PARTIAL PURIFICATION OF AND ATTEMPTED MONOCLONAL ANTIBODY PRODUCTION AGAINST THE VERVET MONKEY HEPATIC OESTROGEN-BINDING PROTEIN

by

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ABSTRACT

Hepatic cytosols from vervet monkeys (Cercopithecus aethiops pygerythrus) were found to contain high affinity oestrogen-binding proteins, In view of the interest in the action mechanism of steroid hormones in mammalian target organs, the vervet monkey was employed as an experimental model in this investigation.

Multipoint saturation analyses revealed an equality in the levels of HEBP present in male and female animals. An additional high capacity, moderate affinity oestrogen-binder has been identified. This binder appears to be more prominent in male animals. SDG analyses established the presence of a 3,8S together with an 8,1S oestrogen-binding component in the hepatic cytosol of both sexes. Two oestrogen-binding components (400 000 and 42 000 daltons) were identified in hepatic cytosol with GPC.

The vervet monkey HEBP was partially purified (approximately 7 435-fold) by a combination of DES-Agarose and heparin-Sepharose affinity chromatography. This procedure yields an average of 42 µg HEBP from 50 g vervet monkey liver. Following affinity chromatography the partially purified HEBP migrated as three bands (30 000, 38 000 and 68 000 daltons) on an SDS polyacrylamide gel. Isoelectric focussing demonstrated pI values of 6,8 and 6,9 for the partially purified HEBP.

Partially purified HEBP was employed to immunize C57 Black/6 mice. The serum from the immunized animals contained antibodies against the partially purified preparation as determined by ELISA. Splenic lymphocytes from the immunized mice were fused with cells of the Sp2 myeloma cell line to yield hybridoma cultures, 43% of which produced antibodies against the immunized preparation. After cloning of the positive polyclonal cell cultures on agar, the selected clones were screened. No antibody activity could, however, be detected in the spent media of the monoclonal cell cultures.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

L Bornman

28 th day of April, 1988

This dissertation is dedicated
with much love to my husband,
Koos

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CONTENTS

PREFACE

The mechanism of action of oestrogenic hormones in target tissues has been extensively studied during the past two decades. Theories originated from the research to define the substantial role of steroid hormone receptors in target tissues. These theories were applied in order to aid the knowledge of hormone responsive tumours and the subsequent development of a sufficient drug for endocrine therapy of e.g. breast cancer patients. However, the practical evaluation of the theories postulated was generally unsatisfactory. An animal model which resembles that of man closely can aid the complete understanding of the *in vivo* action mechanism of steroid hormones in order to overcome the problems experienced in humans.

In view of the interest in the action mechanism of steroid hormones in mammalian target organs and the search for a model closer to man, the non-human primate, *Cercopithecus aethiops pygerythrus*, commonly called the vervet monkey, was employed as an experimental model in this study. On account of the limited availability of uterine tissue the liver of the vervet monkey was the target organ used to investigate the oestrogen-binding protein. Because the complete understanding of the ER action mechanism is hampered by the lack of a pure receptor this study was aimed at the development of a purification protocol for the oestrogen-binding protein and the production of monoclonal antibodies. The latter may provide a powerful tool in revealing the poorly understood hormone receptor mechanism.

Chapter 2 will be published as an article in the **Journal of Steroid Biochemistry** in August 1988 under the title: 'Hepatic estrogen-binding proteins in male and female vervet monkeys'.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
B_{max}	Binding capacity
BSA	Bovine serum albumin
CBG	Corticosteroid binding globulin
cm	centimetre
DCC	Dextran coated charcoal
DES	Diethylstilboestrol
DHEA	Dehydroepiandrosterone
d.H_2O	Distilled water
DHT	Dehydrotestosterone
DMBA	Dimethylbenzanthracene
DMEM	Dulbecco's modified Eagle's medium
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
E_1	Oestrone
E_2	Oestradiol
E_3	Oestriol
EDTA	Ethylenediaminotetraacetate
EE_2	Ethinyloestradiol
ELISA	Enzyme linked immunosorbent assay
ERs	Oestrogen receptor(s)
fmol	femtomol
FNH	Focal nodular hyperplasia
g	gram
g	gravitational constant
GPC	Gel permeation chromatography
GR	Glucocorticoid receptor
h	hour
HA	Hepatic adenoma
HAT	Medium containing hypoxanthine, aminopterine and thymidine
HC	Hydrocortisone

HCC	Hepatocellular carcinoma
HCLA	High capacity low affinity
$^{3}H\text{-}E_2$	Tritiated oestradiol
HEBPs	Hepatic oestrogen-binding protein(s)
HPLC	High performance liquid chromatography
HT	Medium containing hypoxanthine and thymidine
IEF	Isoelectric focussing
Ig	Immunoglobulin
IgY	Chicken anti-mouse Ig
K_a	Associat constant
K_d	Dissociation constant
M	Molar
mA	milliampere
McAb	Monoclonal antibody
mg	milligram
µℓ	microlitre
µM	micromolar
min	minute
mℓ	millilitre
mM	millimolar
m/v	mass per volume
NE	Norethindrone
nm	nanometre
n.m.	not measurable
nM	nanomolar
NSB	Non-specific binding
OC	Oral contraceptive
OCS	Oral contraceptive steroids
P	Progesterone
PBS	Phosphate buffered saline
PcAb	Polyclonal antibody
PEDGP	Phosphate buffer (Section 2.2.2)
PEG	Polyethyleneglycol
pH	Hydrogen ion concentration
pI	Isoelectric point
Ppt	Precipitate

PR	Progesterone receptor
R5020	Promogestone
RBA	Relative binding affinity
S	Svedberg unit (10^{-13}sec)
SA	Specific activity
SB	Specific binding
SBP	Specific binding protein
SDG	Sucrose density gradient
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SM	Sodium molybdate
Supn	Supernatant
T	Testosterone
TB	Total binding
TEDAG	Tris buffer (Section 2.2.2)
TEDAGM	Tris buffer (Section 2.2.2)
TEDAGMT	Tris buffer (Section 4.2.2)
TEDMg	Tris buffer (Section 3.2.2)
TEDMgM	Tris buffer (Section 3.2.2)
TEM	Tris buffer (Section 3.2.2)
Tris	Tris(hydroxymethyl)aminomethane
UEBP	Unusual oestrogen-binding protein
V	Volt
VMUC	Vervet monkey uterine cytosol
V_o	Void volume
V_t	Total column volume
v/v	volume per volume
W	Watt

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF STEROID HORMONE RECEPTORS

A correlation between the phase of the menstrual cycle and the size of breast tumours as well as a relationship between an increased risk of breast cancer and multi-parity were observed as early as 1835 (1). In 1889 Schinzinger (2) suggested an oophorectomy to increase the life expectation in young patients, following his observation that the fatality among premenopausal breast cancer patients appeared to be higher than that recorded for postmenopausal patients. The advantageous effect of an oophorectomy in the fight against advanced breast cancer was demonstrated by Beatson (3) in two premenopausal patients in 1896. It became, however, evident that a positive response of the patient upon oophorectomy could not be guaranteed (4,5). Although irradiation castration (6), rather than surgical oophorectomies was popular at one stage, the latter procedure was employed again in the 1950's (7). The isolation of oestrogen from sow ovaries was a great step forward in the process of understanding the chemical principles of endocrine response (8). Charles Huggins made a very important contribution with his studies on the effect of steroid hormones on cancer. Although, at first his studies were focussed on carcinoma of the prostrate (9-11), he eventually got involved in research on the causes of mammary carcinoma (7, 12, 13). He demonstrated that a relapse after an oophorectomy can often be overcome by adrenalectomy (7, 12). The modern concept of endocrine therapy is formulated on Huggins' theory that tumour growth is influenced by the hormonal function of the host (13). Luyt and Olivectrona (14) reported that hypophysectomy effected a decrease in the frequency of metastatic breast cancer, similar to that

achieved by oophorectomy and adrenalectomy. This observation was late confirmed by Pearson et al. (15).

The synthesis of tritium labelled anti-oestrogen (16) and oestradiol (17) made it possible to illustrate the specific accumulation of oestrogens in target tissues. In 1966 Mobbs (18) illustrated the retention of labelled oestradiol in dimethylbenzanthracene (DMBA) - induced rat mammary tumours. Autonomous tumours did not appear to retain labelled oestradiol. With the aid of radioactive labelled hormones, two general classes of breast cancer were identified, those that demonstrated uptake of oestradiol and those that showed no hormonal uptake (19, 20). Sucrose density gradient centrifugation studies done by Toft and Gorski (1966) established the presence of a specific, high affinity, low capacity oestrogen-binding protein, termed the oestrogen receptor (ER) in target tissue cytosol (21). With the aid of sucrose density gradients it was possible to determine that tumours, responding positively to hormonal therapy, contain higher levels of the ER. Hormone-dependent tumours and autonomous tumours were therefore determined prior to therapy by this method (19).

In 1968 Jensen et al. (22) and Gorski et al. (23) proposed a two-step model for the action mechanism of steroid hormones. According to their model oestrogen diffuses into the cytoplasm, where it binds to the ER. The resultant complex is 'activated' and translocated to the nucleus where it binds to chromatin with a high specificity and affinity and subsequently induces the synthesis of specific m-RNA molecules. In confirmation of this model, Stumpf (24) observed the movement of the steroid hormone to the nucleus by employing autoradiography.

Different forms of the ER were observed in subsequent studies. One form had a relative sedimentation coefficient of 8S under low ionic strength conditions and which dissociated into 4S complexes under high ionic strength conditions (21, 25).

Jensen et al. (26) reported that a hormone-dependent, temperature-induced process appears to transform the cytosolic 8S receptor into a 5S form. It was subsequently proved that the transformed receptor exhibits a higher affinity for polyanions like DNA (27, 28). This transformed receptor was therefore termed the 'activated' form and the foregoing process 'activation'.

In 1974 the general agreement between 14 institutions was made that patients with receptor-negative cancers would not benefit from hormonal manipulations while those with receptor-positive cancers would. This conformity was established at a workshop held under the protection of the Breast Cancer Task Force.

1.2 MECHANISM OF ACTION OF THE STEROID HORMONE RECEPTOR

The mechanism of steroid hormone action was investigated over the last three decades by a great number of multifarious techniques. Initially, analytical techniques established to examine the steroid hormone receptor depended upon cell disruption, extraction of receptors and subsequent binding to radioactive labelled hormone. These early techniques have provided valuable information, but to clarify the subcellular effects and mechanism of hormone action via the receptor needed new approaches. More advanced techniques are needed for the investigation of subcellular compartmentalization and receptor recycling or resynthesis in intact cells. Recently developed techniques include the use of labelled mono- and polyclonal antibodies (29-32), cytochalasin B-induced enucleation (33), radioactive covalent antioestrogen affinity labels (34) and autoradiography (35, 36). Investigation of steroid-induced events in mutant cell lines that contain depressed levels or abnormal receptor (37) or resistent cell lines, provides excellent means for revealing some of the more complex aspects of the molecular biology of the oestrogen receptor and steroid hormone action mechanisms (38, 39).

1.2.1 Proposed models for steroid hormone receptors

As stated previously Jensen et al. (22) and Gorski et al. (23) proposed the well known 'two-step' model in 1968. It appeared as if the receptor was localized in the uterine cytosol (21). By employing cell fractionation and autoradiography it could be illustrated that the receptor actually accumulates in the nucleus of target cells, following hormone administration (22). In this model the ER is described as a cytosolic protein that becomes activated after binding to oestrogen. The hormone-receptor complex is then translocated to the nucleus by a temperature- dependent 'activation' process. A change in sedimentation coefficient (4S to 5S) is established by the 'activation' process and nuclear receptors can exist only as steroid filled entities. With the aid of immunocytochemical and enucleation experiments the nuclear localization of the ER was observed and as a consequence the nuclear model was proposed (29, 33, 40, 41). Receptors not occupied with hormone were in fact found to be present in nuclei of normal (42-44) and tumourous oestrogen target tissues (45, 46). These unoccupied receptors appear to exhibit characteristics that resemble those of the classical nuclear ER (47), except that they are unoccupied. The presence of oestrogen receptor binding sites in nuclear preparations has previously been reported (48, 49). An equilibrium situation with respect to the intracellular distribution of intracellular steroid receptors has been suggested following autoradiography studies of the uptake of tritiated oestradiol by target cells (50). Apparently an equilibrium is established between the cytoplasm and nucleus, which can be shifted in favour of the cytoplasm during homogenization. Thus the unoccupied receptor which is quantitively assayed in the cytosol, obtained by employing conventional cytosol preparation techniques, only makes up a small fraction of the total receptor contents of the target cell (50).

Welshons et al. (33) employed cytochalasin B-induced enucleation to obtain cytoplast and nucleoplast fractions from receptor-containing GH_3 cells, derived from rat pituitary tumours. This technique appears to overcome the problem of receptor solubilization during the preparation of cell nuclei. The results obtained during this study indicated clearly that the receptor is localized in the target cell nucleus. Very low levels of oestrogen-binding activity could be traced in the cytoplasts. These findings led to the proposal of the nuclear model in which both oestrogen-binding and activation occur within the target cell nucleus. The biological relevance of the cytosolic ER and its so-called nuclear translocation can be seriously questioned following the pioneering work of Welshons and his group. The cytosolic ER can well be seen as an extraction artifact. Furthermore the cytosolic receptor recovered in the low salt fraction of a tissue homogenate is thought to be receptor that was loosely bound to nuclear components and the translocation artifact is attributed to increased nucleotrophy of the ER-oestrogen complex.

It was generally believed that antibodies to the ER might reveal some of the most controversial aspects concerning the mechanism of action of the ER. During immunohistochemical studies it became quite evident that the unliganded ER is mainly localized in the nucleus (29). The group of King and Greene used monoclonal antibodies against the ER to provide information about inter- and intracellular receptor distribution in different tisues by an indirect immunoperoxidase technique (30-32). Regardless of hormonal status, the specific immunoperoxidase staining for the ER was observed in the nuclei of all stained cells. Little, or no cytoplasmic staining could be detected in any of the preparations even when tissues were deprived from exogenous oestrogens. Following in vivo or in vitro treatment of the cells, the staining of nuclei appeared to be even more intense, yet, no cytoplasmic staining could be observed. These

results correlated with the enucleation experiments of Welshons et al. (33), in which unoccupied ER was almost exclusively partitioned into the nucleoplast fraction.
There are, however, some doubts about the immunocytochemical and enucleation evidence for the nuclear localized ER. It has previously been shown that the primary anti-ER monoclonal antibodies of King and Greene are incompetent in recognizing the ER (51, 52). The technique of enucleation is powerful in the study of cytoplasmic/nuclear interaction in cell fusion experiments but its usefulness to localize the ER is uncertain (53). It should also be noted that in contrast to the ER, immunoreactive glucocorticoid and progesterone receptors have been observed both in the cytoplasm and the nuclei of target cells (54-56).

Even though some controversy still remains on the issue of the intracellular localization of the steroid hormone receptor, in the final analysis the steroid receptor complex is still a nuclear regulatory element wherever the receptor resides in the target cell. Until unequivocal evidence concerning the localization of the receptor is produced it may be unwise to discard any of the above-mentioned models.

1.2.2 Binding mechanism of steroid hormone receptors

It is generally agreed that steroids enter cells by diffusion. With double-isotope steady-state studies it was revealed that the accumulation of steroids was the same in target as well as non-target tissue, at a concentration corresponding to the steroid concentration, indicating a diffusion mechanism (57). However, the presence of specific binding sites on the plasma membrane of target cells can not be ruled out. In 1974 Szego (58) proposed a plasma membrane model for steroid action. The model comprises the following steps: Following the binding of oestrogen to a plasma membrane-associated ER the receptor complex is internalized by a process of pinocytosis and then transferred to the nucleus by circulating lysosomes. Zanker

et al. (59) reported on a putative plasma membrane integrated ER with a modulator function. The properties of this high affinity (K_d = 6,4 x 10^{-10} M) binding protein corresponds to a large extent to that of the cytosolic receptor.

The K_d value of a receptor (reciprocal of the association constant, K_a) obtained from a Scatchard plot (60), is approximately equal to the concentration of free steroid at which half of the receptors are saturated. A specific hormone may bind to more than one high-affinity receptor and various hormones may interact with one class of receptor. Baulieu (61) observed that oestradiol interacts with both the androgen and oestrogen receptors at K_d values of 3 nM an 0,1 nM respectively. However, after diffusion or specific internalization of the hormone into a target cell it binds to specific or distinct high-affinity receptors or to the more abundant low-affinity binding sites.

The biochemical-biophysical properties of the ER following internalization is still unclear. A variety of factors, such as ionic strength, temperature, molybdate and enzyme inhibitors have an influence on the molecular form of the ER and may even give rise to multiple forms. The various forms observed under these conditions may, however, not necessarily be identical to the endogenous receptor (61, 62). Following the binding of oestrogen to the ER, the ER-oestrogen complex undergoes transformation and activation. Multiple forms of the ER have previously been ascribed to the degeneration of the native receptor. Sherman et al. (63) have demonstrated that 'meroreceptors' are products resulting from endogenous protease activity, which exhibit high affinity, low capacity hormone binding properties. Several other studies suggest that degradation of native receptors by proteolytic and other factors may be responsible for the generation of multiple forms of the ER (64). Hendry and Danzo (65) characterized a steroid receptor-active protease from the rabbit epididymis. This leupeptin-sensitive protease apparently alters the

sedimentation coefficient of the cytosolic steroid receptors by clipping off a portion of the ER necessary for nucleotide binding. In order to investigate native steroid hormone receptors and other protective factors, protease inhibitors are generally included during purification and characterization (66).

Receptor activation (the capacity of the oestrogen-receptor complex to bind to nucleic acid) precedes transformation (4S to 5S dimerization) and can be measured by the dissociation kinetics of a hormone from the receptor. The dissociation of oestradiol from the receptor appears to be a biphasic process (67). Müller et al. (68) proposed that the 4S receptor monomers exist in two conformational states. Changes in conformation occur after oestradiol binding to the low affinity state and result in stronger interactions between the steroid and the amino acid residues of the oestrogen-binding domain (69). Subsequently the rate of oestradiol dissociation decreases. The equilibrium is shifted from a lower-affinity state to a high-affinity, activated, slow dissociating phase (67).

Steroid hormone receptors are a group of gene regulatory proteins. Hormone ligands trigger their biological activity and they interact with hormone responsive elements in the DNA. Steroid hormone receptors are constructed in domains linked by hinge regions which are rather sensitive to proteolytic degradation (70). Gehring (70) proposed a steroid hormone receptor model that consisted of three distinct domains: a steroid and a DNA binding domain together with a domain exerting a modulating effect on the receptor's interaction with DNA. It is suggested that the latter modulates the extent of hormonal responsiveness by facilitating tne discrimination between specific and unspecific regions on the DNA. The DNA binding domain is a region in the middle of the polypeptide chain, rich in basic amino acids and cysteines. The hormone

binding domain and modulator domain are located in the carboxy- and amino-terminal regions respectively.

The primary structures of most types of steroid receptors have been derived from the respective nucleotide sequences. Amino acid sequences of the complete human and chicken ERs (71-73), human, mouse and rat glucocorticoid receptors (74-76) and rabbit progesterone receptor (PR) (77) as well as partial sequences of the chicken PR (78, 79) are already known. Although the sequences differ significantly in length, there exists a noticeable homology between these different receptors. The central DNA binding domain appears to be the most highly conserved. The homology between hormone binding domains is very high for receptors of the same hormone or structurally related ligands species. However, the homology between the hormone-binding domains of the steroid and thyroid receptors is much lower. The highest degree of diversity in length and sequence is present between the modulator domains (amino-terminal). Gehring (70) mentioned the striking conservation of cysteines and several basic and hydrophobic residues in the DNA binding domain and illustrated the importance of this domain for the steroid hormone mechanism of action. It has been suggested that the DNA binding domain of steroid hormone receptors contain DNA binding fingers, that is stretches of amino acid residues which include pairs of cysteines and histidines forming finger-like structures which make contact with DNA (80). Experiments employing human ER cDNA illustrated that a 252 amino acid sequence is responsible for oestradiol binding (81). Trimmed polypeptides containing only the hormone binding domain are adequate for hormone binding (81, 82). Deletion studies revealed that although small truncations of the carboxy-terminal residues of the GR failed to produce a biological response, longer deletions of the carboxy-terminal or truncation of the amino-terminal led to transcriptional activation even without hormone binding. From these results it was suggested that the hormone binding domain represses receptor function in the unliganded state. It

seems that steroid binding unmasks the DNA binding domain by structural changes with subsequent transcriptional activation. This was also observbed with the ERs (70). Specificity for gene activation resides within the DNA binding domain because one specific hormone binding domain can interact with that of another.

The 'putative acceptor' and 'effector' sites on the chromosomes are sites of oestrogen-receptor complex binding and gene expression (23). Selective binding of steroid hormone receptors to specific DNA sequences within or near hormone-regulated genes has previously been demonstrated (83, 84). With the recognition of DNA sequences within or near normone-regulated genes it was suggested that steroid receptors may modulate transcription. Oestrogen-receptor complexes bind with high affinity to a specific structure of the DNA and non-histone protein (85). Low affinity binding sites are also present in large numbers on chromatin (86). Clark et al. (87) described a type II site which is not translocated to the nucleus, is more abundant and remains elevated for longer periods than the sites (type I) which are translocated from the cytoplasm.

Processing, with a 70% loss of bound nuclear receptor sites over a few hours has been proposed (88). However, Siebert and Lippman (89) stated that processing does not entail disappearance of the receptor complex but a modification process. The step following translocation and nuclear binding (activation) is thought to be dephosphorylation by a nuclear phosphatase (90). In agreement, Auricchio (91, 92) hypothesized that inactive receptors are reactivated through phosphorylation by an ATP-dependent enzyme. Therefore dephosphorylation and rephosphorylation in the nucleus and cytoplasm respectively assure receptor circulation.

1.3 STEROID RECEPTORS IN NORMAL AND PATHOLOGICAL HEPATIC TISSUE

Although the liver is not clasically regarded as an oestrogen-dependant organ, like the breast and uterus, it is an oestrogen-responsive organ (93, 94). Several studies during the past decade have demonstrated specific receptors for oestrogens in the livers of various mammals such as the rat (95, 96), rabbit (97) and green monkey (96). Binding of oestradiol to these receptors is of high affinity, low capacity, saturable and specific. The hepatic oestrogen-receptor complexes undergo nuclear translocation in a cell-free system (98). This correlates with oestrogen responsiveness of the liver which synthesizes a variety of plasma transport proteins (93, 99-101).

Oestrogen-receptors have been demonstrated to be present in the livers of both male and female mammals. In 1979 Aten et al. (102) and Eisenfeld and Aten (103) observed the presence of hepatic oestradiol binding sites in both sexes of the green monkey, the rat and the rabbit. Aten et al. (102) reported a similarity in the binding sites of both sexes except for the two-to-three-fold higher values in the affinity and capacity of the oestradiol binding sites in female liver cytosol. In contrast to the adult male green monkey liver cytosol, Eisenfeld and Aten (103) reported on an additional set of oestradiol binding sites in the adult male rat liver cytosol. These sites were present at higher concentrations (500-to 1000-fold) than that of the putative oestrogen receptor and displayed a lower affinity towards oestradiol. The concentration of this binder was reportedly 200-fold higher in the adult male liver cytosol than in the mature female or immature male rat liver cytosol. Powell-Jones et al. (104) reported that ERs are present in equal levels in male and female liver cytosol. The receptors in both sexes demonstrated similar steroid specificities and affinities. However, an additional binding protein was identified in hepatic cytosol of mature male rats. This protein which is

either absent or present in small amounts in mature female and immature male and female animals, exhibits a high binding capacity for oestrogens and androgens (104). It is designated a male-specific oestrogen-binding protein (male-SBP or MEB) (104, 105) or the unusual oestrogen-binding protein (UEBP) (106). This protein is distinctly different from the ER because it demonstrated a high binding capacity, a 3 - 4S sedimentation coefficient on sucrose density gradients (104) and androgen dependency. Various groups have suggested that the male specific binding protein functions as a scavenger to bind excess oestrogen or oestrogenic metabolites and therefore protects the male hepatocyte from the unwanted effects of excess oestrogen (105, 107). During surgical manipulations it was illustrated that the sex differences with respect to the levels of 4S binding in the liver are controlled by the pituitary (104). Following the examination of the role played by steroids and the hypophysis in the regulation of the UEBP, Smirnova et al. (106) concluded that oestrogen-androgen regulation of the UEBP level led to the maintenance of sex differences in the UEBP content. Lax et al. (108) determined the influence of different factors on the hepatic nuclear ER concentration in the rat. Age - but no sex-dependent variations in the course of life in the nuclei ER concentration of vehicle-treated rats were observed.

The hepatic oestrogen receptor appears to be under endocrine control. Previous investigations have shown that the pituitary regulates the expression of hepatic ER. Hypophysectomy virtually eliminates receptor concentrations in both the male and the female (104, 109, 110). In addition sex differences are eradicated as far as male-SBP levels are concerned in hypophysectomized rats (111). Ovariectomy results in an increased receptor level probably as a result of the elimination of endogenous ligands. Smirnova et al. (106) investigated the effect of sex steroids and the hypophysis on the regulation of the UEBP. They concluded that oestrogen-androgen controlling of the UEBP contents led to the

maintenance of sex differences in the UEBP levels. Rumbaugh et al. (112) performed a study to characterize the complexity of the hormonal regulation of the two classes of oestrogen-binding proteins known to exist in the liver. They found that hypophysectomy on rats at any age, eliminates sex differences with respect to the levels of high capacity, low affinity oestrogen-binding sites. Moreover, hypophysectomy at any age removed the hepatic ER completely. It was suggested that the two classes of hepatic oestrogen-binding protein are under different forms of pituitary control. Their study illustrates multitudinous regulation and age dependence of the hepatic oestrogen-binding protein by the pituitary. The endocrine regulation of the synthesis of ER in the regenerating rat liver was previously investigated (113). This investigation was carried out under in vivo conditions by employing partial hepatectomy, castration and hypophysectomy. Multi-endocrine control by the gonads and pituitary of the ER was indicated by the results obtained. However, no indication of an independent direct influence of oestrogens on ER synthesis in the rat liver was observed. In 1981 Powell-Jones et al. (114) studied the ontogeny of the two oestrogen-binding sites in rat liver cytosol, by determining the effect of gonadectomy on the levels of these sites. It became clear that testicular androgens program the sexual differentiation of high capacity oestrogen-binding sites at birth. By employing isoelectric focussing in polyacrylamide gels Norstedt et al. (115) previously measured ER levels in the liver of ovariectomized female rats. A reduction in hepatic receptor levels was identified for hypophysectomized (10%) and adrenalectomized (42%) rats. Thus it can be concluded that the rat hepatic ER is under multihormonal control.

Since the identification of ERs in a variety of animal and human tissues, comparative studies of the properties of these ERs have been launched (116). Tong et al. (117) compared some physical and chemical properties of the ER proteins from the liver, kidney and uterus of the female rabbit under identical

experimental conditions. The ERs were identical in terms of pH-activity profiles, dependence on incubation temperature, sensitivity to sulfhydryl reagents and steroid specificity. In all three tissues oestradiol binding was saturable with a dissociation constant of 4×10^{-10} M. However, heparin-Sepharose chromatographic behaviour was different for the three ERs. Moreover, the liver and kidney ERs exist as 3-4S entities in low and high ionic strength conditions to the 7-8S and 4-5S sedimentation coefficients of the uterine receptor in low and high ionic strength media respectively. A combination of liver and uterine cytosol fractions resulted in a 3-4S receptor in low ionic strength conditions. Some researchers have, however, reported that the liver ER appears to be very similar to all other steroid receptors so far described (118): a large cytoplasmic form, stablized by molybdate which is transformed to a smaller form.

Following the original discovery of oestrogen receptor in liver (96, 102, 103, 105, 119, 120) many attempts have been made to define a possible role for steroid hormones, particularly oestrogens, in the pathogenesis of various hepatic diseases. This is especially true for hepatic adenoma (HA) (121), focal nodular hyperplasia (FNH) (122), hepatoma and angiosarcoma (123, 124).

Porter et al. (125) demonstrated, with the aid of binding studies that cytosolic and nuclear ERs are present in the case of HA and FNH. They observed higher levels of the ER in neoplastic tissues than in normal tissue and therefore suggested that a greater hormone responsiveness may be possible in neoplastic liver tissue. The androgen and ER have been examined by Ohnishi et al. (126). They reported the presence of ER in the cytosol in one out of seven cancerous tissues. In three out of seven non-cancerous tissues the ER was present in the cytosol and/or nucleosol. The amount of ER in the one positively assayed cancerous tissue was approximately double that of the surrounding non-cancerous

tissues. Although they didn't examine the nucleosol of cancerous tissue for ER, they suggested a suppression of the ER, compared to the androgen receptor, in association with malignant transformation. Molteni et al. (127), Friedman et al. (128) and Iqbal et al. (129) illustrated that the ER is present in the cytosol of human hepatocellular carcinoma (HCC) at levels similar to that in normal liver tissue. The ER levels in the HCC and surrounding tissue have also been assayed by Nagasue et al. (130). The ER was detected in 40% of the HCC and 46% of cirrhotic livers. Two livers with chronic hepatitis were ER-negative. They speculated upon the possibility of employing hormone therapy for the treatment of naturally occurring HCC. Francavilla et al. (124) compared the oestrogen-binding activity in Moris hepatoma 7777 with normal rat liver. A great decrease in the cytosolic oestrogen-binding activity of the hepatoma compared to that in normal hepatic cytosol was reported. Furthermore, the ER has been detected in human adenoma at much lower levels than in normal liver tissue (131). Mays and Christopherson (132) found insignificant cytosolic ER levels in five out of six HAs resected from women exposed to oral contraceptive steroids (OCS). The one ER-positive HA contained less ER than normal liver tissue. Friedman et al. (128) analyzed five livers with HCC for ER. Higher ER levels were observed in the adjacent normal tissue than in the HCC tissue. However, no nuclear ER levels were reported in either of these studies.

The possibility exists that oestrogens may contribute to the formation of liver tumours through oestrogen receptor-mediated mechanisms. Some authors have suggested that oestrogens are carcinogenic (133, 134). Others suggested that oestrogens promote carcinogenic effects of procarcinogens but that they are not intrinsically carcinogenic (135-137). Wanless and Medline (138) showed that oestrogens promote hepatic neoplasm development by a mechanism that increases the mitogenic activity of the hepatocyte. The doses of oestrogen employed in

this study were much greater than those generally used in clinical treatments.

Exogenous oestrogens may also play a role in the development of benign (138) and malignant (137) hepatic tumours. Evidence exists that oral contraceptive steroids (OCS) induce benign (139) and probably malignant (140) tumours of the liver. However, the role of OC in HCC has recently been questioned (141). Administration of norethynodrel and norethisterone, alone or in combination with mestranol, a synthetic oestrogen, leads to an increase in benign and in some cases malignant hepatic neoplasms in male rats. An increase in HCC in female rats has been observed following administration of megestrol and/or ethinyloestradiol. Oestrogens are suggested to be promoters of liver cell growth (142). Mishkin et al. (143) questioned the biological importance of oestrogens during malignant transformation of hepatic tumours. They demonstrated a setback of acetylaminofluorine-induced hepatic hypertrophy, nodular proliferation and malignant transformation with oestrogen and tamoxifen administration. The inhibitors of hepatic neoplasia by oestrogens has also been demonstrated (144) in contrast to reports that illustrate oestrogens as initiators of neoplastic growth (145). Regression of carcinogen-induced hyperplastic nodules and suppression of malignant transformation of tumours by high doses of oestrogen or tamoxifen have been reported (143). At this stage it is uncertain as to which factors control receptor levels, and what the role of the ER is in the disease process. Clearly, much more needs to be learned about the influence of oestrogens and their receptors in the pathogenesis and promotion of liver diseases.

CHAPTER 2

HEPATIC OESTROGEN-BINDING PROTEINS IN MALE AND FEMALE VERVET MONKEYS

2.1 INTRODUCTION

The presence of high affinity oestrogen-binding sites in hepatic cytosols of a number of species, including the green monkey (Cercopithecus aethiops) has been identified (102, 103, 104, 147). Hepatic oestrogen receptors have been found to be present in the liver cytosols of both sexes of the species investigated (102, 103, 120). Recently it was illustrated that two distinct classes of oestrogen-binding proteins exist in hepatic cytosols of rats (104, 106, 148, 149). The one class displayed a high affinity for oestrogen and had a relative sedimentation of 8S, while the other binder exhibited an affinity of approximately an order lower with a relative sedimentation of 4S (104, 105). The 4S binder was reported to be androgen-dependent (104, 105). It has been suggested that the function of this high capacity, low affinity oestrogen-binding component was to regulate the availability of oestrogen for its receptor (104, 105). Oestrogen receptors are reportedly present in equal levels in both male and female livers (104, 150). The so-called male-specific oestrogen-binding protein appears to be regulated by pituitary-dependent sexual differentiation at puberty. This apparently leads to the phenomenon that males contain higher levels of the 4S binder than females (104). In spite of all these speculations there is still no clear understanding of the exact biochemical function of the oestrogen-binding proteins in the mammalian liver.

The vervet monkey is employed as an experimental model in order to investigate the mechanism of action of steriod hormones in mammalian target organs. The distribution and

molecular properties of oestrogen and progesterone receptors in the uterus of the same model have previously been reported (151).

In this chapter the presence and levels of oestrogen-binding proteins in the livers of both male and female vervet monkeys are described. Some of the molecular properties of the crude cytosolic HEBPs are discussed.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

17β [^{3}H] - Oestradiol (168 Ci/mmol) was obtained from Amersham, England. [^{14}C] - Methylated albumin (0,02 mCi/mg) was purchased from New England Nuclear. Radioligands were initially checked for radiochemical purity on reverse phase high performance liquid chromatography (HPLC). Oestrone (E_1), oestradiol (E_2), oestriol (E_3), testosterone (T), dehydrotestosterone (DHT), dehydroepiandrosterone (DHEA), diethylstilboestrol (DES), norethindrone (NE), hydrocortisone (HC) and ethinyloestradiol (EE_2) were obtained from Sigma Chemical Company. Progesterone (P) and promogestone (R5020) were purchased from New England Nuclear. Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminotetraacetate (EDTA), glycerol, sucrose, activated charcoal and sodium azide were obtained from Merck Chemicals. Dithiothreitol and gelatine (Carrageenan, Type 1) were bought from Sigma Chemical Company, while Dextran T 70 was purchased from Pharmacia. Ready-Solv HP scintillation cocktail was obtained from Beckman Instruments.

A Beckman LS 5800 counter was used for scintillation counting at an efficiency of 34-40%.

2.2.2 Buffers

TEDAG buffer: Tris-HCl (10 mM), EDTA (1,5 mM), dithiothreitol (1 mM), sodium azide (1 mM), glycerol (10% m/v), pH 7,4 (4°C). TEDAGM buffer: Prepared as TEDAG buffer with the addition of sodium molybdate (10 mM).

PEDGP (0,15): Sodium phosphate (25 mM), EDTA (1,5 mM), dithiothreitol (1 mM), glycerol (10% m/v), potassium chloride (0,15 M), pH 7,4.

PEDGP (0,40): Prepared as PEDGP (0,15) with a final potassium chloride concentration of 0,40 M.

2.2.3 Dextran coated charcoal

This suspension consisted of pre-washed, activated charcoal (0,5% m/v), gelatine (Carrageenan Type 1) (0,1% m/v), dextran T 70 (0,5% m/v) prepared in TEDAG or TEDAGM buffer.

2.2.4 Ligands and competitors

Concentration ranges (0,25 - 3 nM) of tritiated oestradiol were prepared in TEDAG or TEDAGM in the presence or absence of a 5 000-fold excess of unlabelled hydrocortisone. Identical concentration ranges for the determination of non-specific binding were prepared in the presence of a 1 000-fold excess of unlabelled DES. Competing ligands (E_1, E_2, E_3, T, DHT, DHEA, DES, NE,HC, EE_2, P and R5020) were prepared in TEDAG (0,675 nM - 10 nM) from stock solutions (1 mM) in ethanol.

2.2.5 Liver tissue

Vervet monkey livers, obtained from the National Institute of Virology, Edenvale, South Africa, were immediately placed on ice, before transportation to the laboratory. The livers were trimmed of fat and connective tissue and rinsed once in ice

cold homogenization buffer (TEDAG or TEDAGM). Liver samples not immediately used were quick-frozen with dry ice and stored at -70°C until use.

2.2.6 Preparation of cytosol

Fresh or frozen liver tissue, kept on crushed ice, was cut into thin slices with a scalpel. The slices were then suspended in five volumes of pre-cooled TEDAG or TEDAGM per unit mass of tissue and homogenized (TP 10 Ultra Turrax, Janke and Kunkel). Cytosol was obtained by centrifugation of the tissue homogenates at 105 000 *g* for 30 min. and collecting the clear supernatant. All steps were carried out at 4°C.

2.2.7 Protein determination

Protein concentrations of cytosol were determined by the method of Bradford (152) by employing the Bio-rad standard assay procedure with bovine serum albumin as standard. Samples and standards were analysed in duplicate simultaneously. In Figure 2.1 the standard curve for the Bio-rad standard assay procedure, using bovine serum albumin as standard, is depicted.

2.2.8 Oestrogen receptor assay

Aliquots of hepatic cytosol (100 µℓ) were incubated with varying concentrations of tritiated oestradiol for 16 h at 4°C. Non-specific binding was assayed in the same way except that the tritiated ligand solutions contained a 1 000-fold excess of unlabelled DES. Unbound steroid was removed with the addition of 500 µℓ dextran coated charcoal (DCC). The suspension was left on ice for 10 min. and centrifuged at 2 000 *g* for 15 min. Aliquots (0,5 mℓ) of the supernatant were counted in a Beckman LS 5800 counter, following the addition of scintillation cocktail (4 mℓ).

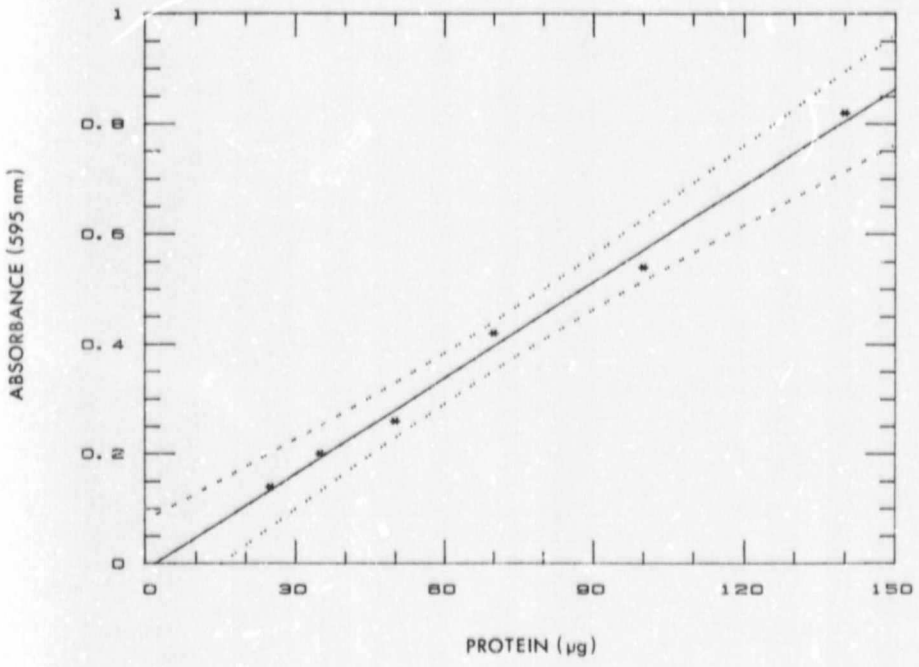

FIGURE 2.1

A typical standard curve for the determination of microgram quantities of protein. The Bio-rad standard assay procedure was employed as outlined in section 2.2.7. Bovine serum albumin (25-140 µg) was used as protein standard. The 99% confidence limits are indicated by the dotted lines.

2.2.9 Ligand specificity of the HEBP

Aliquots of hepatic cytosol (100 µℓ) prepared in the absence of molybdate (TEDAG) were incubated with 50 µℓ of tritiated oestradiol (8 nM final concentration) in the presence of 50 µℓ of increasing concentrations of various competitors (E_1, E_2, E_3, T, DHT, DHEA, DES, NE, HC, EE_2, P and R5020) for 16 h at 4°C. Reactions were terminated by the addition of DCC (500 µℓ) and bound, tritiated oestradiol was determined as described in Section 2.2.8.

2.2.10 Oestradiol binding in liver cytosol prepared from tissue of different areas in the same liver

Liver tissue dissected from three locations in the liver (lobe 1, 2, and 3) was used for the preparation of cytosol in TEDAG at a protein concentration of 5 mg/mℓ. Binding assays were performed as described in Section 2.2.8 with tritiated oestradiol concentration ranges (0,25-3 nM) prepared in TEDAG in the presence and absence of a 1 000-fold excess of unlabelled DES.

2.2.11 Oestradiol binding in liver cytosol prepared at different protein concentrations.

Hepatic cytosol was prepared in TEDAG at three different protein concentrations (5, 10 and 18 mg/mℓ). Binding assays were performed as described in Section 2.2.8 with tritiated oestradiol concentration ranges (0,25-3 nM) prepared in TEDAG in the presence and absence of a 1 000-fold excess of unlabelled DES.

2.2.12 Oestradiol binding in cytosol prepared from liver obtained from male and female vervet monkeys.

Liver tissue dissected from the liver obtained from male and female vervet monkeys was used for the preparation of cytosol

in TEDAG at a protein concentration of 5 mg/mℓ. Binding assays were performed as described in Section 2.2.8 with tritiated oestradiol concentration ranges (0,78 - 50 nM) prepared in TEDAG in the presence and absence of a 1 000-fold excess of unlabelled DES.

2.2.13 Oestradiol binding in liver cytosol in the presence or absence of sodium molybdate and hydrocortisone.

Hepatic cytosol was prepared in TEDAG or TEDAGM. Binding assays were performed as described in Section 2.2.8 with tritiated oestradiol concentration ranges (0,25-3 nM) prepared in the presence or absence of sodium molybdate and hydrocortisone respectively. Non-specific binding was determined in the presence of a 1 000-fold excess of unlabelled DES.

2.2.14 Sucrose density gradient analysis

Tritiated oestradiol (20 nM) was prepared in TEDAG or TEDAGM in the presence or absence of a 5 000-fold excess of unlabelled hydrocortisone. Sucrose density gradients (SDG) (10-35%) were prepared in TEDAG. Linearity of the SDG were monitored by refractometry (Figure 2.2). Unlabelled marker proteins myoglobin (2,0S), ovalbumin (3,6S), bovine serum albumin (4,6S) and aldolase (7,35S) were employed for the calibration of the gradients (Figure 2.3). Aliquots (200 µℓ) of the marker proteins were layered separately onto gradients (1 mg). The duplicate gradients of each marker protein were run and subsequently fractionated and scanned spectrophotometrically at 280 nm. Cytosol was incubated with tritiated oestradiol at a final concentration of 10 nM with and without a 1 000-fold excess of unlabelled DES for 16 h at 4°C. Unbound ligand was removed with DCC treatment. Aliquots of the cytosols (200 µℓ) were layered on top of the gradients and centrifuged at 520 000 *g* for 2 h at 4°C. Protein concentration of cytosols was approximately 10 mg/mℓ. Gradients were