by this insecticide. The possible consequences of this are feminization of male fish, and permanent sterility in the female. Needless to say, these effects could have a dramatic impact on the natural fish populations.

To discover whether DDT has even the potential of affecting the breeding success of rainbow trout, a simple competition assay was performed on liver cytosol preparations. The results showed that o,p'-DDT was able to compete for 30% of specific 17β-estradiol binding sites (Table 5). This means that o,p'-DDT may be estrogenic, although further studies must be performed (its effect <u>in vivo</u>, its ability to translocate the receptor to the nucleus, etc.). The results also showed that p,p'-DDT was able to bind to the estrogen receptor (Table 5). Some studies have demonstrated that p,p'-DDT has an effect on fertility (Lundberg, 1973), and is estrogenic although to a lesser extent than o,p'-DDT (Singhal, Valadares, and

Swark, 1970; Gellert, Heinrichs, and Swerdlaff, 1972). However, other researchers have shown p,p'-DDT to be inactive estrogenically (Bitman, Cecil, Harris, and Fries, 1968; Duby, Travis, and Terrill, 1971). Again further studies must be carried out to determine the effects of these two components of the insecticide DDT on the rainbow trout.

In conclusion, the rainbow trout liver cytosol contains two types of estrogen binding sites, one with a Kd=0.53-5.9 nM, n=14-95 pmoles/g protein, and the other with a Kd=65-265 nM, n=20-180 pmoles/g protein. Both types are bound by ^{3}H-moxestrol as well as ^{3}H-17β-estradiol, which implies that neither class is composed of non-receptor proteins (eg. metabolic enzymes) or is an artifact of binding by metabolites of 17β-estradiol. Characterization of the rainbow trout serum demonstrated that its estrogen binding sites, unlike the cytosolic binding sites, were not able to bind the non-steroidal estrogen, DES. Therefore, it is unlikely that either class of estrogen binding sites in the cytosol is present due to serum contamination. The study of hormone specificity of the cytosolic 17β-estradiol receptors revealed that both estrogens and non-estrogens were able to compete for binding. A study of the size of the two binding components was made using sucrose gradient centrifugation and gel filtration of the liver cytosol. With each technique two estrogen binding peaks were

apparent, one which sedimented at 4.4S, and had a molecular weight of 43,000 daltons, and the other which sedimented at 3.7S and had a molecular weight of 33,000 daltons. The heavier of the two peaks contained sites capable of binding progesterone as well as 17β-estradiol. Therefore, one class of binding sites may be capable of binding non-estrogens as well as estrogens, while the other may be able to bind estrogens alone. The cytosolic estrogen binding sites were shown to have a half life of 60 minutes at 37°C, that is, they are thermolabile. Evidence supports the depletion of type I sites from the cytosol, probably to the nucleus. And finally, components of the insecticide DDT, o,p'-DDT and p,p'-DDT were able to compete for 17β-estradiol bound sites in the rainbow trout liver cytosol, and therefore may be capable of altering the expression of estrogen inducible genes.

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