An operational model for estrogenic action in the presence of sex hormone binding globulin (SHBG)

by

Michael John Vismer

Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch

Study Leaders  Dr. A. Louw
               Prof. J.M. Rohwer

March 2007
Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ..................  Date ..................
Summary

The aim of this study was to build a mathematical model that describes the binding of 17-β-estradiol (E₂) to estrogen receptor α (ER-α) and the influence the sex hormone binding globulin (SHBG) has on this interaction. The influence of SHBG on the transactivation of an estrogen response element, via ligand bound ER-α, was also studied.

COS-1 cells, derived from the kidney of a green african monkey, were used to study the binding of E₂ to ER-α in the absence of SHBG. The influence of SHBG on the binding of E₂ to ER-α was studied using Hep89 cells, human hepatocoma carcinoma, which express SHBG endogenously and are stably transfected with the ER-α gene. Human pregnancy plasma was used to study the interaction of E₂ with SHBG in the absence of ER-α.

The results of this study have shown that the K₅ (E₂) for ER-α was determined as between 3.4nM and 4.4nM in the absence of SHBG. With respect to the binding of E₂ to ER-α it was not possible to determine the K₅ app and B max for ER-α using the Hep89 experimental system. The K₅ (E₂) for SHBG was not determined using the human pregnancy plasma experimental system.

With the aid of mathematical modelling, a model of the Hep89 and human pregnancy plasma experimental systems, was built. The results of the numerical modelling, using mathematical modelling, showed that the presence of albumin together with SHBG was the reason that the K₅ app (E₂) could
not be determined in the Hep89 experimental system. With respect to the use of human pregnancy plasma to determine the $K_d(E_2)$ for SHBG it was shown that if the plasma was diluted 200 times it would have been possible to determine the $K_{d,\text{app}}(E_2)$ for SHBG, in the presence of albumin.

Ligand independent transactivation of an estrogen response element was shown to be a problem in the COS-1 cell system when promoter reporter gene assays were undertaken. As COS-1 cells were used as a control for the absence of SHBG no further promoter reporter gene assays were undertaken using the Hep89 experimental system.
Die doel van hierdie studie was die bou van ‘n wiskundige model wat die verbinding van E\(_2\) met die estrogeenreseptor \(\alpha\) (ER-\(\alpha\)) en die invloed wat die geslagshormoon-verbindingglobulien (SHBG) op hierdie interaksie het, beskryf. Die effek van SHBG op die transaktivering van ‘n estrogeen respon-selement, via die ligandverbonde ER-\(\alpha\), is ook bestudeer.

COS-1-selle uit die nier van ‘n groen afrika-aap is gebruik om die verbinding van E\(_2\) met ER-\(\alpha\) in die afwesigheid van SHBG te bestudeer. Die invloed van SHBG op die verbinding van E\(_2\) met ER-\(\alpha\), is bestudeer deur gebruik te maak van Hep89-selle, die menslike levergeswelkarsinoom, wat SHBG uitwendig afgee en wat stabiel getransfesteer kan word met die ER-\(\alpha\) geen. Menslike swangerskapplasma is gebruik om die interaksie van E\(_2\) met SHBG in die afwesigheid van ER-\(\alpha\) te bestudeer.

Die uitslag van hierdie studie toon aan dat die \(K_d\) (E\(_2\)) vir ER-\(\alpha\) vasgestel tussen 3.4nM en 4.4nM in die afwesigheid van SHBG. Met betrekking tot die verbinding van E\(_2\) met ER-\(\alpha\), was dit nie moontlik om die \(K_d\) (E\(_2\)) en \(B_{\text{max app}}\) vir ER-\(\alpha\) met die gebruik van die Hep89 eksperimentele stelsel vas te stel nie. Die \(K_d\) (E\(_2\)) vir SHBG is nie vasgestel deur die gebruik van die menslike swangerskapplasma eksperimentele stelsel nie.

‘n Model van die Hep89 en menslike swangerskapplasma eksperimentele stelsels is met behulp van wiskundige modellering gebou. Die uitslag van die nu-
meriese modellering, met gebruik van wiskundige modellering, toon dat die teenwoordigheid van albumien, saam met SHBG, die rede was dat die $K_{d_{app}}(E_2)$ nie in die Hep89 eksperimentele stelsel vasgestel kon word nie. Wat betref die gebruik van menslike swangerskapplasma om die $K_d(E_2)$ vir SHBG vas te stel, is daar aangetoon dat, indien die plasma 200 maal verdun was, dit moontlik sou gewees het om die $K_{d_{app}}(E_2)$ vir SHBG in die teenwoordigheid van albumien vas te stel.

Promotor verkilkkergeen toetse het ligandonafhanklike transaktiveering van ‘n estrogeen responselement aangetoon as ‘n probleem in die COS-1-selle stelsel. Omdat COS-1-selle gebruik is as ‘n kontrole vir die afwesigheid van SHBG, is geen verdere promotor verkilkkergeen toetse onderneem met die gebruik van die Hep89 eksperimentele stelsel nie.
My family and friends, thanks for all the support and encouragement.
Acknowledgements

I would like to thank the following people for their help, guidance and support. Without their insight and contributions this particular study would not have been possible.

**Dr. Louw**
I’ll be ever thankful for your continual encouragement, guidance and patience at all times. Without you this would not have been possible.

**Prof. Rohwer**
I’ll be ever thankful for your continual guidance and patience at all times. Without you this would not have been possible.

**Prof. Swart**
For your assistance in this project.

**National Research Fund (NRF)**
For financial support.

**Friends and family**
You know who you are, your support and encouragement has been invaluable over the years.
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<td>Alzheimers disease</td>
</tr>
<tr>
<td>AF-1</td>
<td>Activation function 1</td>
</tr>
<tr>
<td>AF-2</td>
<td>Activation function 1</td>
</tr>
<tr>
<td>AIB</td>
<td>Amplified in breast cancer</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CBP</td>
<td>Creb binding protein</td>
</tr>
<tr>
<td>CEE</td>
<td>Conjugated equine estrogen</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DCC</td>
<td>Dextran coated charcoal</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>E₂</td>
<td>17-β-estradiol</td>
</tr>
<tr>
<td>EAC</td>
<td>Endocrine active compound</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>ERAP</td>
<td>Estrogen receptor associated protein</td>
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<tr>
<td>ERE</td>
<td>Estrogen response element</td>
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<tr>
<td>ERF</td>
<td>Estrogen receptor factor</td>
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<tr>
<td>ERBF</td>
<td>Estrogen receptor β factor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ERK</td>
<td>Serine/Threonine kinase</td>
</tr>
<tr>
<td>ERKO</td>
<td>Estrogen receptor-α knockout</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen related receptor</td>
</tr>
<tr>
<td>ERT</td>
<td>Estrogen replacement therapy</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>GR</td>
<td>Glucorticoid receptor</td>
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<tr>
<td>GRIP1</td>
<td>GR-interacting protein</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary axis</td>
</tr>
<tr>
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<td>Human pregnancy plasma</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IRMSA</td>
<td>Immunoradiometric assay</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LH</td>
<td>Leutinizing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>MAP kinase</td>
</tr>
<tr>
<td>MPA</td>
<td>medroxyprogesterone acetate</td>
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<td>MR</td>
<td>Mineralocorticoid receptor</td>
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<tr>
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<td>Nuclear receptor</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
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<td>Protein kinase A</td>
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</tr>
<tr>
<td>RAC3</td>
<td>Receptor associated coactivator</td>
</tr>
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<td>Trans retinoic acid receptor</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>Rsk</td>
<td>Ribosomal S6 kinase</td>
</tr>
<tr>
<td>RXR</td>
<td>9-cis retinoic acid receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid receptor co-activator</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone-binding globulin</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TIF</td>
<td>Transcription intermediary factor</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid receptor</td>
</tr>
<tr>
<td>VDR</td>
<td>Dihydroxyvitamin D₃</td>
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Chapter 1

Introduction

The steroid hormone 17-β-estradiol (E$_2$), regulates endocrine functions by binding the two estrogen receptor isoforms namely ER-α and ER-β. E$_2$ is found in the plasma in two distinct forms, bound and unbound. In humans the sex hormone binding globulin (SHBG) binds E$_2$ and subsequently regulates the amount of unbound E$_2$ [1]. It has been suggested that the unbound form of E$_2$ is the bio-active form. However, it has also been found that under certain circumstances that protein bound E$_2$ is also available for tissue uptake, this form of E$_2$ thus also being a bio-active form of E$_2$.

Most studies that focus on the effect of estrogenic compounds on the human endocrine system use E$_2$ as a base reference. E$_2$ is used as a prototypical endocrine-active compound (EAC). Endocrine active compounds are a group of chemicals that have been shown to influence the endocrine system, some of which specifically bind to the estrogen receptor.

The primary aim of this study is to determine the effect that SHBG has on the binding of E$_2$ to the human ER-α. The effect that SHBG has on the transactivation of an estrogenically sensitive gene was to also be described. The data that is collected will then be used to build a mathematical model which describes the effect that SHBG had on the binding of E$_2$ to the ER-α.
To achieve the proposed aims of this study an experimental system is required in which the interaction of E$_2$ with ER-α can be investigated in the presence or absence of SHBG. The proposed experimental system consists of two cell lines and human pregnancy plasma. The cell lines being used either lacked SHBG (COS-1 cells) or endogenously expressed SHBG (Hep89 cells). COS-1 cells were derived from the kidney of the green african monkey and don’t contain any endogenous SHBG. Hep89 cells were created by stably transfecting HepG2 cells with pCDNA3-ER-α to create a cell line that expresses ER-α. HepG2 cells were derived from a human liver carcinoma and are known to produce SHBG endogenously. Human pregnancy plasma was being used because it contains SHBG endogenously, however, does not contain ER-α.

To build the operational model of agonism a number of parameters, described in Table 4.1, are required. The kinetic binding constants (K$_d$ and B$_{max}$ (ER-α)) are determined through the use of either competitive or saturation binding assays, while the effect of E$_2$ on an ERE will be determined using promoter reporter gene studies. The K$_d$ (E$_2$) and B$_{max}$ for ER-α will be determined using the COS-1 experimental system. The K$_d$ app (E$_2$) and B$_{max}$ app (ER-α) will be determined using the Hep89 experimental system. Human pregnancy plasma is used to determine the K$_d$ (E$_2$) in the absence of ER-α. The cell volumes for both the Hep89 and COS-1 experimental system will also be determined for use in the model. The metabolism of E$_2$ in the COS-1 and Hep89 experimental systems will also be studied to determine if it is necessary to include this variable in the model. In this thesis a review of the literature concerning ER-α is presented in Chapter 2. As mentioned earlier it is known that SHBG binds E$_2$ and thus a review of SHBG action has been included in Chapter 3. The various models that currently exist to describe the effect of ligands on responsive genes are discussed in Chapter 5.
Chapter 4 describes the development of an experimental system to investigate the effect of E$_2$ in the presence/absence of SHBG. These experimental systems were used to obtain binding data concerning the K$_d$ of ER-α as well as transactivation studies. The binding data for SHBG, obtained using the human pregnancy plasma experimental system, will also be presented.

The design of a mathematical model to describe the effect that SHBG and albumin has on the binding of E$_2$ to the human ER-α, is given in Chapter 6.

The conclusions of this study are presented in Chapter 7.

Table 1.1: Parameters needed to build the mathematical model of agonism

<table>
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<th>Binding constants</th>
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<tbody>
<tr>
<td>hER-α</td>
<td>K$_d$ (E$_2$) (intracellular)</td>
</tr>
<tr>
<td></td>
<td>B$_{max}$ (ER-α) (intracellular)</td>
</tr>
<tr>
<td>hSHBG</td>
<td>K$_d$ (E$_2$)</td>
</tr>
<tr>
<td></td>
<td>B$_{max}$ (SHBG) (intracellular and extracellular)</td>
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<tr>
<th>Volume of compartments</th>
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<td>Cytoplasm</td>
<td>COS-1 and Hep89 cells</td>
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<td>COS-1 and Hep89 cells</td>
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<td>COS-1</td>
</tr>
<tr>
<td>Metabolism of E$_2$</td>
<td>Hep89</td>
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Chapter 2

Estrogen receptor

2.1 Introduction

The estrogen receptor is a ligand activated transcription factor and is part of the nuclear receptor super-family \([2]\), Table 2.1. The members of the nuclear receptor super-family all share common structural features as will be discussed in section 2.2. A number of estrogenic compounds have an influence on the human endocrine system via the estrogen receptor $$\alpha$$. These compounds include natural and synthetic estrogenic compounds such as phytoestrogens, endocrine disruptors and selective endocrine receptor modulators (SERMS).

Phytoestrogens are non-steroidal estrogenic compounds that are produced by plants. SERMS function as agonists or antagonists depending on the tissue in which they are located \([3]\). The prevalence and modulating activity of these compounds has spurred research into the effects, in the human body, and removal of certain compounds from the environment.

$$E_2$$, via the ER-$$\alpha$$ regulates the reproductive system in mammals and has an effect in a number of tissues: liver, bone, brain and cardiovascular system \([4]\).
2.1 Introduction

This review will concentrate on the mechanisms through which the human ER-α modulates the human endocrine system.

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
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<tbody>
<tr>
<td>Class I</td>
<td>The nuclear receptors which belong to this family are the estrogen (ER), glucocorticoid (GR), mineralocorticoid (MR), progestin (PR) and finally the androgen receptor (AR) and have long A/B domains. They have been placed in this group because when no ligand is bound they are found associated with chaperones such as heat shock proteins, immunophilins and others [5, 6]. Once ligand binds the heat-shock proteins disassociate, the receptor dimerizes and binds to hormone response elements in the promoter region of target genes and modulate their transcription [7].</td>
</tr>
<tr>
<td>Class II</td>
<td>This class of receptors has short A/B domains and is composed of the thyroid (TR), dihydroxyvitamin D₃ (VDR), trans retinoic acid (RAR), 9-cis retinoic acid (RXR) and most of the orphan nuclear receptors. These receptors do not associate with heat shock proteins and bind to DNA elements consisting of half sites as homodimers and sometimes monomers.</td>
</tr>
<tr>
<td>Class III</td>
<td>These receptors, for example SF-1, function as monomers and are associated with constitutive transcription.</td>
</tr>
<tr>
<td>Class IV</td>
<td>These receptors have characteristics of both Class I and II receptors.</td>
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</table>
2.2 Structural and functional domains

ER-\(\alpha\) has six functional domains as represented in Table 2.2 and Figure 2.1. These domains are involved in a number of functions which include ligand binding, dimerization, DNA binding and transcriptional activation [8].

Table 2.2: Structural domains of nuclear receptors [8]

<table>
<thead>
<tr>
<th>Domain</th>
<th>Function</th>
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<tbody>
<tr>
<td>A/B</td>
<td>Contains the activation function 1</td>
</tr>
<tr>
<td>C</td>
<td>Involved in DNA binding (DBD)</td>
</tr>
<tr>
<td>D</td>
<td>Involved in the conformational changes of the ligand bound receptor (hinge region)</td>
</tr>
<tr>
<td>E</td>
<td>Involved in ligand binding, contains AF-2 domain (LBD)</td>
</tr>
<tr>
<td>F</td>
<td>Conformation of protein-protein interactions involved in transcription</td>
</tr>
</tbody>
</table>

Figure 2.1: Domain structure of the human ER-\(\alpha\) [9].

**A/B domain** This is also called the N-terminal domain and contains activation function-1 (AF-1). The AF-1 domain is involved in ligand-independent transactivation and interacts with the core transcription machinery [10].

**C domain** Also called the DNA binding domain (DBD), it is a hydrophilic region which contains sequences involved in binding of ER-\(\alpha\) to DNA. The domain contains two zinc type II binding motifs, in which each zinc ion is
2.2 Structural and functional domains

tetrahedrally liganded by four conserved cysteine residues [11]. The discrimination between promoter sequences is controlled by the P-box. For ER-α the P-box is defined as the first three amino acids (CEGCKA) [12] found at the base of the first zinc binding motif [13]. As such the P-box is involved in the specificity and selectivity of the binding of the ligand bound ER-α to the ERE. Another box, termed the D-box, is located in the second zinc binding motif. The D-box, spanning cysteine residues 5-6, is used to differentiate between ERE’s that have similar sequences, of which, the half site spacing is different [14]. Other functions that are related to the DBD include weak dimerization properties in the absence of ligand. The DBD also contains ligand independent nuclear translocation signals which are used in the transport of the unliganded ER-α to the nucleus [15, 16].

**D domain** This domain is also known as the hinge region and its functions are to provide flexibility to the receptor protein when altering conformation upon ligand binding [17].

**E domain** Also referred to as the ligand binding domain (LBD) and as such is involved in the binding of ligand to the ER [18]. Regions found in this domain are involved in:

Dimerization of monomers

Interaction with heat shock proteins (Hsp)

Interaction with transcriptional co-regulators

Ligand dependent activation and nuclear translocation

The LBD is made up of 12 α helices and two β sheets as well as secondary structures that are arranged in a α helical “sandwich” [7]. The AF-2 function contains a highly conserved amphipathic α helix, called helix 12 (H12) also called the AF-2 AD core. This α helix has been found to be essential in the ligand inducible AF-2 function [18]. Amino acids in the region 521-528 of
the ER-α are involved in the recognition and binding of ligands [19]. The binding of stereochemically different compounds alters the tertiary conformation that the ER-α adopts. This alteration of conformation affects the co-regulators that can bind the ER-α [18].

The role that the LBD plays with respect to dimerization is controlled by a leucine zipper type mechanism [20] which is involved in the conversion of 4S to 5S ER [21]. This function is only active in the presence of ligand as in the absence of ligand the DBD controls dimerization.

**F domain** This region possibly plays a role in the conformation of protein-protein interactions necessary for effectiveness of transcription [22].

### 2.3 Subtypes

A number of ER isoforms exist, however, only the ER-α and ER-β isoforms will be discussed with the emphasis on the ER-α isoform. ER-α which was first isolated and cloned from MCF-7 cells in the late 1980’s. ER-α, classified as the primary isoform, is a 66kDa which is found in its unliganded form in the nucleus [23, 24, 25]. A 46kDa isoform of ER-α, has been isolated and characterized [26]. This isoform lacks the first 173 amino acids of the wild type ER-α and negatively regulates ER-α [26]. A number of splice variants of ER-α are known, however, it is not known whether they are expressed as functional proteins [27].

ER-β, an isoform of ER, was first isolated and cloned from rat prostrate in 1996 [28]. Since then the mouse [29] and human ER-β [30, 31] isoforms have also been cloned. The full length ER-β protein is expressed as a 59.2 kDa protein and shows homology with ER-α. The A/B domain of ER-β is 30% homologous to the same domain in the ER-α. The DNA binding domain (C domain) shares 96% homology with that of ER-α whilst the D and
2.4 Distribution and physiological role

E/F domains share 30% and 53% homology, respectively, with the same domains found in ER-α [32]. Even though there are differences in the domains it has been found that ER-α and β have a similar affinity for E$_2$, however, there are differences in affinity for other ligands such as phytoestrogens and antiestrogens [33]. In addition ER-α and ER-β are located in different tissues and ER-β is considered to exert a dominant negative effect on ER-α signaling.

ER-α is located in the nucleus when no ligand is bound, however it has also been found that ER-α can be found in the plasma membrane [34]. In the membrane ER-α appears to be localize mainly in discrete domains, known as caveole. The exact mechanism of how this small pool of ER translocates to the membrane is currently unknown [35].

Estrogen related receptors (ERR) are orphan nuclear receptors and form part of a sub-family that share amino acid homology with the ER [36]. Both ERR-α and β were first isolated in 1988 [37]. A third ERR, the estrogen related receptor gamma (ERR-γ), was isolated in 1999 [38]. When comparing the DBD ERR’s have a 60% homology to ER-α and when comparing the LBD’s the homology is found to be less than 35%.

2.4 Distribution and physiological role

The highest concentration of estrogen receptors are located in tissues with reproductive functions. These tissues include the mammary glands; ovaries; vagina; uterus, however, estrogen receptors can also be found in a number of other tissues, Table 2.3. Many physiological conditions, such as arteriosclerosis, osteoporosis and degenerative processes of the central nervous system are linked to the presence of estrogenic compounds. The following discussion will center on the distribution of and the role that estrogen receptors play in various tissues.
2.4 Distribution and physiological role

Table 2.3: Tissue distribution of ER-α [4]

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovary</td>
<td>Kidney</td>
</tr>
<tr>
<td>Vagina</td>
<td>Islets of Langerhaan</td>
</tr>
<tr>
<td>Uterus</td>
<td>Liver</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>Bone</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>Cardiovascular system</td>
</tr>
<tr>
<td>Prostate</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>Thymocytes</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Lymphoid cells</td>
</tr>
<tr>
<td>Leydig cells</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td></td>
<td>Osteoblastic cells</td>
</tr>
<tr>
<td></td>
<td>Glia cells</td>
</tr>
<tr>
<td></td>
<td>Schwann cells</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
</tr>
</tbody>
</table>

The importance of estrogen in the development of female breast tissue has been known for a long time and is well documented. Using ER-α knockout mice (ERKO) it has been shown that the development of the mammary gland tissue is impeded [39]. This is because of the lack of ER-α. In addition, it has been shown that over 70% of breast cancers are sensitive to the presence of estrogens acting via the ER-α.

In the urogenital tract it has been found that both ER-α and β are expressed in a number of tissues including the ovaries, uterus, testes and prostrate. In these tissues the ER isoforms are involved in the regulation of sexual development as well as fertility [4].

Osteoporosis has been linked to a deficiency of estrogen, normally after menopause. Estrogens are known to be involved in the maintenance of bone resorption and bone formation [40]. It has been proposed that ER-α regu-
lates the effects of estrogens on bone tissue because ER-α is localized in both the bone forming osteoblasts [41] and bone resorbing osteoclasts [42].

With respect to the role that estrogens play in the cardiovascular system it has been shown that women are less likely to develop cardiovascular disease at an early age [43]. Both ER-α and ER-β have been linked to a number of effects seen in vascular endothelial cells [44], smooth muscle cells [45], and myocardial cells [46]. These effects include: non-genomic vasodilation [47] and nitric-oxide synthesis [48]. Estrogens have also been found to have an indirect influence on the cardiovascular system, via ER-α [49]. The indirect action of estrogens on the cardiovascular system can be linked to the effects of estrogen in the liver, where it is known that estrogens regulate the serum levels of lipids and cholesterol [43].

Estrogens are reported to have a number of functions in the central nervous system (CNS) such as learning, memory, awareness, fine motor skills, temperature regulation, mood and reproductive function. The hypothalamic-pituitary axis (HPG) regulates overall endocrine homeostasis in the body. Estrogen, through effects on the HPG-axis, modulates expression and secretion of several hormones from the anterior pituitary gland such as leutinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH) and prolactin (PRL). Both ER-α and ER-β are expressed in the pituitary, however, ER-α predominates in the gonadotrophs and lactotrophs. Both isoforms of the ER are also expressed in the pre-optic area of the hypothalamus and are believed to be involved in the regulation of expression of the pituitary hormones. The serum levels of LH and FSH are directly controlled by the hypothalamic gonadotropin releasing hormone (GnRH). The most important physiological determinants of the serum gonadotropin levels are circulating estrogen, other sex steroids and glycoproteins. There is a strong inverse correlation between the circulating levels of inhibin, a protein which inhibits the action of FSH synthesis and secretion, in females and FSH. The main source
2.4 Distribution and physiological role

of inhibin (inhibin A and B) production in females is in the ovary. Inhibin B is expressed in the early follicular phase with a peak at the mid-follicular phase whilst inhibin A is expressed by the dominant follicle and the corpus luteum with a peak in the late follicular and in the mid luteal phase. The endocrine system is controlled, in part, by both negative and positive feedback, Figure 2.2. It has been found that estrogen has a negative feedback on the release of FSH from the pituitary, which in turn controls ovarian estrogen production [50].
Figure 2.2: Feedback processes which control the concentration of estradiol [50] (+) positive feedback, (-) negative feedback
2.5 Molecular mechanism of estrogen action

The ER’s are transcriptional factors that bind to the regulatory regions of genes, specifically estrogen response elements (ERE). When the ER-α is bound by agonists it undergoes a conformational change. This conformational change induces the disassociation of intra-cytoplasmic chaperones such as heat shock proteins Hsp 70 and 90 [51]. This having been done the ligand bound receptor is now free to bind to DNA after which the transcriptional process is controlled by the recruitment of various cofactors and local transcription machinery.

2.5.1 Ligand-dependent genomic actions

One of the mechanisms through which estrogenic compounds have an effect in the human body is through the interaction of ligand bound ER-α with an estrogen response element (ERE). The ERE is a region of DNA where the ER-α is known to interact directly with DNA. There are a number of known ERE’s, however, the most responsive ERE is the vitellogenin ERE. This ERE was isolated from the African clawed frog *Xenopus laevis* and has been found to contain the consensus palindromic ERE sequence [52]. In mammals most estrogenically sensitive genes contain non consensus ERE sequences [53].

It has been found that three specific amino acids within the proximal P-box of the first zinc finger in the ER-α are used to bind to the ERE in a sequence specific manner [49]. The second zinc finger is involved in receptor molecule dimerization as well as ERE half-site spacing recognition. It has been found that the structure of the ERE has an influence on the cofactors that are recruited to the ER-ERE complex. It has, however, also been noted that the type of ligand also plays a role in the afore mentioned function. When E$_2$ is bound to ER-α the ERE is the major determinant of which cofactors are recruited to the ER-ligand-ERE complex. However, when antiestrogens are bound the ERE loses it’s influence over which AF-2 dependent cofactors
2.5 Molecular mechanism of estrogen action

are recruited [11]. There are two main ways in which ER-α can interact with ERE’s. The first mechanism is the classical mechanism through which ER-α associates directly with the promoter sequence of the ERE. Another mechanism through which the ER-α can interact with the ERE is known as tethering. The tethering mechanism is defined, in the case of ER-α, as the interaction of the ER-α with the ERE via an intermediary protein. An example of this mechanism is the indirect interaction, via c-fos/c-jun, between ER-α and AP-1, Figure 2.3. It has been found that ER-α can interact in a hormone dependent manner with the Sp1 transcription factor [54].

Both ER-α and ER-β can interact with the fos/jun transcription factor complex on AP1 sites which results in the stimulation of gene expression in the absence of E2 [55].

Interaction of ER-α with basal transcription factors

The initiation of transcription is controlled by RNA polymerase II, however, a number of basal transcription factors, TFIIA; TFIIB; TFIID; TFIIE; TFIIF and TFIIH, must first assemble at the core promoter. The transcription factor TFIID consists of the TATA binding protein and at least eight tightly associated factors [57]. ER-α is known to interact directly with TFIIB, TATA binding protein (TBP), and various transcription activation factors (TAF’s) of TFIID directly [52]. ER-α interacts with the TBP using both AF-1 and AF-2 domains as interaction surfaces [58]. The interaction of ER-α with hTAF1130 is controlled by the ligand binding domain of ER-α. Although this interaction is ligand independent it is required for the ligand dependent ER-α mediated activation of transcription [59]

Interaction with co-activators

ER-α interacts with a number of proteins, some of which have been shown to have an influence on transcription. These proteins that interact with ER-α have been termed co-activators and have been shown to interact with other
2.5 Molecular mechanism of estrogen action

Figure 2.3: Models which suggest how ER-α controls gene expression [56].
steroid receptors. By definition co-activators are proteins that interact directly with steroid receptor and enhance transcription [60]. The following discussion will concentrate on the interaction of ER-α with the p160 family, RIP-140, TIF-α, CBP/p300 and p68 co-activators.

The p160 family of co-activators are separated into three separate groups namely steroid receptor co-activator 1 (SRC-1), steroid receptor co-activator 2 (SRC-2) and steroid receptor co-activator 3 (SRC-3).

ERAP-140 and ERAP-160 were the first proteins shown to interact directly with ER-α. Using an MCF-7 cell experimental system and GST pull-down assays it was shown that ERAP-160 interacts with the ligand binding domain of ER-α, when ER-α is bound by E2 [61]. ERAP-160 has since been cloned and is now named SRC-1 [62]. The functions of SRC-1 include the stimulation of E2 mediated gene transcription and enhancing the interaction between the N and C terminal domains of ER-α. It has been suggested that SRC-1 integrates the AF-1 and AF-2 functions and allows full ER-α activation after ligand binding [63]. SRC-1 has histone acetyltransferase activity, which is specific to histone H3 and H4. Using this activity SRC-1 is able to acetylate lysine residues on the N-terminal tails of histones H3 and H4 in chromatin. The acetylation of lysine residues in histones H3 and H4 results in the alteration of nucleosomal formation and stability of the chromatin and enhances the formation of a stable pre-initiation complex. The end result of this process is to facilitate transcriptional activation by RNA polymerase II [64, 65, 66]. SRC-1 can bind both Fos and Jun [67] as well as basal transcription factors TBP and TFIIB [68], because of this it has been suggested that SRC-1 may act as a bridge between the ERE and ligand activated ER-α-Ap-1 complex and the DNA polymerase II initiation complex [69].

GRIP1, NCoA-1 and TIF-2 are now all referred to as SRC-2. It has been shown through the use of GST pull down assays that SRC-2 interacts with
the LBD of ER-α in the presence of E₂ [70] as well as the AF-1 domain [71]. Through the use of a transient transfection system, in HeLa cells over expressing ER-α, it has been shown that SRC-2 stimulates the expression of the vitellogenin ERE in the presence of E₂ [70]. SRC-2 has been shown to interact with the AF-2 domain and is involved in the ligand mediated transcription of hormone dependent genes [71].

The following cofactors, ACTR/RAC₃/p/CIP/AIB₁, are all now referred to as SRC-3. SRC-3 forms complexes with p300/CBP-associated factor (PCAF) and may possibly form part of a larger complex containing ER-α, PCAF and CBP/p300. As with SRC-1 and SRC-2 SRC-3 has an innate HAT activity [72] and is known to enhance reporter gene expression for many NR’s including ER-α (reviewed in [73]).

RIP-140 was first identified in cell extracts from HeLa and COS-1 cells as a protein that interacted with the ER-α LBD in the presence of E₂ [74]. One of the interesting properties of RIP-140 is that it has a bi-phasic effect of E₂ activated ERE driven reporter genes. At low concentrations RIP-140 stimulates transcription whilst at high concentrations it has a repressive effect on the transcription, all of which is in the presence of E₂ [75].

TIF1α was first cloned in mice and interacts with many NR’s including ER-α in a ligand dependent manner and has been shown to interact with ERE bound ER-α in the presence of E₂ [76]. COS-1 cells that were exposed to E₂ co-expressing ER-α and TIF1α revealed that there was ligand dependent phosphorylation of TIF1α and that this required binding to transcriptionally active ER-α [77]. TIF1α also possesses intrinsic kinase activity and selectively phosphorylates TF₁₁Ealpha, TF₁₁28 and TF₁₁55 in vitro. TIF1α may act as a ER-α co-activator, in part by phosphorylating and modifying the activity of components of the transcriptional machinery [77].
2.5 Molecular mechanism of estrogen action

p300 and CREB binding protein (CBP) have been identified as co-activators for class I and class II nuclear receptors [78]. Both CBP (interacts with SRC-1 [79], SRC-2 [80] and SRC-3 [81]) and p300 interact with SRC-1 and synergistically enhance ER-α activated reporter gene in transiently transfected cells [79, 82]. CBP is known to interact with the basal transcription factor TFIIB [83] and interacts with RNA pol II in HeLa extracts which indicates that CBP interaction with NR co-activator complex may recruit RNA pol II initiation complex to the promoter [84]. Both p300 and CBP are known to be acetyltransferases and acetylate all four core histones in nucleosomes [85, 86]. It has been found that p300/CBP specifically hyper-acetylates histone H4 of the promoter of E2 responsive genes (PS2, cathepsin D, c-Myc and EB1) and that after 1 hour acetylation decreases in parallel with RNA pol II engagement with the promoter. These results indicate that histone (de)acetylation plays an important role in the E2 induced expression of sensitive genes [85, 86]. CBP has been known to acetylate SRC-3 and that this acetylation decreases SRC-3 interaction with E2-ER-α complex in vitro, which may provide a mechanism in which gene transcription could be attenuated [77]. CBP interacts with the LBD of ER-α, in the presence of E2, as well as SRC-1 which suggests that CBP may serve to integrate multiple signaling pathways in the nucleus [85].

2.5.2 Ligand-independent genomic actions

It is now well established that ER-α mediated transcription can also be stimulated by ligand independent mechanisms involving second messenger signaling pathways, which result in the phosphorylation of the ER-α. It has also been shown that there are cell specific differences in the ability of secondary messenger pathways to enhance ER-α mediated transcription. This may be one of the reasons why there are differences in ER-α action in various cells [87]. This ligand-independent ER-α mediated transcription has been linked to the AF-1 region of the ER-α [88]. AF-1 activity is enhanced by the activity of secondary messenger signaling pathways, which supposedly
2.5 Molecular mechanism of estrogen action

relieves the inhibition caused by the LBD. It has also been shown that in response to ligand, AF-1, synergizes with AF-2 in the LBD and thus also plays a role in enhancing ligand-dependent, as well as, ligand-independent transcription via the ER-α.

Phosphorylation sites

Apart from ligand-independent activation of ER-α by phosphorylation, it has also been shown that the binding of E₂ to the ER-α is enhanced by the phosphorylation of the ER-α through secondary messenger pathways [89, 90, 91]. There is currently a debate as to whether ER-α is only phosphorylated on serine residues [89, 90, 91, 92] or whether tyrosine residues are also phosphorylated [93, 94]. It does, however, seem that there is not much evidence for the phosphorylation of tyrosine residues. Though a few cases have been reported, others have been unable to show tyrosine phosphorylation.

Serine 104, 106, 118 The Serine (Ser) residues 106 and 118 are highly conserved residues found in many species, whilst Ser 104 is only found in mammals. All of these residues are located in the AF-1 domain of the ER-α. It has already been shown that Ser 118 is the major phosphorylation site in response to E₂. It has also been shown that Ser 118 is phosphorylated in response to MAPK activation by EGF. It has been speculated that Ser 104 and 106 are phosphorylated by different kinases as compared to Ser 118. This is because the former are phosphorylated by Cyclin A-dependent kinase 2 (Cdk2) whereas the latter is not.

Serine 167 Reports have shown that Ser 167 is a major phosphorylation site in response to E₂ [95], however, controversy surrounds this issue as there are also reports which do not find this [89]. Serine 167 located in the AF-1 domain is, however, phosphorylated in response to MAPK activation [96] and it has been found that p90 Ribosomal S6 kinase (Rsk) is responsible for the phosphorylation [97].
2.5 Molecular mechanism of estrogen action

**Serine 236** In *vitro* it has been shown that Ser 236, located in the DBD of ER-α, can be phosphorylated by protein kinase A [98].

**Tyrosine 537** It has been reported that Tyr 537, located in AF-2 domain, is phosphorylated and that this phosphorylation is not dependent on the presence of ligand [93].

**Influence on ER-α function**

It would seem that the phosphorylation of the serine residues in the AF-1 domain is involved in influencing the recruitment of co-activators which enhances ER-α mediated transcription [99, 100, 101]. It has also been shown that when Ser118 is mutated there is a decrease in the level of transactivation via the ER-α [97]. Another one of the pathways that is stimulated by E$_2$, and has important influences on cell biology, is the activation of proline directed serine/threonine kinase (ERK). ERK is a member of the MAP kinase (MAPK) family. It has been shown that growth factors EGF and IGF can activate ERK which leads to the phosphorylation of Ser118 in the nuclear ER-α [102, 103]. Growth factors are also known to activate pp90$^{rsk{-1}}$ via ERK, which results in Ser167 phosphorylation of ER-α [97]. The AF-2 domain plays an important role in mediating transcriptional activation by cAMP [104]. The importance of this function with respects to ER-α mediated transcription is still being studied. When PKA phosphorylation sites were removed from ER-α there was no interference of ER-α transcription via cAMP [105]. This might imply that PKA regulation of the cofactors is more important than ER-α phosphorylation when looking at ER-α mediated transcription. It has been noted that in some cases the phosphorylation of Tyr 537 can result in the ligand independent recruitment of co-activators thus resulting in constitutively active reporters [106].
2.6 Factors influencing the levels of transactivation

There are a number of factors that can influence the level of transactivation of estrogenically sensitive genes. These include modulation of the levels of hormones, estrogen receptor levels as well as interaction of co-activators and co-repressors with the ER-α. In the following section factors that modulate the levels of ER-α will be discussed.

One of the mechanisms through which the expression of genes can be controlled is through chromatin remodeling. It has been found that the chromatin has to be unwound before the genes can be transactivated [107].

Another of the mechanisms through which the expression of genes are controlled is through the epigenetic regulation of afore mentioned genes. As an example the levels of ER-α expression are down regulated via the methylation of the ER-α gene in a number of tissue including colon [108], lung [109], heart [110], prostate [111], breast [112] and ovary [113]. In studies performed using breast cancer tissue it has been found that the level of ER-α expression is inversely proportional to the level of methylation of ER-α gene [114]. There is evidence that methylation is not the sole mechanism by which ER-α levels are controlled. It has been shown that methylation also involves the assistance of histone deacetylases (HDAC) which remove acetyl groups from lysine residues on histones H3 and H4, which results in the compaction of chromatin [64, 66, 115, 116, 117].

The interaction of cofactors with the promoter region of ER-α is another mechanism through which the expression of ER-α is regulated. Two transcription factors ER factor 1 (ERF-1) and ER-β factor 1 (ERBF-1) have been discovered which regulate the levels of ER expression via interaction...
2.6 Factors influencing the levels of transactivation

with the promoter of ER-α [118, 119, 120].

The effects that are seen in various tissues can be described by differences in pharmaco-kinetics, or differential ligand metabolism. It can thus be said that the same hormone may be present in various tissues, however, the relative amounts of the hormone in the tissue differ because of altered uptake or metabolism [107]. All of the above mechanisms can have an indirect regulation of estrogenic compounds on the transactivation of an estrogenically sensitive gene.

The role that SHBG plays in regulating the bioavailable levels of estrogen in the human body will be discussed in the next chapter.
Chapter 3

Sex hormone binding globulin

3.1 Introduction

The bio-availability of sex steroids are regulated via a number of mechanisms, one of them being the binding of steroids to transport proteins in plasma. There are two main proteins which bind steroids in human plasma sex hormone-binding globulin and albumin. Albumin binds sex steroids with a low affinity and high capacity, while SHBG binds sex steroids with a high affinity and low capacity. This review will concentrate on the role that SHBG has in the human body.

It has traditionally been thought that SHBG only regulates the bio-availability of steroids, however, it has recently been found that SHBG also modulates hormone action [121].

3.2 Structure

SHBG is a dimeric glycoprotein and was first identified as a β-globulin which binds E\textsubscript{2} and testosterone with a high affinity in human plasma [122]. SHBG is synthesized in hepatocytes [123] after which it is secreted into plasma where it binds sex steroids. The SHBG precursor polypeptide consists of...
3.2 Structure

29 amino acids and a hydrophobic leader sequence followed by 373 residues that contain two disulfide bridges. In human plasma SHBG is found as a 90 kDa homodimer [124]. SHBG is composed of two laminin G-like domains which do not require glycosylation to form homodimers. The steroid binding and dimerization sites of SHBG reside in the amino terminal domain of the laminin G-like domain. Each monomer of the SHBG homodimer contains a steroid binding site [125].

The laminin G-like domain of SHBG is comprised of a β-sandwich formed by seven stranded β sheets. The steroid binding pocket of SHBG, Figure 3.1, is covered by a loop segment which is formed by amino acid residues 130-135 (Pro130, Leu131, Thr132, Ser133, Lys134 and Arg135). This loop segment functions as a flap that may regulate ligand access to the steroid binding pocket. In this flap residues 130 and 131 are in an extended conformation. Residues 131 to 134 form a single 3_{10} helical turn with a hydrogen bond between the main chain carbonyl oxygen atom of Leu131 and the main chain nitrogen NH group of Lys134. The helical loop segment in this region of SHBG provides the flexibility needed to allow unhindered access of the ligand to the ligand binding pocket [125]. The presence of a 17-β hydroxy group and a planar C-19 steroid with an electro negative group at C3 has been found to be essential for the optimal binding of a steroid to SHBG, Figure 3.2. In line with with these criteria, SHBG has the highest affinity for dihydrotestosterone (DHT), followed by testosterone and finally E_2 [126]. Grishkovskya [127] proposed a model for the formation of the SHBG homodimer. This model places the β strand 7 of one homodimer next to the β strand 10 of the second homodimer, which results in the formation of eight hydrogen bonds being formed within the steroid binding interface. The contact area in the steroid binding pocket is hydrophobic in nature and contains amino acid residues Ala85, Leu87, Val89, Leu122 and Leu124 [128], Figure 3.1. It has been proposed that only a single steroid molecule is bound by each SHBG homodimer [129] which would be achieved by the formation of a
3.2 Structure

single steroid binding site by two monomeric units [130]. Hammond [131] et al have proposed that because the steroid binding sites are located so close to each other, the binding of one site may sterically hinder the binding of steroid to the other binding site [128]. When observing the SHBG-steroid bound crystal structure Grishkovskaya [127] noted that both binding sites were bound, however, also commented that both steroid binding sites may have been artificially saturated.

Figure 3.1: Steroid binding site of SHBG [125].
3.3 Regulation

Initially after birth the plasma levels of SHBG in humans are very low but after a couple of weeks these levels increase dramatically in males and females. However, at the onset of puberty the levels of plasma SHBG increases even more dramatically in females [132].

Hormone replacement therapy (HRT) is often used in post-menopausal women to alleviate the symptoms normally associated with menopause. It is known that estrogen replacement treatment (ERT) reduces the risk of developing cardiovascular disease and osteoporosis [133, 134]. There is, however, evidence that there may be a linkage between ERT and breast cancer [135, 136].

It has been shown in HepG2 cells, a human hepatocarcinoma cell line which expresses SHBG, that 17-β-OH steroids have the ability to increase the levels of SHBG secreted [137]. Clinical trials indicated that, when estrogen production is increased endogenously or exogenously, there is a concomitant increase in the levels of SHBG [132, 138, 139], Table 3.2 and Table 3.1.
3.3 Regulation

Table 3.1: Changes in concentration plasma SHBG in women using various HRT regimes [140].

<table>
<thead>
<tr>
<th></th>
<th>Concentration of plasma SHBG (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>CEE/MPA (2.5mg)</td>
<td>36-119</td>
</tr>
<tr>
<td>CEE/MPA (5mg)</td>
<td>25-129</td>
</tr>
<tr>
<td>Tibolone</td>
<td>25-116</td>
</tr>
</tbody>
</table>

CEE: Conjugated equine estrogens. MPA: medroxyprogesterone acetate. Tibolone is an estrogenic compound that is used in hormone replacement therapy. The effect of the compounds on the production of SHBG over a period of 1 year was studied.

Table 3.2: Effect of long term continuous oral and transdermal estrogen replacement on levels of SHBG [141].

<table>
<thead>
<tr>
<th></th>
<th>Concentration of plasma SHBG (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Oral</td>
<td>38.6 ± 29.0</td>
</tr>
<tr>
<td>Transdermal</td>
<td>42.1 ± 23.4</td>
</tr>
</tbody>
</table>
3.3 Regulation

Studies in HepG2 cells [123] argue against the direct influence of steroids on the production of SHBG in the liver. Both androgens and estrogens increase, or have little effect, on the levels of SHBG production. The estrogen induced increase in SHBG levels is more pronounced inside the cell than in the medium. It is hypothesized that this is due to the production of alternative transcripts and gene products that lack a secretion signal [142]. It would seem that although sex steroids do not have the ability to affect SHBG plasma concentration by direct transcriptional mechanisms, they may modify the carbohydrate composition of the two sub-units [143]. The influence that sex steroids have on SHBG may thus be to regulate the clearance of SHBG from plasma [144].

It is known that by eating food high in fiber the levels of SHBG are also increased because of the presence of phytoestrogens and isoflavonoids, that have been shown to increase the levels of SHBG in HepG2 cells [142]. In contrast it has also been shown that, by eating a high fat diet, the levels of SHBG are decreased [145]. In men there is a correlation between the SHBG and serum high density lipoproteins (HDL) whilst women, who have low SHBG plasma levels, are more susceptible to cardiovascular disease.

The relationship between thyroid hormone levels and SHBG is well known. Patients suffering from thyrotoxicosis have abnormally high thyroid hormone levels and an enhanced hepatic SHBG production [146]. The elevated plasma SHBG level is currently being used as one of the possible markers to detect this disease [146].

Low levels of SHBG are associated with increased triglycerides, decreased HDL, cholesterol, obesity and other cardiovascular factors. There are conflicting results as to how exercise affects serum SHBG levels, Table 3.3. In some studies no influence [147] has been found on the levels of SHBG while in other studies an increase [148] or decrease [149] in SHBG levels has been
3.3 Regulation

found. The following study was designed to determine the effect that a 20-week endurance exercise program has on the plasma levels of SHBG as well as familial SHBG levels [150]. It is well known that SHBG transports sex hormones and that the circulating levels might be regulated differently in men and women. In the study conducted by Ping et al. [150], levels were positively associated with estradiol while testosterone and insulin levels had a negative influence. General adiposity has been shown to increase the production of pancreatic insulin, whilst upper body obesity increases the amount of free testosterone and decreases the clearance of insulin from the liver. This increase of testosterone and insulin correlates negatively with plasma SHBG concentrations [151]. In females the concentrations of SHBG and insulin can be correlated and this relationship can be used to predict the occurrence of non-insulin dependent diabetes [152]. In males the correlation between SHBG and insulin is an inverse correlation [153]. Clinical studies have shown that there is a correlation between hyperinsulinemia, insulin resistance and SHBG [154]. HepG2 cells have been used to study the regulation of SHBG production in vitro [155, 156] and it has been shown that both insulin and insulin-like growth factor had a negative influence on the levels of SHBG production, Table 3.4, and that this influence was at the RNA level.

Table 3.3: Concentration of SHBG (nM) before and after endurance exercise [150].

<table>
<thead>
<tr>
<th></th>
<th>Father</th>
<th>Mother</th>
<th>Son</th>
<th>Daughter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Exercise</td>
<td>44 ± 17.6</td>
<td>83.8 ± 45.3</td>
<td>35.1 ± 15.0</td>
<td>87.6 ± 47.4</td>
</tr>
<tr>
<td>After Exercise</td>
<td>-0.1 ± 8.3</td>
<td>-7.9 ± 25.3</td>
<td>-0.1 ± 8.0</td>
<td>-3.4 ± 43.5</td>
</tr>
</tbody>
</table>

(-) indicates a decrease in the levels of SHBG. The levels of SHBG were measured before and after a 20 week program of endurance exercise.
### 3.4 Physiological function

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>IGFBP-1</th>
<th>P-value</th>
<th>SHBG</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF I (30nM)</td>
<td>4</td>
<td>60±7.3</td>
<td>0.009</td>
<td>75 ± 3.3</td>
<td>0.025</td>
</tr>
<tr>
<td>IGF-II (60nM)</td>
<td>3</td>
<td>48±3.4</td>
<td>0.013</td>
<td>90 ± 3.8</td>
<td>0.20</td>
</tr>
<tr>
<td>Insulin (120nM)</td>
<td>2</td>
<td>48±15</td>
<td>0.034</td>
<td>75 ± 2.9</td>
<td>0.81</td>
</tr>
</tbody>
</table>

1Cited from [157]

**Table 3.4: IGFBP-1 and SHBG RNA calculated in proportion with ribosomal RNA**

### 3.4 Physiological function

Sex hormone-binding globulin has a high affinity and low capacity for androgens and estrogens [158]. It is believed that one of the main functions of SHBG is to regulate the amount of free sex steroids in the plasma, and accordingly the metabolic clearance of these steroids is also influenced. Thus if SHBG has a high affinity for a sex steroid, the rate at which this steroid will be cleared from the plasma will be lower than that of a steroid for which SHBG has a lower affinity for [159, 160].

There are numerous schools of thought as to the function that SHBG plays in the bio-availability of steroids in the human body. The free hormone hypothesis states that only the portion of steroid that is free, i.e. unbound, is biologically active as only the free steroids can enter the cell by simple diffusion [1]. This hypothesis has been challenged by many authors such as Siiteri [159] and Padridge [161] with respect to the role that extracellular binding proteins such as SHBG play. There are two arguments which have been used both of which argue against the hypothesis that only free steroids are biologically active. The first argument proposed by Siiteri [162] states that when considering the concentration of SHBG and circulating amount of E2 at any stage during the menstrual cycle, there would not be enough free steroid to fully occupy cellular estrogen receptors, which is a requirement
for maximal estrogen response. The second argument proposed by Padridge [161] states that if the pool of free hormone accounted for all the action of sex steroids, then all tissues would be exposed to the same level of hormones. Padridge also suggested that the SHBG bound steroid is available to cells and that SHBG could release steroids at the site of action and amplify the steroid effect where needed. As a result of this research it is now known that SHBG can bind a membrane bound receptor in cells that are sensitive to the presence of sex steroids. This binding of the SHBG to the membrane receptor is thought to play a role in guiding sex steroids to the site of their action [163].

Epidemiological evidence has shown that the risk of developing breast cancer is related to the amount of time that a woman is exposed to ovarian estrogens and progestins [164, 165]. Post-menopausal estrogen replacement therapy (ERT) or hormone replacement therapy (HRT), which contains estrogens and progestins, is related to the development of breast cancer [166, 167]. Both obesity [168] and hyperinsulinemia have been identified as risk factors for developing breast cancer [169] because they affect estrogen metabolism and decrease the binding of estrogens to SHBG.

A membrane SHBG-receptor has been identified which is thought to be involved in the control of proliferation of breast cancer cells, via a cAMP cascade pathway [170, 171, 172]. A New York Women’s Health study has shown that an increase in free and total E$_2$, with a decrease in SHBG-bound E$_2$, results in an increase of breast cancer incidences in post-menopausal women [168, 173, 174].

It is well known that coronary heart disease (CHD) is often positively associated with serum levels of DHEAS and free testosterone in women. The association of SHBG with coronary heart disease is not well known but it is known that low levels of SHBG are associated with low levels of high density lipoproteins (HDL), cholesterol and high levels of triglycerides, apoprotein B
3.4 Physiological function

and free testosterone, all of which are known to coincide with coronary heart disease [175, 176]. In support of this it has been found that post-menopausal women with CHD have a low serum concentration of SHBG [177].

The role that SHBG plays in the regulation of the bioavailable levels of estrogens in the human body has been described. The next chapter describes the results obtained using the experimental systems described in Chapter 1.
Chapter 4

The influence of SHBG on E₂ binding by the ER-α: An experimental approach

As stated in the introduction the aim of this project was to develop an experimental system to describe the binding of E₂ to the human ER-α in the absence or presence of SHBG. The secondary aim was to determine what effect SHBG had on the transactivation of an ERE, via the ligand bound ER-α. The final aim of this project was to build a mathematical model that would describe the above mentioned conditions. A number of variables are needed to build the mathematical model. The variables that are needed are shown in Table 4.1.

To achieve these aims a number of experimental systems are needed so as to obtain the binding constants (K₅ₐₓ (E₂) and Bₘₐₓ) for ER-α in the absence and presence of SHBG, as well as data from the transactivation studies. The binding constants for SHBG binding E₂, K₅ and Bₘₐₓ in the absence of ER-α are also required.

The experimental systems used for this approach were three fold:
• COS-1 cells were used as an experimental system in which data on the binding of E\(_2\) to ER-\(\alpha\), in the absence of SHBG, as well as data from transactivation studies could be collected, Figure 4.1.

• Hep89 cells were used for similar experiments except that SHBG is endogenous in these cells. As such the data collected would show the effect that SHBG has on the binding of E\(_2\) to ER-\(\alpha\) and the transactivation of an ERE, via the ligand bound ER-\(\alpha\). As Hep89 cells were stably transfected with ER-\(\alpha\) it was not necessary to transf ect these cells with the human ER-\(\alpha\) gene, Figure 4.2.

• Human pregnancy plasma contains high levels of SHBG. By using saturation binding assays it should be possible to determine the kinetic binding constants (\(K_d\) and \(B_{\text{max}}\)) for the binding of E\(_2\) to SHBG.

| Table 4.1: Parameters needed to build the mathematical model of agonism. |
|---|---|
| Binding proteins | Binding constants |
| hER-\(\alpha\) | \(K_d (E_2)\) (intracellular) \(B_{\text{max}} (ER-\alpha)\) (intracellular) |
| hSHBG | \(K_d (E_2)\) \(B_{\text{max}} (SHBG)\) (intracellular and extracellular) |

<table>
<thead>
<tr>
<th>Volume of compartments</th>
<th>Experimental system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>COS-1 and Hep89 cells</td>
</tr>
<tr>
<td>Medium</td>
<td>COS-1 and Hep89 cells</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolic studies</th>
<th>Experimental system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism of E(_2)</td>
<td>COS-1</td>
</tr>
<tr>
<td>Metabolism of E(_2)</td>
<td>Hep89</td>
</tr>
</tbody>
</table>
Figure 4.1: Diagram of the experimental system when only $E_2$ and ER-$\alpha$ are present. In this experimental system $E_2$ can be transferred across the cell membrane and bind to ER-$\alpha$. The nucleus and cytoplasm have been defined as one compartment.
Figure 4.2: Diagram of the experimental system when E$_2$, ER-\(\alpha\) and SHBG present. This system describes the situation where E$_2$ can bind to both ER-\(\alpha\) as well as SHBG. The binding of E$_2$ to SHBG has been defined as occurring in the medium and inside the cell, while E$_2$ can be transported into the cell and bind ER-\(\alpha\). The nucleus and cytoplasm have been defined as one compartment.
4.1 Development of an experimental system for E₂ binding

4.1 Development of an experimental system for E₂ binding

4.1.1 COS-1 cell system

Optimisation of binding

The aim of the following experiments was to determine the binding constants, \( K_d \) and \( B_{\text{max}} \) of hER-\( \alpha \) when binding E₂ in COS-1 cells in the absence of SHBG. The experimental approach used to obtain the required data was to transflect COS-1 cells with the hER-\( \alpha \) gene and then to perform competitive binding experiments. The experimental system was initially optimised by varying the amount of hER-\( \alpha \) that the COS-1 cells were transfected with. After the amount of hER-\( \alpha \) to be used was optimised the amount of \(^3\)H-E₂ that would be used in the experimental procedure had to be optimized. The reason that the amount of \(^3\)H-E₂ had to be optimized was as follows: To determine the \( K_d \) using the competitive binding experimental system the amount of \(^3\)H-E₂ used had to be between two and ten times lower than the IC\(_{50}\) E₂. The IC\(_{50}\) E₂ is defined as the concentration of unlabelled ligand that reduces the Specific binding of labelled ligand by 50%. By using this information the relationship between the \( K_d \) (E₂) and IC\(_{50}\) E₂ can be mathematically defined.

\[
0.5 \frac{B_{\text{max}} \times [^3\text{H-E}_2]}{[^3\text{H-E}_2] + K_d (E_2)} = \frac{B_{\text{max}} \times [^3\text{H-E}_2]}{[^3\text{H-E}_2] + [E_2] + K_d (E_2)}
\] (4.1)

Equation 4.1 can be simplified to:

\[
\text{IC}_{50} = [^3\text{H-E}_2] + K_d (E_2)
\] (4.2)

As can be seen when determining the \( K_d \) if the IC\(_{50}\) E₂ is less than the amount of labelled compound (\(^3\)H-E₂) the results of this calculation would yield a negative \( K_d \), which is not possible.

The aim of the following experiments was to determine minimal and maximal
4.1 Development of an experimental system for E\textsubscript{2} binding

amounts of pcDNA3-ER-\textit{\alpha} to be used in binding experiments and the levels of Specific binding that could be expected. COS-1 cells were transiently transfected with either 0.012\(\mu\)g or 0.102\(\mu\)g of pcDNA3-ER-\textit{\alpha} and exposed to 20nM \(^3\)H-E\textsubscript{2}. In determining the Specific binding the transfected COS-1 cells were exposed to two conditions:

- COS-1 cells were exposed to 20nM \(^3\)H-E\textsubscript{2} whilst using 1\(\times\)10\(^{-5}\)M E\textsubscript{2} as a competitor. This would allow the determination of Non Specific binding.

- COS-1 cells were exposed to 20nM \(^3\)H-E\textsubscript{2} whilst using ethanol as a competitor. This would allow the determination of Total binding.

Specific binding, that is the binding was determined as follows:

\[ \text{Specific binding} = \text{Total binding} - \text{Non Specific binding} \quad (4.3) \]

The results of this experiment, Figure 4.3, show that maximal specific binding was obtained when COS-1 cells were transfected with 0.102\(\mu\)g pcDNA3-ER-\textit{\alpha} and minimal specific binding (about 2000 cpm) when 0.012\(\mu\)g of pcDNA3-ER-\textit{\alpha} was used. It was decided that in future 0.52\(\mu\)g of pcDNA3-ER-\textit{\alpha} would be used to transfect COS-1 cells as it would then be possible to transfect with a \(\beta\)-galactosidase and ERE reporter gene when performing transactivation studies. This method would enable the transfection of COS-1 cells and use these cells for both binding and transactivation studies.
4.1 Development of an experimental system for E₂ binding

Figure 4.3: Optimization of amount of pcDNA3-ER-α to use in binding experiments. COS-1 cells were transiently transfected with either 0.012µg (12%) or 0.102µg (96%) pcDNA3-ER-α plasmid as described in Appendix C.3.2. COS-1 cells were then exposed to 20nM[^3]H-E₂ in the presence of either ethanol (Total binding) or 1×10⁻⁵M E₂ (Non Specific binding) for 10 hours. Ligand binding was determined as described in Appendix C.5.2. Results are shown as the standard error of the mean of triplicate samples. Specific binding was calculated as Total binding - Non Specific binding.
4.1 Development of an experimental system for E$_2$ binding

**Competitive binding assay**  The aim of the following experiment was to determine the amount of $^3$H-E$_2$ that should be used in competitive binding assays. Competitive binding assays were used to determine the K$_d$ and B$_{max}$ of hER-$\alpha$ when binding E$_2$ in the absence of SHBG. The experimental conditions used were optimised by varying the amount of $^3$H-E$_2$ (10nM, 1nM, 0.5nM and 0.1nM). From the results of this experiment, Figure 4.4, it can be seen that maximal Specific binding was obtained when 10nM $^3$H-E$_2$ was used and that minimal specific binding was obtained when 0.1nM $^3$H-E$_2$ was used. When examining the results it was noted that when 1nM of $^3$H-E$_2$ was used a Specific binding of roughly 1800 cpm was obtained and it was decided that in future 1nM of $^3$H-E$_2$ would be used in future competitive binding assays.
4.1 Development of an experimental system for E_2 binding

Figure 4.4: Optimisation of the amount of ^3^H-E_2 used in binding experiments. COS-1 cells were transfected using 0.52µg pcDNA3-ER-α, 0.10µg ERE-vit, 2.34µg pGL-2-Basic and 0.12µg pCMV-β-galactosidase as described in Appendix C.3.2. COS-1 cells were then exposed to varying amounts concentration of ^3^H-E_2 (10nM, 1nM, 0.5nM and 0.1nM) in the presence of either ethanol (Total binding) or 1×10^{-5}M E_2 (Non Specific binding) for 10 hours. Ligand binding was determined as described in Appendix C.5.2. Results are shown as the standard error of the mean of triplicate samples. Specific binding was calculated as Total binding - Non Specific binding.
4.1 Development of an experimental system for E₂ binding

Whole cell binding of E₂ to the hER-α in COS-1 cells

The aim of the following experiments was to determine the K<sub>d</sub> and B<sub>max</sub> for hER-α binding E₂ in the absence of SHBG. This was accomplished performing competitive binding assays, using COS-1 cells, to determine the IC<sub>50</sub> E₂ and to use this to determine the K<sub>d (E₂)</sub> for hER-α.

COS-1 cells were transfected with pcDNA3-ER-α, as described in Appendix C.3.2, after which competitive binding assays were performed using 1nM ³H-E₂ in the presence of varying amounts of E₂ (between 1×10⁻⁵ M to 1×10⁻¹³ M) or ethanol. From the results of this experiment, Figure 4.5, it was determined that the IC<sub>50</sub> E₂ was determined as 2.35×10⁻⁹ M, using a one site binding equation [178]. As the IC<sub>50</sub> E₂ was greater than twice the amount of ³H-E₂, 1nM, used the K<sub>d</sub> could be determined using these results. The K<sub>d (E₂)</sub> was determined as between 3.45nM and 4.4nM using a homologous competitive binding model equation [178]. The K<sub>d (E₂)</sub> that was determined using this experimental system is not similar to that which Kupier [49], K<sub>d (E₂)</sub> = 0.1nM, determined using a pure ER-α solution, however is similar to that which Pederson [179] determined, between 2nM and 4nM, from human fat samples. With respect to the determination of B<sub>max (ER-α)</sub> it was determined that the B<sub>max (ER-α)</sub> was between 1.42nM and 2.62nM a one site binding equation [178] and the quick calculations from the Graphpad website (http://www.graphpad.com/quickcalcels/radcalcform.cfm).
4.1 Development of an experimental system for E$_2$ binding

Figure 4.5: Competitive binding assay using COS-1 cells. COS-1 cells were transfected using 0.52µg pcDNA3-ER-α, 0.10µg ERE-vit, 2.34µg pGL-2-Basic and 0.12µg pCMV-β-galactosidase as described in Appendix C.3.2. COS-1 cells were then exposed to 1nM $^3$H-E$_2$ in the presence of either ethanol (Total binding) or varying amount of E$_2$ ($1\times10^{-5}$M to $1\times10^{-13}$M, Non Specific binding) for 10 hours. Ligand binding was determined as described in Appendix C.5.2. Results are shown as the standard error of the mean of triplicate samples. Specific binding was calculated as Total binding - Non specific binding. The IC$_{50}$ E$_2$ was determined as $2.35\times10^{-9}$M and the B$_{max}$ (ER-α) as between 1.42nM and 2.62nM.
4.1 Development of an experimental system for E$_2$ binding

4.1.2 Optimisation of E$_2$ binding in Hep89 cells

The aim of the following experiment was to determine the effect that SHBG had on the binding of E$_2$ to hER-α. To accomplish this, competitive binding and saturation binding assays were undertaken to determine the B$_{\text{max\ app}}$ (ER-α) and K$_{\text{d\ app}}$. The experimental approach was to use Hep89 cells which have been stably transfected with hER-α and endogenously produce SHBG. Initially competitive binding assays were used, however, as will be explained problems were encountered and saturation binding assays performed.

Competitive binding assay

As stated the aim of these experiments was to determine the B$_{\text{max\ app}}$ and K$_{\text{d\ app}}$ for E$_2$ binding ER-α in the presence of SHBG. Hep89 cells were seeded and grown as described in Appendix C.5.3 after which the cells were exposed to 1nM $^3$H-E$_2$ in the presence of varying concentrations E$_2$ ($1\times10^{-5}$M to $1\times10^{-13}$M) or ethanol. From the results of this experiment, Figure 4.6, it was determined that the IC$_{50\ \text{app\ } E_2}$ was $7.52\times10^{-12}$M. As has been stated the IC$_{50\ \text{E}_2}$ has to be between two and ten times more than the amount of $^3$H-E$_2$ or the K$_d$ would be negative. As the IC$_{50\ \text{app\ } E_2}$ was about 132 times smaller than the amount of $^3$H-E$_2$ used it was not possible to determine the K$_{\text{d\ app}}$ from the results of these experiments. There are two suggestions as suggested by literature [178] in which this problem can be overcome. The first possibility being that the labelled ligand ($^3$H-E$_2$) and the unlabelled ligand (E$_2$) bind the receptor (hER-α) with different affinities. This is not a likely option as both the labelled and unlabelled ligand are both 17-β-estradiol. The second option is to lower the amount of labelled ligand used in the experiment significantly. It should be noted from the results, Figure 4.6, that maximal binding occurred at roughly 925 cpm. If the amount of $^3$H-E$_2$ used in the experiment was lowered then the number of cpm at maximal and minimal binding would be so low that Poisson distribution would have to be taken into account. Poisson distribution is a term that is used to describe the decay of a population of radioactive atoms and the sampling error that
4.1 Development of an experimental system for $E_2$ binding

arises during measuring the decay. The sampling error can be determined through the following equation:

\[
\frac{100 \times 1.96 \times \sqrt{C}}{C} = \frac{196}{\sqrt{C}}
\] (4.4)

Where $C = \text{cpm}$ multiplied by the number of minutes that the sample was counted. Using this data it is possible to calculate that if the number of cpm is below 500 there is a large error intrinsically present in sampling [178]. Because of the problems associated with this experimental system it was decided that saturation binding assays would be used to determine $B_{\text{max app}} (E_R-\alpha)$ and $K_{d \text{ app}} (E_2)$. 

4.1 Development of an experimental system for E\textsubscript{2} binding

Figure 4.6: Competitive binding assay using Hep89 cells to determine the effect that SHBG has on the B\textsubscript{max app (ER-\textalpha)} and K\textsubscript{d app (E\textsubscript{2})}. Hep89 cells were seeded and grown as described in Appendix C.5.3. The cells were then exposed to 1nM \textsuperscript{3}H-E\textsubscript{2} in the presence of ethanol (Total binding) or varying concentrations E\textsubscript{2} (1\times10^{-5}M to 1\times10^{-13}M). Ligand binding was determined as described in Appendix C.5.2. The IC\textsubscript{50 app E\textsubscript{2}} was determined as 7.52\times10^{-12}M using a one site competitive binding function [178]. Results are shown as the mean and standard error of the mean for triplicate samples.
4.1 Development of an experimental system for E$_2$ binding

Saturation binding assay

The aim of the following experiment was to determine the K$_{d \text{ app}}$ and B$_{\text{max app}}$ for E$_2$ binding hER-α in the presence of SHBG. The experimental approach used was to perform a saturation binding assay using Hep89 cells as the experimental system. Hep89 cells were seeded and grown as described in Appendix C.5.3 after which they were exposed to varying amounts of $^3$H-E$_2$ (between 0.25nM and 30nM) in the presence of 1×10$^{-5}$M E$_2$ (Non Specific binding) or ethanol (Total binding). The results of this experiment, Figure 4.7, showed that there was ligand depletion, Table 4.2.

Table 4.2: Ligand depletion encountered during saturation binding using Hep89 cells.

<table>
<thead>
<tr>
<th>Total added (cpm)</th>
<th>Total binding (cpm)</th>
<th>Percentage ligand depletion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>73460</td>
<td>23544</td>
<td>29</td>
</tr>
<tr>
<td>60540</td>
<td>12649</td>
<td>19</td>
</tr>
<tr>
<td>31060</td>
<td>4350</td>
<td>15</td>
</tr>
<tr>
<td>17700</td>
<td>2262</td>
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<td>12300</td>
<td>1897</td>
<td>14</td>
</tr>
<tr>
<td>8520</td>
<td>1226</td>
<td>15</td>
</tr>
<tr>
<td>7760</td>
<td>763</td>
<td>9</td>
</tr>
<tr>
<td>4200</td>
<td>618</td>
<td>13</td>
</tr>
<tr>
<td>3000</td>
<td>429</td>
<td>13</td>
</tr>
</tbody>
</table>

Ligand depletion calculated as (Total binding/total added)×100.

Ligand depletion is defined as the condition under which more than 10% of the labelled ligand, $^3$H-E$_2$, is bound in the experimental system in the absence of competitor. Because of the presence of ligand depletion the results of this experiment had to be analyzed using an equation that accounts for the presence of ligand depletion equation 4.5 [178]. The results of this
4.1 Development of an experimental system for E\textsubscript{2} binding

analysis showed that it was not possible to determine the B\textsubscript{max app} (ER-\textalpha) and K\textsubscript{d app} (E\textsubscript{2}).

\[
\begin{align*}
\text{Total binding} &= \frac{-b + \sqrt{b^2 - 4ac}}{2a} \\
\text{Non Specific binding} &= X \left( \frac{\text{NS}}{\text{NS} + 1} \right)
\end{align*}
\]

Where:

\[
\begin{align*}
a &= -1 - \text{NS} \\
b &= X(2-\text{NS}+1) + K_d(\text{NS}+1) + B_{\max} \\
c &= -X(\text{NS}(K_d + X) + B_{\max})
\end{align*}
\]

\text{NS} = \text{Non Specific binding (cpm) in the presence of an excess of unlabelled compound}

\text{X} = \text{Concentration labelled ligand (cpm)}

K\textsubscript{d} = \text{Equilibrium disassociation constant (cpm)}

B\textsubscript{max} = \text{Maximal density of receptor binding sites for radioligand (cpm)}
4.1 Development of an experimental system for E₂ binding

Figure 4.7: Saturation binding assay using Hep89 cells to determine the effect that SHBG has on the B\textsubscript{max} \text{app} (ER-α) and K\textsubscript{d} \text{app} (E₂). Hep89 cells were seeded and grown as described in Appendix C.5.3. The cells were then exposed to varying amounts of $^{3}$H-E₂ (between 0.25nM and 30nM) in the presence of ethanol (Total binding) or $1 \times 10^{-5}$M E₂ (Non Specific binding). Ligand binding was determined as described in Appendix C.5.2. The results were fitted to an equation that accounted for the presence of ligand depletion equation 4.5 [178]. Results are shown as the mean and standard error of the mean for triplicate samples.
4.1 Development of an experimental system for E₂ binding

4.1.3 Binding of E₂ to SHBG in human pregnancy plasma

The aim of the following experiments was to determine the $K_d$ and $B_{\text{max}}$ (SHBG) for SHBG binding E₂. To determine the $K_d$ (E₂) competitive and saturation binding experiments were performed using human pregnancy plasma as an experimental system. Human pregnancy plasma was used because it contains high amounts, up to 420nM [180], of SHBG. The experimental system used was based on that of Hammonds [180].

Optimisation of experimental system

The aim of the following experiment was to determine whether Dextran coated charcoal (DCC) could be used to remove endogenous and unbound steroids from human pregnancy plasma. The removal of endogenous steroids was important because if any endogenous steroids remained they might have a negative influence on the binding of E₂ to SHBG.

The effect of the presence of endogenous steroids was studied by comparing samples that had been exposed to DCC before and after the addition of $^3$H-E₂ to samples that had only been exposed to DCC after the addition of $^3$H-E₂. The ability of DCC to remove any unbound steroids was studied by comparing samples that contained no SHBG but either contained $^3$H-E₂ or no ligand and then exposed to DCC. The results of this experiment are shown in Figure 4.8. With respect to the ability of the effect of endogenous steroids it can be seen that the removal of these endogenous steroids does have an effect. This can be seen when comparing the total binding of the samples labelled “DCC before/after”, maximal binding 24309 cpm to “SHBG + DCC after”, maximal binding 24524 cpm as well as the samples “SHBG + DCC before”, maximal binding 163604 cpm, and “No DCC”, maximal binding 164001 cpm. By comparing the samples “No SHBG and DCC”, maximal binding to “No SHBG and No DCC” it is possible to see that DCC does
4.1 Development of an experimental system for E₂ binding

remove any unbound steroids.

Figure 4.8: Optimisation of conditions for binding of \(^3\)H-E₂ using human pregnancy plasma as an experimental system. Human pregnancy plasma was prepared as described in Appendix C.6. The samples were then exposed to 20nM \(^3\)H-E₂ and either 10\(^{-5}\)M E₂ (Non Specific binding) or EtoH (Total binding) was added to the samples after which the samples were incubated for 10 hours. Error bars indicate the standard error of the mean for triplicate determinations.
4.1 Development of an experimental system for E\textsubscript{2} binding

Competitive binding assay

The aim of the following experiment was to determine the K\textsubscript{d} for E\textsubscript{2} binding SHBG in the absence of ER-\textalpha. Human pregnancy plasma was prepared as described in Appendix C.6 after which the samples were exposed to 1nM $^{3}$H-E\textsubscript{2} in the presence of varying amounts of E\textsubscript{2} (between $1 \times 10^{-5}$M and $1 \times 10^{-13}$M) or ethanol.

From the results of this experiment, Figure 4.9, it was determined that the IC\textsubscript{50} E\textsubscript{2} was $1.09 \times 10^{-7}$M. As has been described before, to determine the K\textsubscript{d} using this method the IC\textsubscript{50} E\textsubscript{2} has to be between two and ten times the amount of $^{3}$H-E\textsubscript{2}, in this case 1nM, used. As this was not the case it was decided to determine the K\textsubscript{d} (E\textsubscript{2}) using a saturation binding assay.
4.1 Development of an experimental system for E₂ binding

Figure 4.9: Competitive binding assay using human pregnancy plasma as a source of SHBG. Human pregnancy plasma was prepared as described in Appendix C.6 after which 1nM ³H-E₂ was added to the samples in the presence of varying amounts (between 1×10⁻⁵M and 1×10⁻¹₃M) or ethanol and the samples incubated for a further 10 hours. Ligand binding was determined as described in Appendix C.5.2. The IC₅₀ E₂ was determined as 1.09×10⁻⁷M by fitting a one site binding equation [178] to the data. Results are shown as the mean of the triplicate samples with standard errors.
4.1 Development of an experimental system for E\textsubscript{2} binding

Saturation binding assay

The aim of the following experiments was to determine the K\textsubscript{d} for SHBG binding E\textsubscript{2} in the absence of ER-\textalpha. Initially the experimental system was optimized for the amount of SHBG present by varying the dilution ratio of the human pregnancy plasma. This optimisation was aimed at determining how much the plasma should be diluted to obtain the highest Specific binding.

The optimisation of the system was accomplished by diluting human pregnancy plasma 100, 50 or 25 times, as described in Appendix C.6, whilst maintaining the same ratio between DCC and plasma. It is known that the K\textsubscript{d} for SHBG is 1.5nM [158] and thus SHBG should be saturated in the presence of 15nM E\textsubscript{2}. Using this information it was decided that 30nM of \textsuperscript{3}H-E\textsubscript{2} would be used as this would ensure the saturation of SHBG binding sites. The results of this experiment, Figure 4.10, showed that the highest Specific binding roughly 7500 cpm was obtained when the pregnancy plasma was diluted 25 times.
4.1 Development of an experimental system for E₂ binding

Figure 4.10: Optimisation of the conditions for saturation binding using human pregnancy plasma. Human pregnancy plasma was diluted, as described in Appendix C.6, either 100; 50 or 25 times, whilst maintaining the same ratio between DCC and plasma. After the samples had been prepared 30nM of ^3^H-E₂ was added in the presence of 1×10⁻⁵M E₂ (Non Specific binding) or ethanol (Total binding) and the samples incubated for 10 hours. Ligand binding was determined as described in Appendix C.5.2. Specific binding = Total binding - Non Specific binding. The results are shown as the average mean with standard errors for triplicate samples.
4.1 Development of an experimental system for E₂ binding

The aim of the following experiment was to determine the Kₐ for SHBG binding E₂ in the absence of ER-α. Human pregnancy plasma was diluted 25 times and prepared as described in Appendix C.6. The samples were then exposed to 30nM ³H-E₂ in the presence of either 1×10⁻⁵M E₂ (Non Specific binding) or ethanol (Total binding). The results of this experiment, Figure 4.11, show that there was ligand depletion, Table 4.3.

Table 4.3: Ligand depletion encountered during saturation binding using human pregnancy plasma.

<table>
<thead>
<tr>
<th>Total binding (cpm)</th>
<th>Total Added (cpm)</th>
<th>Percentage ligand depletion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130628</td>
<td>371600</td>
<td>35</td>
</tr>
<tr>
<td>71220</td>
<td>194817</td>
<td>37</td>
</tr>
<tr>
<td>27947</td>
<td>75232</td>
<td>37</td>
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<td>10784</td>
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<td>4658</td>
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<td>32</td>
</tr>
<tr>
<td>1878</td>
<td>4338</td>
<td>43</td>
</tr>
</tbody>
</table>

Ligand depletion calculated as (total binding/total added)×100.

As has been described it is possible to determine the Kₐ for SHBG binding E₂ in the presence of ligand depletion by fitting a function which accounts for the presence of ligand depletion. It was, however, not possible to determine the Kₐ (E₂) for SHBG using this function. It should be noted that in other publications it has been possible to determine the Kₐ for SHBG binding E₂ using a solid phase assay [158]. Hammond [181] used a similar experi-
4.1 Development of an experimental system for E₂ binding

mental system, to the one employed in these experiments, to determine the K_d (dihydrotestosterone) for SHBG as was successful. The possible reasons why this experimental system did not work will be discussed later in Chapter 6.

Figure 4.11: Saturation binding assay using human pregnancy plasma.
Human pregnancy plasma was diluted 25 times and 30nM ^3H-E₂ in the presence of either 1×10^{-5}M (Non Specific binding) or ethanol (Total binding) was added. Ligand binding was determined as described in Appendix C.5.2. Due to the presence of ligand depletion Table 4.3 data was fitted with a function that accounts for ligand depletion and results graphed as Total binding (cpm) vs Total added (cpm). Results are shown as the mean of triplicate samples with standard errors.
4.2 Development of experimental system for promoter reporter gene assay studies

As stated in Chapter ?? one of the aims of this project was to determine the effect that SHBG has on the transactivation of an ERE via the ligand bound ER-α. To determine the effect of SHBG on the above mentioned interaction the following experimental approach was used:

- COS-1 cells were used to determine the transactivation of an ERE, via the ligand bound ER-α, using promoter reporter gene assays.
- Hep89 cells were used to determine the effect that SHBG had on the transactivation of an ERE, via ligand bound ER-α.

The aim of the following experiment was to determine the level of transactivation of an ERE in COS-1 cells. The experimental system was optimized for the use of various medium supplements; fetal calf serum, stripped fetal calf serum, no fetal calf serum. The rationale for the use of these supplements was to determine whether fetal calf serum contained any endogenous steroids that might interfere with the assay. The use of a DMSO during transfection was also studied as it has been suggested that a DMSO shock may increase transfection efficiency [182].

The results of this experiment, Figure 4.12, showed that DMSO did not have a significant effect as can be seen when comparing the samples exposed to a DMSO shock to those not exposed. With respect to the use of various medium supplements it was noted that there highest level of transactivation was obtained when COS-1 cells were not DMSO shocked and fetal calf serum was used as a medium supplement. The lowest levels of transactivation were obtained when no fetal calf serum was used. This would seem to indicate that endogenous steroids present in fetal calf serum may have an influence on the transactivation of an ERE. The promoter reporter gene assays performed in COS-1 cells were to be the control for the absence of SHBG. As
4.2 Development of experimental system for promoter reporter gene assay studies

there was ligand independent transactivation, compare samples exposed to E_2 to those exposed to ethanol, it was decided to not continue with the promoter reporter studies in the presence of SHBG. The presence of ligand independent transactivation in these experiments is in contradiction with the results of Pennie et al [183] who stated that there was no ligand or receptor independent transactivation of a vitellogenin ERE in COS-1 cells. One of the possible reasons may be the use of the specific ERE in these experiments, which was designed for use in HepG2 cells.

![Graph showing optimization of transactivation in COS-1 cells](image)

**Figure 4.12: Optimization of transactivation in COS-1 cells.** COS-1 cells were transfected with an ER-α, ERE-vitellogenin reporter gene and a β-galactosidase (ratios used were those of Stoica C.3 in Appendix C.3.1) reporter using the DEAE-Dextran transfection method as described in Appendix C.4. The use of various medium supplements (fetal calf serum, stripped fetal calf serum and no fetal calf serum) was studied as well as the role of a DMSO shock during transfection. Results are shown as the average of triplicate samples with standard errors.
4.3 Metabolic studies

The aim of the following experiments was to determine whether there was any metabolism of E₂ in COS-1 cells and Hep89 cells. The reason for this is Hep89 cells, which were derived from a liver carcinoma, may have enzymes present that could metabolize E₂.

4.3.1 Separation of steroids using thin layer chromatography

The experimental protocols that were used to determine whether E₂ was metabolized in COS-1 cells or Hep89 cells relied on exposing the cells to ³H-E₂ for a period of either 1 or 10 hours as described in Appendix C.7. The cells were then lysed and the steroids extracted using dichloromethane as a solvent. As a control for extraction efficiency the samples were “spiked” with a known amount of ³H-Dexamethasone. After the extraction the amount of ³H-Dexamethasone could be compared to the amount added and this would give an indication of the extraction efficiency. The results of these experiments are shown in Figure 4.13 and Figure 4.14. From these results it was concluded that there was no metabolism of E₂ after either 1 hours or 10 hours. This was determined by comparing the samples from COS-1 or Hep89 cells after either 1 or 10 hours.
4.3 Metabolic studies

Figure 4.13: Metabolic studies in COS-1 and Hep89 cells COS-1 and Hep89 cells were exposed to 1 µl of $^3$H-E$_2$ for a period of 1 hour. The cells were lysed after which 1ml of the samples was added to 5ml dichloromethane. To account for extraction efficiency the samples were “spiked” with 1 µl of $^3$H-Dexamethasone. The samples were prepared as described in Appendix C.7, after spotting on a thin layer chromatography plate the samples were run for one and a half hours in the presence of a 70% chloroform and 30% acetone solution. The amount of $^3$H-E$_2$ and $^3$H-Dexamethasone was determined as described in Appendix C.7 and the results normalized using the extraction efficiency of $^3$H-Dexamethasone. Extraction efficiency for $^3$H-Dexamethasone was calculated as the amount of $^3$H-Dexamethasone retrieved over amount added. Results are shown as the average with standard errors for duplicate samples.
4.3 Metabolic studies

Figure 4.14: Metabolic studies in COS-1 and Hep89 cells The experiment was performed as described in Figure 4.13. Results are shown as the average with standard errors for duplicate samples.
4.4 Determination of amount of SHBG in Hep89 cells

Hep89 cells were seeded and grown as described in Appendix C.8. The results of this experiment were inconclusive because no detectable SHBG was found in either the extracellular or intracellular samples. Although SHBG has been found in HepG2 cells (± 21.4 nmol/L) [137], as of yet there has been no determination of SHBG levels in Hep89 cells. One of the reasons for the failure to detect SHBG in either the extracellular or intracellular samples may have been that the protein in the samples may not have been in solution, however, further experimentation will be needed to determine this.

4.5 Determination of cell volumes

The volume of the extracellular medium and intracellular medium was determined for COS-1 cells as described in Appendix C.9. From the results of this experiment it was determined that the extracellular volume was 0.001L and the intracellular volume was $5.96 \times 10^{-6}$L.

The various models that currently exist to describe the interactions of estrogenic compounds with the estrogen receptor will be described in Chapter 5.
Chapter 5

Mathematical and descriptive models

5.1 Operational model of agonism

Bio-assays are used in quantitative pharmacology to estimate the pharmacological properties of compounds. These bio-assays measure and quantify the physiological response to a pharmacologically active compound. To quantify the pharmacological properties of a pharmaco-active substance the bio-assays have to be designed in such a way that they are comparative, or “null methods”, so as to eliminate secondary physiological information.

A brief history of the events that led to the development of the operational model of agonism follows. One of the simplest bio-assays developed, to measure physiological response, was a dilution assay in which a compound, known to have a physiological response, was titrated against a known standard of the same compound. This method was useful to determine the concentrations, of a pharmaco-active compound, that was needed to initiate a physiological response. When analyzing simple competitive antagonism, which is also a form of a dilution assay, a number of assumptions about the nature of the action of agonist and antagonist are needed. It is assumed, in these bio-assays,
5.1 Operational model of agonism

that both agonist and antagonist interact with common tissue components and receptors. These interactions are said to be governed by the law of mass action and the antagonist receptor interaction can be characterized by a single binding parameter.

The physiological effect can be equated with fractional occupancy and a single binding parameter, which itself is characterized by the receptor agonist complex. This information has led to the concept of “full” and “partial” agonism [184]. The concept of “full” and “partial” agonists was first noted when it was seen that different agonists would elicit different maximal responses when associating with the same receptor whilst still in the same tissue. In response to this Arins introduced a proportionality term between effect and fractional occupancy. This proportionality term was assumed to be agonist determined, however Stephenson [185] altered the model when it was noted that the pharmacological effect was not necessarily proportional to fractional occupancy.

In his model Stephenson proposed that agonists produced equal effects by evoking equal stimuli in a tissue. The concept of stimulus, the product of traditional occupancy and a parameter efficacy was introduced. Efficacy was defined as the power of the agonist to elicit a response. Stephenson also related effect to stimulus through a monotonic function that was dependent on the tissue and the agonist. Using this concept it was thus possible to describe why the same agonist had a different response in various tissues and that a maximal response could be elucidated at sub-maximal receptor occupancy. The model that Stephenson proposed was revised and modified by Furchgott [186].

In the model Furchgott introduced the concepts of initial concentration of active receptors and intrinsic efficacy. Intrinsic efficacy is defined as the purely agonist dependent part of efficacy and thus the traditional theory of efficacy
5.1 Operational model of agonism

was divided up into two parts:

- The agonist dependent part which is defined by the affinity $K_A$ and intrinsic efficacy ($\varepsilon$).

- The tissue dependent part which is defined by receptor concentration ($R_o$) and the stimulus effect (E/S) relation.

There are however a number of problems with the current traditional model. In the assays it is assumed that tissue dependent information is canceled out and thus the $K_A$ and $\varepsilon$ would become estimable. Mackay [187] has shown that it is theoretically impossible to estimate absolute values of $\varepsilon$ and that only relative values, for different agonists, are measurable. With respect to the determination of $K_A$ for partial agonists, using full agonists in the same system, it is only possible to do so if an extreme ratio of intrinsic efficacies is assumed.

With these problems in mind Black and Leff [188] developed a general operational model of agonism which expressed pharmacological effect as an explicit function of agonist concentration.

5.1.1 General operational model of agonism

The effect of hormones, and other agonists, can only be recognized in physiological, intact tissue, systems. A hormone-tissue pair can be characterized by determining response as a function of agonist concentration and is quantified using the $E/[L]$ function, where $E$ is the effect and $[L]$ is the concentration of ligand. Most $E/[L]$ curves are monotonically increasing functions of $[L]$ that involve a minimum of three parameters:

- Maximal response generated by saturating $[L]$

- $IC_{50}$ is the value of ligand at half maximal response

- The slope of the $E/[L]$ curve at half maximal response
5.1 Operational model of agonism

Black and Leff developed a model for operationally hyperbolic E/[L] curves.

In the traditional theory it is assumed that an agonist, L, binds to receptors, R, and that this reaction obeys the laws of mass action. At equilibrium the concentration of occupied receptors depends on the [L] and $K_d$.

Using Furchgott’s [186] convention it is possible to determine that the concentration of liganded receptors $[LR]$, depends on the total concentration of receptors $[R_{tot}]$, the equilibrium concentration of ligand, [L], and the $K_d$.

$$[LR] = \frac{[R_{tot}][L]}{K_d + [L]} \quad (5.1)$$

The chemical reaction can be represented as follows:

$$R + L \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} RL \quad (5.2)$$

The law of mass action as formulated by Guldberg and Waage states that the rate of a chemical reaction is proportional to the concentration of the individual species involved in the chemical reaction [189]. Using the chemical reaction, equation 5.2, it is possible to determine the $K_d$ via derivation of the rate at which the ligand receptor complex is formed, $k_{on}$, and disassociates, $k_{off}$, if one assumes equilibrium.

$$V_{on} = k_{on}[R][L] \quad (5.3)$$

$$V_{off} = k_{off}[LR]$$

At equilibrium $V_{on} = V_{off}$

$$\frac{k_{off}}{k_{on}} = \frac{[R][L]}{[LR]} = K_d \quad (5.4)$$

The total amount of receptor can be determined in terms of bound and unbound receptor.

$$[R_{tot}] = [LR] + [R] \quad (5.5)$$
5.1 Operational model of agonism

By combining the equations 5.5 and 5.4 it is possible to describe the amount of bound receptor as follows, which is the same as equation 5.1:

\[ [LR] = \frac{[R_{tot}][L] - [LR][L]}{K_d} \]

\[ [LR].K_d = [R_{tot}][L] - [LR][L] \]

\[ [LR].K_d + [LR].[L] = [R_{tot}][L] \]

\[ [LR] = \frac{[R_{tot}][L]}{K_d + [L]} \] (5.6)

When \([L] \gg [R_{tot}]\) it is assumed that the amount of free ligand at equilibrium is the same as the amount of ligand added initially. By using this assumption it is possible to derive a function that relates the concentration of ligand bound receptor to a physiological response (E).

\[ E = z([LR]) \] (5.7)

By combining equations 5.9 and 5.6 it is possible to mathematically describe the E/[L] relationship.

\[ E = z \left( \frac{[R_{tot}][L]}{K_d + [L]} \right) \] (5.8)

A function for \(z\) must still be found which can be used to describe experimental hyperbolic curves. The function \(z\) can be regarded as a transducer function that conveys occupancy ([LR]) into an effect, E. Black and Leff were able to show that there were only two choices available to describe the \(E/[LR]\) relation: either linear or hyperbolic.

If the transducer function, \(z\), were a straight line then \(E\) would be proportional to [LR]. If this was so then IC\(_{50}\) would equal \(K_d\) for every agonist and no receptor reserve would exist. This option has been ruled by experimental results in which different agonists exert maximal pharmacological effect under different occupancy values. The only other choice is to describe the transducer function as a rectangular hyperbolic function of [LR].

\[ \frac{E}{E_{maximal}} = \frac{[LR]}{K_E + [LR]} \] (5.9)
Where:

- $E_{\text{maximal}}$ is the maximal response
- $K_E$ is the value of [LR] that elicits half maximal response

By combining equations 5.6 and 5.9 it is possible to express equation 5.8 as follows:

$$\frac{E}{E_{\text{maximal}}} = \frac{[R_{\text{tot}}][L]}{K_dK_E + ([R_{\text{tot}}] + K_E)([L])} \quad (5.10)$$

Equation 5.10 can be simplified by defining the transducer ratio $\tau$, $\tau = \frac{[R_{\text{tot}}]}{K_E}$:

$$E = \frac{(E_{\text{maximal}})(\tau)([L])}{K_d + ([L])(\tau + 1)} = \frac{([L])(E_{\text{maximal}})(\tau)}{K_d + ([L])(\tau + 1)} \quad (5.11)$$

By using equation 5.11 it is possible to determine two factors about the interaction of agonist and receptor namely:

- **Potency** - Defined as the ability of the ligand to bind the receptor
- **Efficacy** - The effect that the ligand bound receptor has on the transactivation of sensitive genes

The operational model of agonism describes the ability of the ligand to bind the receptor as well as the ability of the ligand bound receptor to initiate the transcription of sensitive genes. By incorporating the function $\tau$ it is possible to mathematically account for the observation that ligands have different effects in various tissues without accounting for the mechanism of action. Experimentally it is possible to determine the affinity of the ligand by determining the $K_d$, whilst it is possible to determine the efficacy of the ligand bound receptor complex using promoter reporter gene studies.

### 5.2 Tripartite and Bipartite models

It is now known that hormones can have different effects depending on the tissue where they are found and which receptors they interact with. These
5.2 Tripartite and Bipartite models

effects cannot be explained only using the ligand and receptor concentration and thus new models had to be developed which could explain these observations. A new model proposed by Katzenellenbogen [107] states that the selectivity of steroids for nuclear hormones may be mediated by three distinct mechanisms:

- Ligand based selectivity
- Receptor based selectivity
- Effector site based selectivity

Ligand based selectivity The effects that are seen in various tissues can be described by differences in pharmaco-kinetics, or differential ligand metabolism. It can thus be said that the same hormone may be present in various tissues, however, the relative amounts of the hormone in the tissue differ because of altered uptake or metabolism.

Receptor based selectivity The receptor based mechanism of selectivity states that tissues respond differently to the same hormones because the tissues contain different levels of receptors. These differences in receptor levels may be due to concentration or ratios of receptors, subtypes, isoforms, splice variants or that the receptor have different states of covalent modification (eg. phosphorylation).

Effector site based selectivity It has been noted that under certain conditions, even in the absence of ligand and receptor based selectivity, hormones can have different effects in tissues. These differences can be explained by the presence of co-factors and the role that these co-factors play in the transactivation of genes.

There are currently two types of models which describe the interaction of ligands with their respective receptors and the transactivation of genes via
5.2 Tripartite and Bipartite models

the ligand bound receptor complex. These models are the bipartite and tripartite models.

5.2.1 Pharmacology of bipartite and tripartite systems

Bipartite systems

The original model that was proposed to describe the interactions of ligands with their receptors, and any further physiological responses, accounted for the presence of ligand and receptor. This was an operational model and has been refined by Black and Leff [188]. The operational model related the affinity of the ligand to a receptor using the term potency, whilst the ability of the ligand receptor complex to initiate transcription was described by the term efficacy. In this model it was the ligand which controlled the ability of the ligand receptor complex to initiate a response. As such the implications of the bipartite model is that the ligand controlled the shape and function of the receptor directly and thus it was possible to assign a unique potency and efficacy to each ligand.

Tripartite systems

The tripartite model is composed of three components namely the ligand, receptor and effector (co-regulators). It has been proposed that the tripartite model describes steroid hormone pharmacology more accurately in that the ligand binding and the response initiation functions are assigned to separate functions: ligand binding to the receptor and response initiation to the effector.

The tripartite model differs to the bipartite model in a number of ways which include the definition of potency and efficacy. The tripartite model states that the ligand binding to the receptor forms a complex, and that the affinity of this binding is the determining factor in ligand potency. The ligand receptor interaction does not, however, control the response and is
5.3 The physiologically based pharmacological model

Therefore not a direct determinant of ligand efficacy. The interactions of the ligand receptor complex with the effector is the determining factor of the efficacy of the ligand receptor complex. These effectors are commonly part of the co-factor family and can thus be either co-activators or co-repressors.

Using the tripartite model it is possible to explain why the same ligand may have different effects in various tissues, when interacting with the same receptor. The reason for these differing effects may be that each tissue type has its own milieu of effectors; such as co-factors, DNA sequences flanking the response element, consensus and non-consensus response element; which the tripartite model accounts for.

5.3 The physiologically based pharmacological model

The physiologically based pharmacokinetic model was developed by Plowchalk [190]. The aim of this model was to provide a quantitative tool with which to evaluate the importance of physiological parameters on \( E_2 \) blood and tissue concentrations in rats and humans.

Estradiol is found in plasma in two forms, those being bound and unbound. In humans both SHBG and albumin can bind estradiol and in so doing restrict the free fraction of estradiol in plasma. It is presumed that the free form of estradiol is pharmacologically active, and is capable of passing through the lipid bilayer and equilibrating with tissues. However, it has also been found that under specific conditions, protein bound \( E_2 \), is also available to tissue uptake [1, 191].

In recent years interest has been expressed in using estradiol as a prototypical endocrine-active compound. Endocrine active compounds are a group of chemicals that have been found to have an effect on the endocrine system,
5.3 The physiologically based pharmacological model

some of which are mediated via the estrogen receptor. The effects of these endocrine active compounds are normally compared to those of estradiol in the same system. The external dose of these compounds cannot be used as the pharmacologically active concentration. The tissue concentration and the potency of these compounds are determined by a number of factors which include binding affinity to ER-α and plasma binding proteins, ligand-receptor competition, metabolic and total clearance rates and finally tissue and blood partition coefficients. The physiologically based model attempts to take all of these factors into account to determine which specific factors play a role in the function of estrogenic compounds in the human physiological system.

In setting up the model number of assumptions were made: 1) ER-α concentration in tissues were time and treatment independent, 2) Estrogen receptors existed as a single subtype, 3) Type II estrogen estrogen binding sites [192] were not taken into account, 4) The cytoplasm and nuclear ER-α were both equally available in tissue. With respect to the disassociation constants a $K_d (E_2)$ of 0.25nM was used for ER-α, consistent with literature values of between 0.1 and 0.5nM [193, 194]. The $K_d (E_2)$ for SHBG was defined as 1.5nM [158] and the $B_{max}$ was 19.36-38nM (males), 37-70nM (females), 400nm (pregnant females) [158]. The binding constants for albumin were $K_d (E_2) = 1.7 \times 10^4$nM and $B_{max} = 5 \times 10^5$nM [158]. The metabolic clearance of $E_2$ was attributed solely to metabolism, which was assumed to take place in the liver and central plasma compartment. It was also assumed that there was no endogenous production of $E_2$ either through de novo synthesis or regeneration from conjugates.

The results of the modelling for $E_2$ binding and fractional distribution between SHBG and albumin predicted that albumin would bind most of the $E_2$ in males and females, Table 5.1.

These results are in concurrence with the literature where it was shown that
only a small percentage of E₂ would be free in plasma [158]. The results of the hepatic extraction for E₂ were not similar to those found in the literature. It was concluded that the values were greater than would be expected for the uptake of unbound E₂ alone. Padridge [195] has demonstrated that the *in vitro* binding constants for E₂ are not predictive of *in vivo* binding constants in the liver, brain and uterus. As a result of these findings it has been proposed that an apparent Kₐ₄ for a single organ would better reflect the bio-available E₂ fraction in plasma. With respect to the metabolic clearance of E₂ it was found that the hepatic blood flow was one of the most important factors in experimental variability. The E₂ concentration in plasma under equilibrium conditions showed that only the infusion and clearance rates had an influence, while other parameters such as partition coefficients, receptor binding, blood flow, diffusional clearance rates and organ volumes only had an impact on the time it took to reach an equilibrium state.

The following chapter will deal with the modelling of the Hep89 and human pregnancy plasma experimental systems. As it is not possible to determine the equilibrium concentration of all the binding proteins and their respective bound forms respectively, a numerical approach is used to calculate the equilibrium concentrations.
5.3 The physiologically based pharmacological model

Table 5.1: Simulation results of predicted estradiol distribution in plasma containing various protein compositions [190].

<table>
<thead>
<tr>
<th>Protein free plasma</th>
<th>Free (%)</th>
<th>Total bound (%)</th>
<th>Albumin bound (%)</th>
<th>SHBG bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma with albumin and SHBG (male)</td>
<td>2.5</td>
<td>97.5</td>
<td>73.8</td>
<td>23.7</td>
</tr>
<tr>
<td>Plasma with albumin and SHBG (female)</td>
<td>1.9</td>
<td>98</td>
<td>58.3</td>
<td>39.7</td>
</tr>
<tr>
<td>Plasma with albumin and SHBG (pregnant)</td>
<td>0.4</td>
<td>99.6</td>
<td>10.6</td>
<td>89</td>
</tr>
</tbody>
</table>
Chapter 6

The influence of SHBG on $E_2$ binding by ER-$\alpha$: An *in silico* approach

As was stated in the Chapter ?? the aim of this project was to build a mathematical model that would describe the effect that SHBG has on the binding of $E_2$ to ER-$\alpha$ and thus influence SHBG has on this interaction. As the results have shown there was difficulty when determining the binding constants $B_{\text{max app}}$ and $K_{\text{d app}} (E_2)$ for ER-$\alpha$ in the presence of SHBG, however, it was possible to determine the $B_{\text{max}}$ and $K_{\text{d}} (E_2)$ for ER-$\alpha$ in the absence of SHBG. With respect to determining the $K_{\text{d}} (E_2)$ for SHBG it was shown that this could not be achieved. As a result of these setbacks the proposed mathematical model, was not built, and instead models that describe the experimental systems, those being the Hep89 and human pregnancy plasma, were built. These model are being built in an attempt to determine why the binding constants for ER-$\alpha$ and SHBG were not determined in these systems.
6.1 Model of the Hep89 experimental system

The aim of modelling the Hep89 experimental system was to provide an explanation as to why it was not possible to determine the binding constants for ER-α, $K_{d\,\text{app}}(E_2)$ and $B_{\text{max\,app}}$. An example of the experimental system is shown in Figure 6.1. It should be noted that neither SHBG nor albumin were included in the extracellular compartment. This is in keeping with the experimental system where the medium was removed before the binding studies were performed. The presence of albumin has been included because the Hep89 cells were derived from a liver heptatoma and thus should contain albumin.

Figure 6.1: Diagram of the Hep89 experimental system. This system describes the binding of $E_2$ to ER-α in the presence of intracellular albumin and SHBG.

The variables that are needed to build this model are shown in Table 6.1. As
there is no information on the amount of ER-α expressed in Hep89 cells it was decided to use the B_{max} values that were determined in the COS-1 cells system. These values were used because both these cell lines were transfected with the same plasmid containing the ER-α gene.

The amount of cytostolic SHBG was calculated by using the ratio of intracellular SHBG to extracellular SHBG (shown as pg/µg DNA [142]) and then using this ratio to calculate the cytostolic SHBG from the extracellular SHBG concentration (20nM) as reported by Brown-Martin et al [137].

The concentration of albumin in Hep89 cells was estimated on the basis of the amount that is normally present in plasma and applying the same ratio that was used to determine the cytostolic concentration of SHBG. This was done as there is no literature reporting on the concentration of albumin in Hep89 cells.

The mathematical model was consisted of two compartments, the cytoplasm and nucleus were treated as one compartment (cytostolic) while the extracellular medium was treated as a separate compartment (medium). The transfer of E_2 across the membrane was governed by mass action kinetics as was the binding of E_2 to the binding proteins (ER-α, SHBG and albumin). The role that the K_d (E_2) for ER-α had on the binding kinetics was studied by varying the K_d (E_2) between 0.25nM and 4.4nM, Table 6.1.

The following chemical equilibria were used to describe the binding of E_2 to binding proteins and the transfer of E_2 across the membrane.

\[
\begin{align*}
E_2_{\text{medium}} & \rightleftharpoons E_2_{\text{cytosol}} \\
\text{ER-α}_{\text{cytosol}} + E_2_{\text{cytosol}} & \rightleftharpoons \text{ER-α}_{\text{cytosol}.E_2_{\text{cytosol}}} \\
\text{SHBG}_{\text{cytosol}} + E_2_{\text{cytosol}} & \rightleftharpoons \text{SHBG}_{\text{cytosol}.E_2_{\text{cytosol}}} \\
\text{albumin}_{\text{cytosol}} + E_2_{\text{cytosol}} & \rightleftharpoons \text{albumin}_{\text{cytosol}.E_2_{\text{cytosol}}}
\end{align*}
\]
6.1 Model of the Hep89 experimental system

<table>
<thead>
<tr>
<th>B&lt;sub&gt;max&lt;/sub&gt; nM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-α 1.2</td>
<td>experimental (section 4.1.1)</td>
</tr>
<tr>
<td>SHBG 0.63 - 0.98</td>
<td>calculated from [142] and [137]</td>
</tr>
<tr>
<td>albumin 1.9×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>estimated using ratio determined for SHBG concentration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K&lt;sub&gt;d (E&lt;sub&gt;2&lt;/sub&gt;)&lt;/sub&gt; nM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-α 0.25</td>
<td>[190]</td>
</tr>
<tr>
<td>SHBG 1.5</td>
<td>[158]</td>
</tr>
<tr>
<td>albumin 1.9×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt; transfer 1</td>
<td>in silico</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volumes (L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 0.001</td>
<td>experimental (section 4.5)</td>
</tr>
<tr>
<td>Cytosol 5.96×10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>experimental (section 4.5)</td>
</tr>
</tbody>
</table>
6.1 Model of the Hep89 experimental system

As it is not possible to analytically solve all of the above equations simultaneously so as to calculate the equilibrium concentration of all the species involved it was decided to use numerical modelling to solve these equilibria. The numerical modelling of this system was accomplished using Gepasi [196]. The initial concentration of $E_2$ medium was varied between $1 \times 10^{-13}$ nM and 30nM and the equilibrium concentrations of the various species 6.1 was calculated.

Initially only ER-$\alpha$ was included so as to determine the $K_d (E_2)$ in the absence of SHBG and albumin. The results obtained, that is the concentration of $E_2$ bound ER-$\alpha$, was fitted to a one site binding equation 6.2. The one site binding equation was used to calculate the $K_d$ and $B_{max}$ for ER-$\alpha$ binding $E_2$ in the presence of SHBG and albumin.

$$[\text{ER-}\alpha \cdot E_2] = \frac{[\text{ER-}\alpha_{\text{tot}}] \cdot [E_2_{\text{cytosol}}]_{\text{eq}}}{K_d + [E_2_{\text{cytosol}}]_{\text{eq}}} \quad (6.2)$$

The following discussion will concentrate on the binding of $E_2$ to ER-$\alpha$ and the results obtained when a one site binding, equation 6.2, was fitted to the equilibrium concentrations of ER-$\alpha_{\text{cytosol}}$ $E_2_{\text{cytosol}}$ in the presence or absence of SHBG and albumin.

With respect to the binding of $E_2$ to ER-$\alpha$ in the absence of SHBG and albumin it was noted that the dilution of $E_2$, by the cytoplasmic compartment, did not influence the $K_d (E_2)$. This was noticed when comparing the $K_d$ as published by Plowchalk [190] which was determined as 0.25nM, to that obtained via modelling 0.25nM, Figure 6.2. In general it was noted that when the $K_d (E_2)$ for ER-$\alpha$ was increased the equilibrium concentration of $E_2$ bound ER-$\alpha$ decreased. The decrease in equilibrium concentration of $E_2$ bound ER-$\alpha$ can be seen when the $K_d (E_2)$ was increased from 0.25nM to 3.4nM and 4.4nM , Table 6.2 and Figure 6.2. This is to be expected as when
6.1 Model of the Hep89 experimental system

the $K_d(E_2)$ is lowered ER-$\alpha$ has a lower affinity for $E_2$ and thus equilibrium will only be reached at higher concentrations of $E_2$.

![Graph showing the influence of $K_d(E_2)$ on the binding of $E_2$ to ER-$\alpha$ in the absence of SHBG and albumin.](image)

**Figure 6.2:** The influence that the $K_d\text{ app}(E_2)$ has on the binding of $E_2$ to ER-$\alpha$ in the absence of SHBG and albumin. The equilibrium concentration of $E_2$ bound ER-$\alpha$ was determined for a system where $E_2$ can cross a membrane and bind to ER-$\alpha$. The $K_d(E_2)$ for ER-$\alpha$ was varied between 0.25, 3.4 and 4.4nM.
6.1 Model of the Hep89 experimental system

When determining the influence that SHBG has on the binding of E₂ it was noted that there was an apparent increase, though very little, in the $K_d (E_2)$ ($K_{d\,app} (E_2)$), Table 6.2. In the presence of 0.63nM SHBG the $K_d (E_2)$, defined as 0.2500nM was increased from 0.2500nM to 0.2551nM, while in the presence of 0.98nM of SHBG increased the $K_{d\,app} (E_2)$ to 0.2553, Table 6.2. The same trend was noticed when the $K_d (E_2)$ was defined as 3.4nm or 4.4nM Table 6.2. The apparent maximal binding stayed the same in the presence of 0.63nM SHBG and 0.98nM of SHBG 6.2. From these results, Figure 6.3 it was shown that the right shift in the $K_{d\,app}$ because of the presence of SHBG affecting the equilibrium concentrations of free E₂ Table 6.3. In the absence of SHBG at least 94% of E₂ was unbound while in the presence of 0.98nM SHBG the amount of unbound E₂ was decreased to 93.35%. The results pertaining to SHBG would thus seem to indicate that SHBG binds E₂, which effects the levels of unbound E₂. This decrease in the levels of unbound E₂ in turn affects the $K_{d\,app} (E_2)$ for E₂ binding ER-α.
6.1 Model of the Hep89 experimental system

Figure 6.3: The influence that SHBG has on the binding of E$_2$ to ER-$\alpha$. SHBG concentrations were varied between 0.63nM and 0.98nM. The use of various $K_d$ (E$_2$) values (0.25nM) was also studied. The equilibrium concentration of ER-$\alpha$ bound E$_2$ was numerically determined using Gepasi when the concentration of E$_2$ was varied between $1 \times 10^{-13}$ nM and 30 nM. (a) $K_d$ (E$_2$) for ER-$\alpha = 0.25$nM, (b) $K_d$ (E$_2$) for ER-$\alpha = 0.25$nM, [SHBG] = 0.63$nM, (c) $K_d$ (E$_2$) for ER-$\alpha = 0.25$nM, [SHBG] = 0.98$nM.
6.1 Model of the Hep89 experimental system

The influence that albumin has on the binding of E\textsubscript{2} to ER-\(\alpha\) can be seen when determining the \(K_{d \text{ app}}(E_2)\) and apparent maximal binding, Table 6.2. In the presence of both albumin and SHBG it can be seen that the \(K_{d \text{ app}}(E_2)\) (0.2569nM), is increased from 0.2553nM in the presence of 0.98nM SHBG, and 0.2547nM in the presence of ER-\(\alpha\) alone, if the \(K_d(E_2)\) was initially defined as 0.25nM. Once again it was noted that the maximal binding calculated at equilibrium was not affected, Table 6.2. The apparent shift in \(K_d\) is once again related to the lower concentrations of E\textsubscript{2} that are available at equilibrium, Figure 6.4 and Table 6.4. The influence of SHBG and albumin together to limit the binding of E\textsubscript{2} to ER-\(\alpha\), as well as the influence that the \(K_d(E_2)\) for ER-\(\alpha\) can be seen in Figure 6.5.
6.1 Model of the Hep89 experimental system

Figure 6.4: The influence that SHBG and albumin have on the binding of E$_2$ to ER-$\alpha$. SHBG concentrations were varied between 0.63nM and 0.98nM while the concentration of albumin was $1.9 \times 10^4$ nM. The use of various K$_d$ (E$_2$) values (0.25nM) was also studied. The equilibrium concentration of ER-$\alpha$ bound E$_2$ was numerically determined using Gepasi when the concentration of E$_2$ was varied between $1 \times 10^{-13}$ nM and 30 nM. (a) K$_d$ (E$_2$) for ER-$\alpha = 0.25$nM, (b) K$_d$ (E$_2$) for ER-$\alpha = 0.25$nM, [SHBG] = 0.63nM, (c) K$_d$ (E$_2$) for ER-$\alpha = 0.25$nM, [SHBG] = 0.98nM.
# 6.1 Model of the Hep89 experimental system

Table 6.2: Results of numerical modelling for Hep89 cells.

<table>
<thead>
<tr>
<th>Binding proteins</th>
<th>Concentration (nM)</th>
<th>$K_d$ app</th>
<th>$B_{max}$ app (ER-$\alpha$ only nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d(E_2) = 0.25\text{nM}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>0.25</td>
<td>1.20</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>0.25</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>0.25</td>
<td>1.20</td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $\times 10^4$</td>
<td>0.25</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.98 $\times 10^4$</td>
<td>0.25</td>
<td>1.20</td>
</tr>
<tr>
<td>$K_d(E_2) = 3.4\text{nM}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>3.40</td>
<td>1.20</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>3.43</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>3.42</td>
<td>1.20</td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $\times 10^4$</td>
<td>3.44</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.98 $\times 10^4$</td>
<td>3.45</td>
<td>1.20</td>
</tr>
<tr>
<td>$K_d(E_2) = 4.4\text{nM}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>4.42</td>
<td>1.20</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>4.43</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>4.43</td>
<td>1.20</td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $\times 10^4$</td>
<td>4.45</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.98 $\times 10^4$</td>
<td>4.46</td>
<td>1.20</td>
</tr>
</tbody>
</table>

The concentration of $E_2$ was 30nM in the presence or absence of SHBG and albumin. The equilibrium concentration of ER-$\alpha$ bound $E_2$ (ER-$\alpha$.E$_2$) was calculated and fitted to a one site binding equation.
6.1 Model of the Hep89 experimental system

Table 6.3: Results for the distribution of $E_2$ between the binding proteins in the Hep89 experimental system, expressed as a percentage.

<table>
<thead>
<tr>
<th>Binding proteins</th>
<th>Concentration (nM)</th>
<th>% $E_2$ unbound</th>
<th>% $E_2$ bound to ER-$\alpha$</th>
<th>% $E_2$ bound to SHBG</th>
<th>% $E_2$ bound to albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_d$ ($E_2$) = 0.25nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>96.16</td>
<td>3.83</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>94.22</td>
<td>3.76</td>
<td>2.02</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>93.35</td>
<td>3.73</td>
<td>2.92</td>
<td>0.00</td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $1.9 \times 10^4$</td>
<td>45.93</td>
<td>1.85</td>
<td>0.99</td>
<td>51.24</td>
</tr>
<tr>
<td></td>
<td>0.98 $1.9 \times 10^4$</td>
<td>45.72</td>
<td>1.84</td>
<td>1.44</td>
<td>51.01</td>
</tr>
<tr>
<td>K$_d$ ($E_2$) = 3.4nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>96.51</td>
<td>3.49</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
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<td>94.59</td>
<td>3.42</td>
<td>2.02</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>93.68</td>
<td>3.38</td>
<td>2.93</td>
<td>0.00</td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $1.9 \times 10^4$</td>
<td>46.02</td>
<td>1.67</td>
<td>0.99</td>
<td>51.33</td>
</tr>
<tr>
<td></td>
<td>0.98 $1.9 \times 10^4$</td>
<td>45.81</td>
<td>1.66</td>
<td>1.44</td>
<td>51.10</td>
</tr>
<tr>
<td>K$_d$ ($E_2$) = 4.4nM</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ER-$\alpha$</td>
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<td>96.63</td>
<td>3.39</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>94.68</td>
<td>3.32</td>
<td>2.03</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>93.81</td>
<td>3.29</td>
<td>2.94</td>
<td>0.00</td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $1.9 \times 10^4$</td>
<td>46.04</td>
<td>1.62</td>
<td>0.99</td>
<td>51.35</td>
</tr>
<tr>
<td></td>
<td>0.98 $1.9 \times 10^4$</td>
<td>45.83</td>
<td>1.62</td>
<td>1.44</td>
<td>51.12</td>
</tr>
</tbody>
</table>

The concentration of $E_2$ was 30nM in the presence or absence of SHBG and albumin. The percentages of $E_2$ bound to each protein was calculated as the concentration of $E_2$ bound protein over total internal $E_2$ concentration and then expressed as a percentage.
6.1 Model of the Hep89 experimental system

Table 6.4: Results for the distribution of $E_2$ between the binding proteins in the Hep89 experimental system.

<table>
<thead>
<tr>
<th>Binding proteins</th>
<th>Concentration (nM)</th>
<th>$[E_2]$ nM Total</th>
<th>$[E_2]$ nM unbound</th>
<th>$[E_2]$ nM bound to ER-$\alpha$</th>
<th>$[E_2]$ nM bound to SHBG</th>
<th>$[E_2]$ nM bound to albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d (E_2) = 0.25\text{nM}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>31.01</td>
<td>29.82</td>
<td>1.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>31.64</td>
<td>29.81</td>
<td>1.19</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>31.93</td>
<td>29.81</td>
<td>1.19</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $1.9 \times 10^4$</td>
<td>64.49</td>
<td>29.62</td>
<td>1.19</td>
<td>0.64</td>
<td>33.04</td>
</tr>
<tr>
<td></td>
<td>0.98 $1.9 \times 10^4$</td>
<td>64.78</td>
<td>29.61</td>
<td>1.19</td>
<td>0.93</td>
<td>33.04</td>
</tr>
<tr>
<td>$K_d (E_2) = 3.4\text{nM}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>30.89</td>
<td>29.82</td>
<td>1.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>31.53</td>
<td>29.81</td>
<td>1.08</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>31.82</td>
<td>29.81</td>
<td>1.08</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $1.9 \times 10^4$</td>
<td>64.37</td>
<td>29.62</td>
<td>1.08</td>
<td>0.64</td>
<td>33.04</td>
</tr>
<tr>
<td></td>
<td>0.98 $1.9 \times 10^4$</td>
<td>64.67</td>
<td>29.62</td>
<td>1.08</td>
<td>0.93</td>
<td>33.04</td>
</tr>
<tr>
<td>$K_d (E_2) = 4.4\text{nM}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-$\alpha$</td>
<td>1.20</td>
<td>30.86</td>
<td>29.82</td>
<td>1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>31.50</td>
<td>29.81</td>
<td>1.05</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>31.79</td>
<td>29.81</td>
<td>1.05</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>SHBG and albumin</td>
<td>0.67 $1.9 \times 10^4$</td>
<td>64.34</td>
<td>29.62</td>
<td>1.05</td>
<td>0.64</td>
<td>33.04</td>
</tr>
<tr>
<td></td>
<td>0.98 $1.9 \times 10^4$</td>
<td>64.63</td>
<td>29.62</td>
<td>1.05</td>
<td>0.93</td>
<td>33.04</td>
</tr>
</tbody>
</table>

The concentration of $E_2$ was $30\text{nM}$ in the presence or absence of SHBG and albumin. The amount of $E_2$ bound to each protein was calculated mathematically with the aid of Gepasi. The data represented in this table shows the absolute concentration (nM) of $E_2$ bound to each protein.
6.1 Model of the Hep89 experimental system

![Graph showing the influence of SHBG and albumin on the binding of E<sub>2</sub> to ER-α.](image)

Figure 6.5: The influence that SHBG and albumin have on the binding of E<sub>2</sub> to ER-α. SHBG concentrations were varied between 0.63nM and 0.98nM while the concentration of albumin was 1.9×10⁴ nM. The use of various K<sub>d</sub> (E<sub>2</sub>) values (0.25nM and 4.4nM) was also studied. The equilibrium concentration of ER-alpha bound E<sub>2</sub> was numerically determined using Gepasi when the concentration of E<sub>2</sub> was varied between 1×10⁻¹³ nM and 30 nM. (a) and (f) are ER-α only, K<sub>d</sub> (E<sub>2</sub>) = 0.25 and 4.4nM respectively. (a) through (e) K<sub>d</sub> = 0.25nM, (f) through (j) K<sub>d</sub> (E<sub>2</sub>) = 4.4nM. (b,c,g,h) [SHBG] = 0.63nM. (e,f,i,j) [SHBG] = 0.98nM. (c,e,h,j) [albumin] = 1.9×10⁴ nM.
6.1 Model of the Hep89 experimental system

The following discussion will center around the results that could be expected in an experimental situation. For the purposes of this discussion the maximal binding in the presence of SHBG is calculated as the sum of the ER-α and SHBG bound E₂. In the presence of SHBG and albumin the same principle is applied. Maximal binding is calculated using this principle because in an experimental situation it would not be possible to distinguish the ER-α bound E₂ from either SHBG or albumin bound E₂, in this specific experimental system.

The results of the modelling of saturation binding experiments using Hep89 cells showed that it was not possible to reach saturating conditions if SHBG and albumin were both present and 30nM of E₂ was used. In the presence of only ER-α and SHBG it is possible to reach saturating conditions, Figure 6.6. The only difference between the absence of SHBG and presence of SHBG the maximal binding in higher is in the presence of SHBG. There is also a slight right shift of the K_{d,app} (E₂) as has already been shown. When both albumin and SHBG are present it can be seen that saturating conditions are not reached, Figure 6.7. Using this information it should be possible to see that the presence of albumin is interfering with the binding of E₂ to ER-α and that saturating conditions are not met.
6.1 Model of the Hep89 experimental system

Figure 6.6: The influence of SHBG on the binding of $E_2$ to ER-α. The concentration of ER-α used was 1.2nM, SHBG concentration was set as 0.98nM. The $K_d$ for $E_2$ binding ER-α was set as 0.25nM. The equilibrium concentration of ER-α and SHBG bound $E_2$ was numerically determined using Gepasi when the concentration of $E_2$ was varied between $1 \times 10^{-13}$ nM and 30nM. Maximal binding was calculated as the sum of $E_2$ bound ER-α and SHBG, in the presence of SHBG. In the absence of SHBG maximal binding was defined as the equilibrium concentration of $E_2$ bound ER-α.
6.1 Model of the Hep89 experimental system

Figure 6.7: The influence of SHBG and albumin on the binding of E₂ to ER-α. The concentration of ER-α used was 1.2nM, SHBG concentration was set as 0.98nM while the concentration of albumin was $1.74 \times 10^4$ nM. The $K_d$ for E₂ binding ER-α was set as 0.25nM. The equilibrium concentration of ER-α, SHBG and albumin bound E₂ was numerically determined using Gepasi when the concentration of E₂ was varied between $1 \times 10^{-13}$ nM and 30nM. Maximal binding was calculated as the sum of E₂ bound ER-α and SHBG, in the presence of SHBG and as the sum of ER-α, SHBG and albumin bound E₂, in the presence of SHBG and albumin. In the absence of SHBG or albumin maximal binding was defined as the concentration of E₂ bound ER-α at equilibrium.
6.2 Model of human plasma experimental system

The aim of modelling the binding of \( E_2 \) in human pregnancy plasma was to determine why saturating conditions were not reached. To explain these results the experimental system of Hammond [180] will be modelled after which the experimental system as was used in this study will be modelled. Both of these systems were being modelled to highlight the differences between the experimental systems, so as to give an explanation as to why saturating conditions were not reached in the experiments performed in human pregnancy plasma.

6.2.1 Binding of dihydrotestosterone to SHBG in human pregnancy plasma

An experimental system to determine the binding constants, \( K_d \) and \( B_{\text{max}} \), for SHBG binding dihydrotestosterone (DHT) was proposed by Hammond [180]. The presence of albumin was not explicitly mentioned by Hammond although it is known that albumin is present in human pregnancy plasma [158], and as such albumin has been included in this model.

The experimental approach used by Hammond relied on SHBG and CBG both binding DHT and cortisol. As CBG binds cortisol with a higher affinity than SHBG, cortisol was included to counteract the effect that CBG binding DHT would have on SHBG binding DHT. The Specific binding of DHT to SHBG was calculated from the Total binding of DHT minus the non specific binding of DHT. Total binding was determined as the binding of \(^3\)H-DHT in the presence of 66.67 nM of cortisol. Non Specific binding was determined as the binding of \(^3\)H-DHT in the presence of 1666.67 nM DHT and 66.67 nM cortisol.
Figure 6.8: Experimental system that was used by Hammond [180]. The experimental system used by Hammond when binding dihydrotestosterone (DHT) and cortisol to SHBG and corticosteroid binding protein (CBG) in the presence of albumin.
6.2 Model of human plasma experimental system

The chemical equilibria used to describe the experimental system are as follows for Total binding:

\[
\begin{align*}
\text{SHBG} + \text{DHT}_{\text{labelled}} & \rightleftharpoons \text{SHBG.DHT}_{\text{labelled}} \\
\text{CBG} + \text{DHT}_{\text{labelled}} & \rightleftharpoons \text{CBG.DHT}_{\text{labelled}} \\
\text{albumin} + \text{DHT}_{\text{labelled}} & \rightleftharpoons \text{albumin.DHT}_{\text{labelled}} \\
\text{SHBG} + \text{cortisol} & \rightleftharpoons \text{SHBG.cortisol} \\
\text{CBG} + \text{cortisol} & \rightleftharpoons \text{CBG.cortisol} \\
\text{albumin} + \text{cortisol} & \rightleftharpoons \text{albumin.cortisol}
\end{align*}
\]

(6.3)

where DHT_{labelled} is $^3$H-DHT.

and for Non Specific binding:

\[
\begin{align*}
\text{SHBG} + \text{DHT}_{\text{labelled}} & \rightleftharpoons \text{SHBG.DHT}_{\text{labelled}} \\
\text{CBG} + \text{DHT}_{\text{labelled}} & \rightleftharpoons \text{CBG.DHT}_{\text{labelled}} \\
\text{albumin} + \text{DHT}_{\text{labelled}} & \rightleftharpoons \text{albumin.DHT}_{\text{labelled}} \\
\text{SHBG} + \text{cortisol} & \rightleftharpoons \text{SHBG.cortisol} \\
\text{CBG} + \text{cortisol} & \rightleftharpoons \text{CBG.cortisol} \\
\text{albumin} + \text{cortisol} & \rightleftharpoons \text{albumin.cortisol}
\end{align*}
\]

(6.4)

The kinetic binding constants for all of the interactions are shown in Table 6.5 as are the values that were used to initialize the model.
6.2 Model of human plasma experimental system

Table 6.5: Kinetic constants for SHBG, CBG and albumin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Steroid</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHBG</td>
<td>DHT</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>cortisol</td>
<td>625</td>
</tr>
<tr>
<td>CBG</td>
<td>DHT</td>
<td>1204.82</td>
</tr>
<tr>
<td></td>
<td>cortisol</td>
<td>13.16</td>
</tr>
<tr>
<td>albumin</td>
<td>DHT</td>
<td>4×10^4</td>
</tr>
<tr>
<td></td>
<td>cortisol</td>
<td>3×10^3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (nM)</th>
<th>200x dilution</th>
<th>100x dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>CBG</td>
<td>1.77</td>
<td>3.53</td>
<td></td>
</tr>
<tr>
<td>albumin</td>
<td>833.33</td>
<td>1587.30</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>^3H-DHT</td>
<td>0.33 - 16.67</td>
</tr>
<tr>
<td>DHT</td>
<td>1666.67</td>
</tr>
<tr>
<td>cortisol</td>
<td>66.7</td>
</tr>
</tbody>
</table>

The values that were used to initialize the model, these include binding constants (B_{max} and K_d) as well as protein and steroid concentrations.
6.2 Model of human plasma experimental system

As it is not possible to analytically determine the equilibrium concentration of all the species mentioned numerical modelling, with the aid of Gepasi, was used. The amount of $^3$H-DHT was varied between $1 \times 10^{-13}$ nM and 16.67 nM, and the equilibrium concentrations of the various species calculated.

The binding curves for Total binding, Non Specific binding and Specific binding are shown in Figure 6.9, for plasma diluted 200 times, and Figure 6.10, for plasma diluted 100 times. Total binding was calculated as the amount of SHBG, albumin and CBG bound $^3$H-DHT while non specific binding was calculated as the amount of albumin and CBG bound $^3$H-DHT in the presence of 1666.67 nM DHT. From these results it was shown that when plasma was diluted 200 and 100 times saturating conditions were reached. These results are in concordance with those of Hammonds [180].
6.2 Model of human plasma experimental system

Figure 6.9: Modelling results for the binding of DHT in plasma diluted 200 times. The binding of DHT to SHBG, CBG and albumin in plasma that was diluted 200 times was modelled. The concentration of protein bound by $^3$H-DHT under total, non specific and specific binding conditions was calculated with the aid of Gepasi. The Specific binding was calculated as the binding of $^3$H-DHT in the presence of 66.67 nM cortisol and absence of DHT minus the binding of $^3$H-DHT in the presence of 1666.67 nM DHT and 66.67 nM cortisol at equilibrium. Maximal binding was calculated as the sum of SHBG, albumin and CBG bound $^3$H-DHT, at equilibrium, for any given concentration of $^3$H-DHT.
6.2 Model of human plasma experimental system

![Graph showing the binding of DHT in plasma diluted 100 times.](image)

**Figure 6.10: Modelling results for the binding of DHT in plasma diluted 100 times.** The binding of DHT to SHBG, CBG and albumin in plasma that was diluted 100 times was modelled. The concentration of protein bound by \( ^3 \text{H-DHT} \) under total, non specific and specific binding conditions was calculated with the aid of Gepasi. The Specific binding was calculated as the binding of \( ^3 \text{H-DHT} \) in the presence of 66.67 nM cortisol and absence of DHT minus the binding of \( ^3 \text{H-DHT} \) in the presence of 1666.67 nM DHT and 66.67 nM cortisol at equilibrium. Maximal binding was calculated as the sum of SHBG, albumin and CBG bound \( ^3 \text{H-DHT} \), at equilibrium, for any given concentration of \( ^3 \text{H-DHT} \).
6.2 Model of human plasma experimental system

To explain these results the percentage of free ligand as well as the percentage bound to SHBG, CBG and albumin was calculated. The percentage bound was calculated as the ratio between the concentration of bound protein to the total amount of the steroid present initially. This ratio was then converted to a percent of 100, Table 6.6.
Table 6.6: Results for the distribution of $^3$H-DHT between the binding proteins, expressed as a percentage.

<table>
<thead>
<tr>
<th>Total (nM)</th>
<th>% Free</th>
<th>% bound to SHBG</th>
<th>% bound to albumin</th>
<th>% bound to CBG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total binding, 200x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-DHT</td>
<td>16.67</td>
<td>94.19</td>
<td>3.97</td>
<td>1.84</td>
</tr>
<tr>
<td>cortisol</td>
<td>66.67</td>
<td>77.70</td>
<td>0.1</td>
<td>20.20</td>
</tr>
<tr>
<td><strong>Non Specific binding, 200x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-DHT</td>
<td>16.67</td>
<td>98.12</td>
<td>0.04</td>
<td>1.84</td>
</tr>
<tr>
<td>DHT</td>
<td>1666.67</td>
<td>98.10</td>
<td>0.04</td>
<td>1.84</td>
</tr>
<tr>
<td>cortisol</td>
<td>66.67</td>
<td>78.68</td>
<td>0.1</td>
<td>19.65</td>
</tr>
<tr>
<td><strong>Total binding, 100x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-DHT</td>
<td>16.67</td>
<td>88.61</td>
<td>7.88</td>
<td>3.47</td>
</tr>
<tr>
<td>cortisol</td>
<td>66.67</td>
<td>63.08</td>
<td>0.1</td>
<td>32.90</td>
</tr>
<tr>
<td><strong>Non Specific binding, 100x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-DHT</td>
<td>16.67</td>
<td>96.27</td>
<td>0.08</td>
<td>3.62</td>
</tr>
<tr>
<td>DHT</td>
<td>1666.67</td>
<td>96.25</td>
<td>0.08</td>
<td>3.62</td>
</tr>
<tr>
<td>cortisol</td>
<td>66.67</td>
<td>64.54</td>
<td>0.1</td>
<td>32.37</td>
</tr>
</tbody>
</table>

The concentration of $^3$H-DHT was varied between $1 \times 10^{-13}$ nM and 16.67 nM in the presence of SHBG, CBG and albumin. The percentages of $^3$H-DHT bound to each protein was calculated as the concentration of $^3$H-DHT bound protein over Total $^3$H-DHT concentration (16.67 nM) and then expressed as a percentage.
6.2 Model of human plasma experimental system

Table 6.7: Results for the distribution of the binding proteins (SHBG, albumin and CBG) expressed as a percentage.

<table>
<thead>
<tr>
<th></th>
<th>Total (nM)</th>
<th>% Free</th>
<th>% bound to $^{3}$H-DHT</th>
<th>% bound to DHT</th>
<th>% bound to cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total binding, 200× dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>1.13</td>
<td>98.77</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>albumin</td>
<td>833.33</td>
<td>98.35</td>
<td>0.04</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>CBG</td>
<td>1.77</td>
<td>20.21</td>
<td>0.26</td>
<td>79.53</td>
<td></td>
</tr>
<tr>
<td><strong>Non specific binding, 200× dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>0.01</td>
<td>0.99</td>
<td>99.00</td>
<td>0.0009</td>
</tr>
<tr>
<td>albumin</td>
<td>833.33</td>
<td>94.71</td>
<td>0.04</td>
<td>3.68</td>
<td>1.57</td>
</tr>
<tr>
<td>CBG</td>
<td>1.77</td>
<td>15.73</td>
<td>0.21</td>
<td>21.34</td>
<td>62.70</td>
</tr>
<tr>
<td><strong>Total binding, 100× dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>1.33</td>
<td>1.20</td>
<td>98.71</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>albumin</td>
<td>1587.30</td>
<td>98.58</td>
<td>0.04</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>CBG</td>
<td>3.53</td>
<td>99.71</td>
<td>0.29</td>
<td>75.94</td>
<td></td>
</tr>
<tr>
<td><strong>Non specific binding, 100× dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>1.33</td>
<td>0.01</td>
<td>0.99</td>
<td>99.00</td>
<td>0.0007</td>
</tr>
<tr>
<td>albumin</td>
<td>1587.30</td>
<td>94.80</td>
<td>0.04</td>
<td>3.80</td>
<td>1.35</td>
</tr>
<tr>
<td>CBG</td>
<td>3.53</td>
<td>17.81</td>
<td>0.23</td>
<td>23.71</td>
<td>58.23</td>
</tr>
</tbody>
</table>

The concentration of $^{3}$H-DHT was varied between $1 \times 10^{-13}$ nM and 16.67 nM in the presence of SHBG, CBG and albumin. The concentration of DHT, $^{3}$H-DHT and cortisol bound binding proteins (SHBG, albumin and CBG) was calculated and expressed as a percentage.
6.2 Model of human plasma experimental system

From the results of these calculations it can be seen that the relative distribution of $^3$H-DHT changes when comparing the plasma diluted 200, Figure 6.9, times and 100 times, Figure 6.10. In the plasma that was diluted 100 times it can be seen that there is ligand depletion in that more than 10% of $^3$H-DHT is bound, Table 6.6. With respect to the use of cortisol in the assay it was found that this prevented the binding of $^3$H-DHT to CBG to an extent and in the absence the amount of specific binding is increased minimally, Table 6.7. These results would suggest that it is not the relative distribution of $^3$H-DHT that is the cause for non saturating conditions being reached in plasma that was diluted 100 times. The results of the modelling has shown that when plasma was diluted 100 times the amount of ligand that was bound was more than 10%, and as there is more than one binding protein it is difficult to calculate the binding constants ($B_{max}$ and $K_d$ (DHT) for SHBG). This may have been the reason why it was not possible to determine the binding constants for SHBG using human pregnancy plasma that was diluted 100 times, as stated by Hammond [180].
6.2 Model of human plasma experimental system

6.2.2 Binding of E\textsubscript{2} to SHBG in human pregnancy plasma

The aim of the following section of work is to determine why when saturation binding assays were performed, using human pregnancy plasma, saturating conditions were not reached. The experimental approach was similar to that of Hammond [180] except for the following conditions:

- Saturation binding experiments were performed using \(^3\text{H}-\text{E}\textsubscript{2}\) to determine the \(K_d(\text{E}_2)\) for SHBG binding \(\text{E}_2\).
- Human pregnancy plasma was only diluted 25 times.
- No cortisol was used to prevent the binding of \(\text{E}_2\) to CBG as CBG has no affinity for \(\text{E}_2\) [158].

To determine whether diluting the plasma 200 times has on the final outcome, the model will include a description of conditions that would have been encountered if the plasma was diluted 200 times.

A graphical description of the experimental system can be seen in Figure 6.11. From this diagram it can be seen that \(\text{E}_2\) can bind either SHBG or albumin. The binding of \(^3\text{H}-\text{E}_2\) to SHBG in the absence in the absence of an excess of \(\text{E}_2\) was used to determine the Total binding. Non specific binding was performed under the same circumstances except that an excess, \(1\times10^4\text{nM} \text{E}_2\), was added. Specific binding was calculated as Total binding - non specific binding.

The chemical equilibria that describe this experimental system are shown in equations 6.5 and 6.6. The chemical equilibria that describe Total binding are as follows:

\[
\text{SHBG} + ^3\text{H}-\text{E}_2 \rightleftharpoons \text{SHBG.}^3\text{H}-\text{E}_2
\]
\[
\text{albumin} + ^3\text{H}-\text{E}_2 \rightleftharpoons \text{albumin.}^3\text{H}-\text{E}_2
\] (6.5)
Figure 6.11: Experimental system that was used in saturation binding assays in human pregnancy plasma using $E_2$. The diagram shows the binding of $E_2$ to SHBG and albumin as would have been the case in human pregnancy plasma.
6.2 Model of human plasma experimental system

The chemical equilibria that describe Non Specific binding are as follows:

\[
\begin{align*}
\text{SHBG} + ^3\text{H-E}_2 & \rightleftharpoons \text{SHBG}^3\text{H-E}_2 \\
\text{SHBG} + \text{E}_2 & \rightleftharpoons \text{SHBG.E}_2 \\
\text{albumin} + ^3\text{H-E}_2 & \rightleftharpoons \text{albumin}^3\text{H-E}_2 \\
\text{albumin} + \text{E}_2 & \rightleftharpoons \text{albumin.E}_2
\end{align*}
\]  

(6.6)

The kinetic binding constants for all of the interactions are shown in Table 6.8 as are the values that were used to initialize the model.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Steroid</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHBG</td>
<td>E₂</td>
<td>1.5</td>
</tr>
<tr>
<td>albumin</td>
<td>E₂</td>
<td>1.7×10⁴</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200x dilution</td>
<td>25x dilution</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
</tr>
<tr>
<td>albumin</td>
<td>833.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>^3H-E₂</td>
<td>1×10⁻¹³ - 30</td>
</tr>
<tr>
<td>E₂</td>
<td>1×10⁴</td>
</tr>
</tbody>
</table>

The values that were used to initialize the model, these include binding constants (Bₘₐₓ and Kₐ) as well as protein and steroid concentrations.
6.2 Model of human plasma experimental system

As it is not possible to analytically determine the equilibrium concentration of all the species mentioned numerical modelling, with the aid of Gepasi, was used. The amount of $^3$H-E$_2$ was varied between $1 \times 10^{-13}$ nM and 30nM, and the equilibrium concentrations of the various species calculated.

The binding curves for Total binding, Non Specific binding and Specific binding are shown in Figure 6.12, for plasma diluted 25 times, and Figure 6.13, for plasma diluted 200 times. Total binding was calculated as the amount of SHBG and albumin $^3$H-E$_2$ while non specific binding was calculated as the amount of albumin bound $^3$H-E$_2$ in the presence of $1 \times 10^4$ nM E$_2$.

![Graph showing binding curves](image)

**Figure 6.12: Modelling results for the binding of E$_2$ in plasma diluted 25 times.** The binding of E$_2$ to SHBG and albumin in plasma that was diluted 25 times was modelled. The concentration of protein bound by $^3$H-E$_2$ under total, non specific and specific binding conditions was calculated via the aid of Gepasi. The Specific binding was calculated as the binding of $^3$H-E$_2$ and absence of E$_2$ minus the binding of $^3$H-E$_2$ in the presence of $1 \times 10^4$ nM E$_2$ at equilibrium. Maximal binding is defined as the amount of protein (the sum of SHBG and albumin) bound by $^3$H-E$_2$, at equilibrium, for any given concentration of $^3$H-E$_2$. 

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6.2 Model of human plasma experimental system

Figure 6.13: Modelling results for the binding of E$_2$ in plasma diluted 200 times. The binding of E$_2$ to SHBG and albumin in plasma that was diluted 200 times was modelled. The concentration of protein bound by $^3$H-E$_2$ under total, non specific and specific binding conditions was calculated via the aid of Gepasi. The Specific binding was calculated as the binding of $^3$H-E$_2$ and absence of E$_2$ minus the binding of $^3$H-E$_2$ in the presence of $1\times10^4$ nM E$_2$ at equilibrium. Maximal binding is defined as the amount of protein (the sum of SHBG and albumin) bound by $^3$H-E2, at equilibrium, for any given concentration of $^3$H-E2.
6.2 Model of human plasma experimental system

For the purposes of this discussion maximal binding was calculated as the concentration of SHBG.\textsuperscript{3}H-E\textsubscript{2}. From the results of these models it was concluded that when human pregnancy plasma was diluted 25 times it was not possible to reach saturating conditions, as can be seen when Specific binding was plotted 6.12. When examining the results for human pregnancy plasma diluted 200 times it was noted that when Specific binding was plotted, saturating conditions were also not reached 6.13. To explain these results the percentage of free ligand as well as the percentage bound to SHBG and albumin was calculated. The percentage bound was calculated as the ratio between the concentration of bound protein to the total amount of the steroid present initially. This ratio was then converted to a percent of 100, Table 6.9.
6.2 Model of human plasma experimental system

Table 6.9: Results for the distribution of $E_2$ between the binding proteins, expressed as a percentage.

<table>
<thead>
<tr>
<th></th>
<th>Total (nM)</th>
<th>% Free</th>
<th>% bound to SHBG</th>
<th>% bound to albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total binding, 200x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-$E_2$</td>
<td>30.00</td>
<td>93.22</td>
<td>2.21</td>
<td>4.56</td>
</tr>
<tr>
<td><strong>Non Specific binding, 200x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-$E_2$</td>
<td>30.00</td>
<td>97.11</td>
<td>0.01</td>
<td>2.88</td>
</tr>
<tr>
<td>$E_2$</td>
<td>10000.00</td>
<td>97.11</td>
<td>0.01</td>
<td>2.88</td>
</tr>
<tr>
<td><strong>Total binding, 25x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-$E_2$</td>
<td>30.00</td>
<td>60.87</td>
<td>16.42</td>
<td>22.71</td>
</tr>
<tr>
<td><strong>Non Specific binding, 25x dilution of plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-$E_2$</td>
<td>30.00</td>
<td>79.70</td>
<td>0.05</td>
<td>20.25</td>
</tr>
<tr>
<td>$E_2$</td>
<td>10000.00</td>
<td>79.70</td>
<td>0.05</td>
<td>20.25</td>
</tr>
</tbody>
</table>

The concentration of $^3$H-$E_2$ was varied between $1 \times 10^{-13}$ nM and 30nM in the presence of SHBG and albumin. The percentages of $^3$H-$E_2$ bound to each protein was calculated as the concentration of $^3$H-$E_2$ bound protein over Total $^3$H-$E_2$ concentration (30nM) and then expressed as a percentage.
### Table 6.10: Results for the distribution of bound and unbound binding proteins, as expressed as a percentage.

<table>
<thead>
<tr>
<th></th>
<th>Total binding, 200× dilution of plasma</th>
<th>Non specific binding, 200× dilution of plasma</th>
<th>Total binding, 25× dilution of plasma</th>
<th>Non specific binding, 25× dilution of plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (nM)</td>
<td>% Free</td>
<td>% bound to $^3$H-E$_2$</td>
<td>% bound to E$_2$</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.67</td>
<td>0.84</td>
<td>99.16</td>
<td></td>
</tr>
<tr>
<td>albumin</td>
<td>833.33</td>
<td>99.84</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

The concentration of $^3$H-E$_2$ was varied between $1 \times 10^{-13}$ nM and 30nM in the presence of SHBG and albumin. The distribution of the binding proteins between $^3$H-E$_2$ and E$_2$ was calculated and expressed as a percentage.
6.2 Model of human plasma experimental system

The results of determining the amount of E₂ bound to the SHBG or albumin showed that when human pregnancy plasma was diluted 200 times there was less than 10% ligand depletion. When plasma was diluted 25 times it was noted that there was about 40% ligand depletion. With respect to the apparent right shift in the \( K_d \), it should be noted as there is more SHBG and albumin present in the plasma that was diluted 25 times, as compared to 200 times, equilibrium binding would occur at higher concentrations of E₂.

When examining the binding of \(^3\text{H}-\text{E}_2\) and \(\text{E}_2\) by the various binding proteins it was noted that at least 99% of SHBG was bound by \(^3\text{H}-\text{E}_2\) whilst not much albumin, about 0.3%, was bound by \(^3\text{H}-\text{E}_2\), Table 6.10. This is in keeping with the \( K_{d\ E_2} \) for SHBG and albumin. When examining the binding of \(\text{E}_2\) to SHBG in the absence of albumin when human plasma is diluted 200 times it is noted that it is possible to saturate SHBG, Figure 6.13.

From these results it was concluded that when human pregnancy plasma was diluted 25 times it would not have been possible to saturate SHBG, as was found experimentally. When examining the role of diluting plasma 200 times before performing saturation binding assays it was noted that the only real differences would have been a increase in the \( K_{d\ app\ (E_2)} \) and \( B_{max\ app} \). It was also noted that when plasma was diluted 200 times and no albumin was present it was possible to saturate SHBG using 30nM \(^3\text{H}-\text{E}_2\), Figure 6.13.

When comparing this experimental system to the one Hammond used it should be noted that the \( K_{d\ (\text{DHT})} \) for DHT binding SHBG, \( K_d \) is 0.18nM is significantly lower than the affinity that SHBG has for \( E_2 \), \( K_d \) is 1.5nM. With respect to the presence of cortisol in Hammond’s experimental system it was noted that both albumin and CBG bound cortisol. The binding of cortisol by CBG and albumin would have decreased the Non Specific binding of \(^3\text{H}-\text{DHT} \), and thus there would have been a higher concentration of DHT available for binding to SHBG. In the experimental system described
6.2 Model of human plasma experimental system

In this system there was no cortisol present and thus there would have been a higher amount of non specifically bound $^3$H-E$_2$. In conclusion the modelling of Hammond’s system and the experimental system used in this study has highlighted the differences between the two systems. By comparing these systems it has been shown that when attempting to perform saturation binding assays using E$_2$, in human pregnancy plasma, the presence of albumin has a negative influence.
Chapter 7

Conclusion

The aim of this study was to determine to what extent SHBG modulates the binding of E_2 to ER-α and the subsequent transactivation of an ERE reporter gene. The data obtained from these experiments was to be used to build a mathematical model to describe these interactions. The following discussion will deal with the conclusion of the experimental and modelling results.

From the results of the binding studies in COS-1 cells it was possible to determine that the K_d (E_2) for ER-α was between 3.4 and 4.4nM. These values are in accordance with those reported by Pederson [179]. The results are however not in accordance with those determined by Kuiper et al [49] who determined the K_d (E_2) as 0.5nM when using a pure solution of ER-α protein. One of the factors that was shown to have an effect on the K_d, when working in a whole cell system, was the transfer of E_2 across the cell membrane as shown in Chapter 6. As COS-1 cells were transiently transfected it is not possible to discuss the relevance of the B_max that was determined, as the values determined cannot be compared to the physiological concentrations of ER-α in humans.

The results of the saturation binding assays performed using Hep89 cells
showed that when using 30nM of $^3$H-E$_2$ saturation conditions were not reached. As there is no literature available for saturation binding assays performed in Hep89 cells a mathematical model was built to describe the experimental system. The results of this model showed that the transfer of E$_2$ across the membrane caused a shift in the saturation binding curve. This shift was as a result of a decrease in the maximal binding and an increase in the K$_{d\,\text{app}}$(E$_2$). The reason for the decrease in maximal binding is understandable when one determines the equilibrium concentration of E$_2$ in the extracellular and intracellular compartment in the absence of binding proteins. Initially 30nM of E$_2$ was in the extracellular compartment, after the transfer of E$_2$ across the membrane and equilibrium had been reached there is roughly 29nM of E$_2$ in both the extracellular and intracellular compartments. The apparent decrease in the maximal binding and K$_d$ can thus be explained by the decreased equilibrium concentration of E$_2$ as compared to the total amount of E$_2$ that was initially present. With respect to the role that SHBG had on the binding of E$_2$ to ER-α it was found that even at the highest concentration of SHBG, 0.98nM, it was still possible to saturate ER-α. When albumin was introduced, as would be the case in the experimental system, it was still possible to saturate ER-α but there was an increase in the K$_{d\,\text{app}}$(E$_2$). This was expected because albumin binds E$_2$ and thus lowering the equilibrium concentration of unbound E$_2$. When the maximal binding that would be expected in the case of an experimental system was calculated, that is the sum of ER-α, SHBG and albumin bound E$_2$, it was found that in the presence of SHBG and albumin saturating conditions were not reached. It was also noticed that there was at least 60% ligand depletion. The amount of ligand depletion that was calculated from the model exceeds that encountered experimentally, probably because the defined concentration of albumin in the model is higher than that present in the experimental system. It should be noted, however, that if SHBG was present at the concentrations that have been published for HepG2 cells, between 0.63nM and 0.98nM intracellular [137, 142], it is still possible to reach saturating conditions. From the results
of the modelling it was thus concluded that the reason that saturation conditions were not reached in the Hep89 experimental system was because of the presence of albumin.

The results of the saturation binding assay, performed using human pregnancy plasma diluted 25 times, showed that it was not possible to determine the binding constants for E$_2$ binding SHBG using this experimental system. It was hypothesized that the presence of albumin was interfering with the binding assay.

To determine whether albumin was interfering with the binding of E$_2$ to SHBG, the experimental system proposed by Hammond [180] was first modelled. The results of this model show that when plasma was diluted 200 times, the binding constants for SHBG binding DHT can be determined. If the plasma was diluted 100 times it was not possible to determine the binding constants for SHBG, as Hammond reported. A possible reason for this is that when plasma was diluted 100 times ligand depletion in excess of 10% was encountered.

The results of the model describing the binding of E$_2$ to SHBG in human plasma, diluted 25 times, showed that over 40% of E$_2$ was bound. These results are similar to those of the experimental system where it was found that between 32% and 43% of E$_2$ was bound. Using the model it was possible to calculate that if the plasma was diluted 200 times there would be have been less than 10% ligand depletion, however saturation conditions would not have been reached. With respect to the influence that albumin has on the binding of E$_2$ to SHBG it was possible to show that if no albumin was present then saturating conditions would have been reached if the plasma was diluted 200 times. It should be noted that even in the plasma was diluted 200 times it would not have been possible to determine the K$_d$ for SHBG, only the K$_{d\text{ app}}$ as albumin binds E$_2$ and lowers the equilibrium concentration of E$_2$. 

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The binding of E$_2$ by albumin is supported in the literature as it has been found to bind between 58.3% and 73.8% in females and males respectively in human plasma [158].

If one were to recommend an experimental system to determine the binding constants for SHBG when binding E$_2$, using human pregnancy plasma, it is recommended to rather use the experimental system that Dunn _et al_ [158] used. In this experimental system concanavalin A-Sepharose was used to isolate SHBG from human pregnancy plasma. The pure SHBG solution can then be used to determine the binding constants for SHBG binding E$_2$.

When examining the results of the promoter reporter gene assays it was noted that the ligand independent transactivation of the ERE reporter gene constructs was problematic. This is not a problem that has been reported in the literature surrounding the use of COS-1 cells to perform the same type of promoter reporter gene assays. Korach _et al_ [197] and Pennie _et al_ have both been able to use COS-1 cells as experimental systems in which to determine the effect of E$_2$ on estrogenically sensitive reporter genes. In Korach’s article the role that the ERE plays in changing the conformation that ER-$\alpha$ adapts, when binding the ERE, was elucidated. It was shown that the sequence of the ERE can have an indirect influence on the cofactors that are recruited and in so doing also have an affect on the level of transactivation, via the ligand bound ER-$\alpha$. The work of Pennie [183] showed that the level of transactivation, via the ligand bound ER-$\alpha$, of an ERE depends on the cellular environment as well as the ligand that ER-$\alpha$ bound.

In conclusion it has been shown that the aims of this project as stated were not accomplished due to a number of problems that were encountered. It has been possible to show why the binding constants for ER-$\alpha$ and SHBG were not determined using the Hep89 and human pregnancy plasma experimental systems. With respect to the promoter reporter gene assay studies it is still
not clear why there was ligand independent transactivation in the COS-1 cell experimental system. It has also become clear from this study that it may not just be the influence that SHBG has on the binding of E₂ to ER-α but that other factors will have to be taken into account in further studies.
Appendix A

Optimisation of protocols using the Calcium Phosphate transfection technique

The aim of the experiments described in this section of work was to develop an experimental system in which the effect of E₂ on an estrogenically sensitive reporter gene could be studied.

As COS-1 cells had to be transfected the available transfection processes were evaluated on the basis of reproducibility, cost and availability of reagents. Based on these decisions it was decided that either the calcium phosphate or DEAE-Dextran transfection method could be used to transfect COS-1 cells. Initially it was decided to investigate the calcium phosphate transfection method [198].

A.1 Optimisation of transfection

The aim of the following experiments was to optimize the transfection process with respect to COS-1 cells. To accomplish this the method was optimized with respect to the number of cells seeded and the amount of DNA used in
A.1 Optimisation of transfection

the transfection process. The role of a glycerol shock, as suggested by the protocol [198], was studied to ascertain whether it was necessary for optimal transfection efficiency, Table A.1.

Table A.1: Conditions used for optimisation of calcium phosphate transfection.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells seeded per well</td>
<td>1, 1.5 and 2×10⁵ cells</td>
</tr>
<tr>
<td>Amount of DNA per well</td>
<td>1.73 and 2.41</td>
</tr>
<tr>
<td>Effectiveness of shocking</td>
<td>10% glycerol shock vs no shock</td>
</tr>
</tbody>
</table>

COS-1 cells were seeded at varying densities; 1×10⁵, 1.5×10⁵ and 2×10⁵; cells per well in 24 well plates, transfected using the calcium phosphate transfection method with varying amounts of DNA and finally exposed to a 10% glycerol shock or a PBS wash in place of the shock.
A.1 Optimisation of transfection

As COS-1 cells were transiently transfected the results of the experiments had to be normalized and it was decided to normalize the results using the results obtained from a β-galactosidase reporter that was co-transfected. The use of Neogal and pSV-β-galactosidase β-galactosidase reporter genes were studies as well.

The results of these experiments are shown in Figure A.1. From the results of these experiments it was concluded that optimal transfection efficiency was obtained when COS-1 cells were seeded at a density of $1 \times 10^5$ cells per well in 24 well plates and transfected with 1.73µg DNA or 2.41µg DNA. With respect to the use of a 10% glycerol shock during the transfection process it was found that this was not necessary for optimal transfection. It was also decided that in future pSV-β-galactosidase will be used as a β-galactosidase reporter gene.
A.1 Optimisation of transfection

Figure A.1: Optimisation of calcium phosphate transfection. COS-1 cells were maintained and transfected with either 1.73µg or 2.41µg DNA as described in Appendix C.2.1 and C.3.1. Cells were transfected using either Neogal or pSV-β-galactosidase as β-galactosidase reporter genes. (a) COS-1 cells transfected with Neogal and exposed to a 10% glycerol shock, (b) COS-1 cells transfected with Neogal, the cells were not exposed to a 10% glycerol shock., (c) COS-1 cells transfected with pSV-β-galactosidase and exposed to a 10% glycerol shock, (d) COS-1 cells transfected with pSV-β-galactosidase, the cells were not exposed to a glycerol shock. Results are shown as the average with standard errors for triplicate samples.
A.2 Optimisation of transactivation

The aim of the following experiments was to determine the level of transactivation of an ERE, via the ligand bound ER-α, using COS-1 cells as the experimental system. The experimental system was optimized with respect to the following: plasmid ratios (ERE.tk-luc, β-galactosidase reporter gene, and ER-α), the medium and supplements used.

Initially COS-1 cells were maintained and transfected as described in Appendix C.3.1 with 1.73 µg of DNA, Table C.3. The results of this experiment are shown in Figure A.2. From the results of this experiment it was determined that there was ligand independent transactivation. This can be seen when comparing samples that were exposed to 1 × 10⁻⁵ M E₂ to those only exposed to ethanol. It was also noted that the β-galactosidase reporter gene, pSV-β-galactosidase, seemed to be sensitive to the presence of estrogenic compounds. With this in mind it was decided to search the literature and it was noted that the ratio of plasmids that were used in the experiment, Table C.3 was not the same as that in the literature, Table C.3.
A.2 Optimisation of transactivation

Figure A.2: Optimisation of transactivation using ERE.tk-luc as ERE reporter gene (Normalized results). COS-1 cells were seeded and transfected using the calcium phosphate transfection technique with a total of 1.73 µg of DNA consisting of 1.02 µg ER-α, 0.58 µg ERE.tk-Luc and 0.13 µg β-galactosidase reporter gene as described in Appendix C.3.1. The cells were grown for a further 24 hours after which the 10^{-5}M E_2 was added and the cells incubated for a further 24 hours. The cells were then lysed and frozen overnight at -20°C and the lysate analyzed for the presence of luciferase and β-galactosidase and protein. Control 1 is transfection with only the addition of EtOH as a control, Control 2 is where there was no transfection. Luciferase values were normalized by dividing by the β-galactosidase values. Results are shown as the average with standard errors for triplicate samples.
A.2 Optimisation of transactivation

**Figure A.3: Optimisation of transactivation using ERE.tk-luc as ERE reporter gene (β-galactosidase results).** COS-1 cells were seeded and transfected using the calcium phosphate transfection technique with a total of 1.73 µg of DNA consisting of 1.02 µg ER-α, 0.58 µg ERE.tk-Luc and 0.13 µg β-galactosidase reporter gene as described in Appendix C.3.1. The cells were grown for a further 24 hours after which the 10⁻⁵ M E₂ was added and the cells incubated for a further 24 hours. The cells were then lysed and frozen overnight at -20°C and the lysate analyzed for the presence of β-galactosidase. Control 1 is transfection with only the addition of EtOH as a control, Control 2 is where there was no transfection. Results are shown as the average with standard errors for triplicate samples.
A.2 Optimisation of transactivation

The aim of the following experiment was to determine whether changing the ratios of plasmids used would have an influence on the transactivation of the ERE.tk-luc reporter gene. The secondary aim was to determine whether Neogal, a β-galactosidase reporter, was sensitive to the presence of estrogenic compounds.

COS-1 cells were transfected with a total of 1.73μg DNA using the calcium phosphate transfection method. The ratio of plasmids used is shown in Table C.3. The following controls were also included:

- Control 1- COS-1 cells were transfected with the ERE.tk-luc and β-galactosidase reporter genes as well as the pCDNA-3-ER-α plasmid. The cells were only exposed to ethanol as a test compound. This is a control for the presence of ligand independent transactivation.

- Control 1- COS-1 cells were transfected with the ERE.tk-luc and β-galactosidase reporter genes. The cells were only exposed to ethanol as a test compound. This is a control for the presence of receptor independent transactivation.

- COS-1 cells were not transfected. The cells were only exposed to ethanol as a test compound. This is a control for the constitutive expression of the ERE reporter gene.

As can be seen from the results, Figure A.4, there seemed to be ligand independent transactivation as there was no difference between the samples that were exposed to varying concentrations $E_2$ ($1 \times 10^{-5}$M, $1 \times 10^{-7}$M or $1 \times 10^{-10}$M) or ethanol. With respect to the use of Neogal as a β-galactosidase reporter it did not seem that Neogal was sensitive to the presence of estrogenic compounds, Figure A.5. From the results of these experiments it was concluded that there was either ligand and receptor independent transactivation when using ERE.tk-luc as a luciferase reporter gene or that the calcium phosphate transfection technique could not be used as the transfection effi-
A.2 Optimisation of transactivation efficiency was too low. As a result, it was decided to investigate the use of the DEAE-Dextran transfection technique.

![Graph showing Luciferase ± SEM, n=3 for Neogal, Green Neogal, Stoica Neogal](image)

**Figure A.4: Optimisation of DNA ratios used in transactivation studies (Luciferase values).** COS-1 cells were seeded and transfected using the calcium phosphate transfection technique with a total of 1.73µg of DNA. The ratios of DNA used in the experiments were as follows: Original (1.03µg ER-α, 0.58µg ERE.tk-Luc, 0.12µg pSV-β-galactosidase), Stoica (0.21µg ER-α, 1.08µg ERE.tk-luc, 0.08µg pSV-β-galactosidase, 0.36µg pGL2-Basic), Green (0.216µg ER-α, 1.08µg ERE.tk-Luc, 0.426µg pSV-β-galactosidase). The cells were grown for a further 24 hours after which the 11×10⁻⁵M E₂ was added and the cells incubated for a further 24 hours. The cells were then lysed and frozen overnight at -20°C and the lysate analyzed for the presence of luciferase and β-galactosidase and protein. Control 1 is transfection with only the addition of EtOH as a control, Control 2 is where there was no transfection. Luciferase values were normalized by dividing by the β-galactosidase values. Results are shown as the average with standard errors for triplicate samples.
A.2 Optimisation of transactivation

Figure A.5: Optimisation of DNA ratios used in transactivation studies (β-galactosidase values). COS-1 cells were seeded and transfected using the calcium phosphate transfection technique with a total of 1.73µg of DNA. The ratios of DNA used in the experiments were as follows: Original (1.03µg ER-α, 0.58µg ERE.tk-Luc, 0.12µg pSV-β-galactosidase), Stoica (0.21µg ER-α, 1.08µg ERE.tk-luc, 0.08µg pSV-β-galactosidase, 0.36µg pGL2-Basic), Green (0.216µg ER-α, 1.08µg ERE.tk-Luc, 0.426µg pSV-β-galactosidase). The cells were grown for a further 24 hours after which the 1×10⁻⁵M E₂ was added and the cells incubated for a further 24 hours. The cells were then lysed and frozen overnight at -20°C and the lysate analyzed for the presence of β-galactosidase. Control 1 is transfection with only the addition of EtOH as a control, Control 2 is where there was no transfection. Results are shown as the average with standard errors for triplicate samples.
Appendix B

Optimisation of protocols using DEAE-Dextran transfection technique

The aim of the following experiments was to determine whether the DEAE-Dextran transfection technique was suitable for the transient transfection of COS-1 cells. The DEAE-Dextran transfection protocol was chosen because it was not expensive and had been shown to be reproducible. Once it had been shown that COS-1 cells could be transfected using the DEAE-Dextran transfection technique, promoter reporter studies could be performed.

B.1 Optimisation of transfection

The aim of the following experiments was to optimize the transient transfection of COS-1 cells using the DEAE-Dextran transfection technique.

The protocol was optimized with respect to the amount of DNA used, number of COS-1 cells seeded and the amount of DEAE-Dextran used in the protocol, Table C.5.
B.1 Optimisation of transfection

COS-1 cells were maintained and transfected as described in Appendix C.3.2 after which the cells were exposed to $1 \times 10^{-5}$M or ethanol and assayed for the presence of luciferase and $\beta$-galactosidase. The results of this experiment, Figure B.1, showed that optimal transfection efficiency was obtained when COS-1 cells were seeded at a density of $5 \times 10^4$ cells per well in 24 well plates and transfected using 34.45ng of DNA and 6910ng of DEAE-Dextran. It was noted that the transfection efficiency was not greatly affected by cell number and thus it was decided that in future $6.9 \times 10^4$ would be seeded per well.
B.1 Optimisation of transfection

Figure B.1: Optimisation of DEAE-Dextran transfection in COS-1 cells. COS-1 cells were plated at varying densities per well (5×10⁴, 6.9×10⁴ and 1×10⁵ cells per well in 24 well plates) and incubated at 37°C, 5% CO₂ and 90% humidity. After 24 hours the cells were transfected using the DEAE-Dextran transfection technique whilst varying the amounts of DEAE-Dextran (between 3450 and 13820ng per well) and DNA (between 34.45 and 345.45ng) per well, using the ratios of Stoica, Table C.3, and the cells incubated for a further 24 hours. The cells were then exposed to 1×10⁻⁵E₂ in DMEM containing 10 %fetal calf serum and 1% Pencillin-Streptomycin for 24 hours. The medium was then removed and the cells lysed after which the samples were frozen overnight at -20°C. The samples were then analyzed for the presence of luciferase and β-galactosidase. Results are shown as the average with standard errors for triplicate samples.
B.2 Optimisation of transactivation

The aim of the following studies was to determine the level of transactivation of the ERE.tk-luc, via the ligand bound ER-α, using COS-1 cells as an experimental system. The experimental approach used was to determine the effect that various medium supplements (fetal calf serum, stripped fetal calf serum and no fetal calf serum), as well as the presence of phenol red in the medium, had on the transactivation of an ERE. The time after transfection that the medium was changed was also studied to determine the effect. The use of various β-galactosidase reporter genes, pSV-β-galactosidase, Neogal and pCMV-β-galactosidase, was also studied.

Initially the time after transfection that the medium was changed was studied. The medium was changed from DMEM containing 10% fetal calf serum to one of the following:

- DMEM containing either fetal calf serum, stripped fetal calf serum or no fetal calf serum.
- DMEM containing no phenol red either fetal calf serum, stripped fetal calf serum or no fetal calf serum as supplements.

The medium was changed either 12 or 24 hours after transfection. If the medium was changed 12 hours after transfection then $1 \times 10^{-5}$M E$_2$ or ethanol (0.01%) was added to the cells after a further 12 hours. The same conditions were used if the medium was changed 24 hours after transfection, the only difference being that the test compounds would be added only after a further 24 hours. The reason for these changes was to determine whether there was a difference in the expression levels of luciferase when comparing 12 and 24 hours. The results of this experiment, Figure B.2, showed that there was no difference in the results if the medium was changed 12 or 24 hours after transfection. With respect to the use of DMEM or DMEM containing no phenol red, it was found that higher levels of transactivation were induced in
B.2 Optimisation of transactivation

the presence of phenol red. The results pertaining to the use of the various medium supplements showed that maximal transactivation was reached in the presence of fetal calf serum, whilst when no fetal calf serum was used the levels of transactivation were lower. It was however noted from the results that pSV-β-galactosidase was sensitive to the presence of estrogenic compounds, Figure B.3. With this in mind it was decided to repeat the experiment using either Neogal or pCMV-β-galactosidase as β-galactosidase reporter genes.
B.2 Optimisation of transactivation

Figure B.2: Optimization of transactivation studies in COS-1 cells.
COS-1 cells were seeded and transfected as described in Appendix C.4. The medium was changed to DMEM with or without phenol red containing various supplements (fetal calf serum, stripped fetal calf serum and no fetal calf serum) either 12 or 24 hours after transfection. After a further 24 hours either $1 \times 10^{-5} \text{M} \text{E}_2$ or ethanol was added to the medium and the cells incubated for a further 24 hours. The samples were assayed for the presence of $\beta$-galactosidase and luciferase as described in Appendix C.4. Control 1: Transfection with ERE, ER-\(\alpha\) and ethanol used as test compounds. Control 2: Transfection with ERE, $\beta$-galactosidase reporter and ethanol used as test compounds. Control 3: No transfection and ethanol used as test compounds. Results are shown as the average with standard errors for triplicate samples.
Figure B.3: Optimization of transactivation studies in COS-1 cells, \(\beta\)-galactosidase values. COS-1 cells were seeded and transfected as described in Appendix C.4. The medium was changed to DMEM with or without phenol red containing various supplements (fetal calf serum, stripped fetal calf serum and no fetal calf serum) either 12 or 24 hours after transfection. After a further 24 hours either \(1 \times 10^{-5}\)M E\(_2\) or ethanol was added to the medium and the cells incubated for a further 24 hours. The samples were assayed for the presence of \(\beta\)-galactosidase as described in Appendix C.4. Control 1: Transfection with ERE, ER-\(\alpha\) and ethanol used as test compounds. Control 2: Transfection with ERE, \(\beta\)-galactosidase reporter and ethanol used as test compounds. Control 3: No transfection and ethanol used as test compounds. Results are shown as the average with standard errors for triplicate samples.
B.2 Optimisation of transactivation

The aim of the following experiments was to determine the level of transactivation of the ERE.tk-luc reporter gene, via ligand bound ER-α. The secondary aim was to determine whether pCMV-β-galactosidase or Neogal could be used as β-galactosidase reporters. The experimental procedures used were the same as those reported above, the only difference being the use of different β-galactosidase reporters. The results of these experiments where pCMV-β-galactosidase was used as a β-galactosidase reporter, are shown in Figures B.4 and B.5. The results showed that maximal levels of transactivation, 250 relative light units, were obtained in the presence of DMEM containing using fetal calf serum as a supplement. The minimum level of transactivation, 125 relative light units, was obtained when DMEM containing phenol red and no fetal calf serum was used. With respect to the use of pCMV-β-galactosidase it was noted that there were higher levels of expression in samples that were not transfected with a β-galactosidase reporter than samples that were transfected, Figure B.5. As a result of this not much can be said about the influence of estrogenic compounds on the pCMV-β-galactosidase reporter gene. With respect to the results, Figure B.6, relating to the samples which were transfected with the Neogal reporter gene the following can be said. It was noted that there was receptor independent transactivation. This was noticed when comparing the samples that were transfected with ER-α as compared to those that were not transfected with ER-α. Once again there was a problem with the β-galactosidase values in that higher levels of expression were being reported in the absence of the Neogal reporter than when Neogal was present.
B.2 Optimisation of transactivation

Figure B.4: Optimisation of transactivation using the pCMV-β-galactosidase reporter gene (Luciferase results). COS-1 cells were seeded and transfected with a total of 34.45ng DNA (ratios used were those of Stoica Table C.3) as described in Appendix C.4. After 24 hours the medium was changed to either DMEM containing or not containing phenol red and various supplements and 24 hours later $1 \times 10^{-5}$M E$_2$ or ethanol was added. The cells were lysed and the samples assayed for the presence of luciferase. The type of medium and supplements which the cells were exposed to after transfection were as follows: a) COS-1 cells were grown in DMEM containing phenol red and 10% fetal calf serum, b) COS-1 cells were grown in DMEM containing no phenol red and 10% fetal calf serum, c) COS-1 cells were grown in medium containing phenol red and 10% stripped fetal calf serum, d) COS-1 cells were grown in DMEM containing no phenol red and 10% fetal calf serum. After this the cells were incubated for a further 24 hours after which the medium was removed and the cells lysed. The samples were then frozen overnight after which they were analyzed for the presence of luciferase. Control 1: Transfection with ERE, ER-α and ethanol used as test compounds. Control 2: Transfection with ERE, β-galactosidase reporter and ethanol used as test compounds. Control 3: No transfection and ethanol used as test compounds. Results are shown as the average with standard errors for triplicate samples.
B.2 Optimisation of transactivation

Figure B.5: Optimisation of transactivation using the pCMV-β-galactosidase reporter gene (β-galactosidase results). The same methodology was used as described in Figure B.4, only the β-galactosidase results are shown. Results are shown as the average with standard errors for triplicate samples.
B.2 Optimisation of transactivation

Figure B.6: Optimisation of transactivation using the Neogal \(\beta\)-galactosidase reporter gene (Luciferase results). COS-1 cells were transfected and treated as described in Figure B.5. Neogal was used as a \(\beta\)-galactosidase reporter gene instead of pCMV-\(\beta\)-galactosidase. Results are shown as the average with standard errors for triplicate samples.
B.2 Optimisation of transactivation

In an attempt to determine whether the ligand independent transactivation of the ERE.tk-luc reporter was due to the amount of reporter gene or ER-α expressed it was decided to determine whether using a higher amount of DNA in the transfection process made a difference. The use of DMSO as a solvent was also studied. The results of these experiments are shown in Figure B.7. From these results it was concluded that the time after transfection that the medium was changed did not make a difference and it was decided in future the medium would be changed 24 hours after transfection. With respect to the use of DMSO as a solvent it was found that this did not have an influence as there was still ligand independent transactivation as can be seen when comparing samples that were exposed to E₂ to those exposed to only ethanol.
Figure B.7: Optimisation of amount of DNA to use in transactivation experiments. COS-1 cells were seeded at $7\times10^4$ cells per well and transfected using the DEAE-Dextran transfection technique as described in Appendix C.4. Modifications to the transfection protocol included varying the amount of DNA used, between 150ng and 850ng (ratios used were those of Stoica, Table C.3). The growth medium was changed to DMEM containing no phenol red and 10% stripped fetal calf serum either 6, 12 or 24 hours after transfection. After a further 24 hours the cells were exposed to $1\times10^{-5}$M $E_2$ or DMSO (as a control) after which the samples assayed for the presence of $\beta$-galactosidase and luciferase.
Appendix C

Methods

C.1 Plasmid isolation

C.1.1 Preparation of electro-competent cells

*Escherichia coli* (*E. coli*) cells (JM109) plated on Luria broth (LB) agar plates were incubated overnight at 37°C. A single colony was then picked and incubated in 50ml SOB medium overnight at 37°C shaking at 180rpm. Of the overnight culture, 5ml was added to 500ml of SOB and the resultant culture was incubated at 37° shaking at 180rpm until a cell density with an OD$_{550}$ of 0.8 was reached. These cultures were subsequently transferred to chilled A10 centrifuge bottles and concentrated by centrifugation at 3500rpm for 15 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 200ml of MQ-H$_2$O and centrifuged at 3500rpm, 4°C for 15 minutes. This step was repeated twice after which the cells were washed twice with an ice-cold 10% glycerol solution using the same procedure, resuspended in 4ml of 100% glycerol solution and frozen at -80°C.

C.1.2 Transformation of electro-competent cells

Bacterial cells were transformed using electroporation as previously described [199, 200, 201]. Electro-competent *E. coli* cells were diluted 1:1 with 10%
C.2 Tissue culture

glycerol and 40µl of the prepared solution was added to a chilled eppindorf tube (1.5ml). A total of between 10 and 20ng of DNA was added to the cells and the solution was placed in a electroporation cuvette (model 610,TX). The cuvette was then placed into the gene transformer and a quick pulse was applied to the sample, after which the sample was transferred to 1ml SOC and incubated at 37°C shaking at 225rpm for 1 hour. Cultures were then streaked out on LB-Ampicillin (LB-AMP) plates and incubated overnight at 37°C.

C.1.3 Extraction of plasmid DNA

Concert Maxi Prep

The method followed for this protocol is the same as the Concert®High Purity plasmid Purification systems manual supplied by Life Technologies®.

C.2 Tissue culture

C.2.1 Maintenance of COS-1 cells

COS-1 cells were maintained in DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin. COS-1 cells, grown at 37°C, 90% humidity and 5% CO₂, were split twice a week. To split cells the medium was aspirated and 1ml trypsin was added. After two minutes the flask was lightly tapped to loosen the cells. The suspended cells were then transferred to DMEM (no fetal calf serum) and centrifuged for 5 minutes at 3000g to remove the trypsin. The cells were subsequently suspended in DMEM plus 10% fetal calf serum and again plated in 75cm² tissue culture flasks.

C.2.2 Maintenance of Hep89 cells

Hep89 cells were maintained in DMEM containing 10% fetal calf serum, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate. The cells were
grown at 37°C, 90% humidity and 5% CO₂. Cells were split as described in the previous section. The cells were subsequently resuspended in DMEM containing 10% fetal calf serum, 0.1mM non-essential amino acids and 1.0mM sodium pyruvate and again plated in 75cm² tissue culture flasks.

C.3 Optimisation of transfection

C.3.1 Optimisation of transfection using calcium phosphate as transfection method

The transfection method used in this study was based on a standard calcium phosphate transfection protocol [198].

The protocol was, however, optimised for this study by changing the following conditions: number of cells plated (1×10⁵, 1.5×10⁵, 2×10⁵ cells per well in a 24 well plate), amount of DNA (1.73µg and 2.41µg of DNA per well) and finally the use of a glycerol shock.

The general transfection protocol for 1×10⁵ cells per well and 1.73µg total DNA follows, Table C.2.

COS-1 cells plated in 24 well (Nunc) plates, at a density of 1×10⁵ cells per well, and grown for 24 hours in DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin. Four hours before transfection the medium was replaced with DMEM containing 1% Penicillin-Streptomycin. The following transfection solutions were prepared, Table C.1.

Solution A was slowly added, drop wise, to solution B and the resulting mixture left at room temperature for 45 minutes until a fine precipitate had developed. A total of 34µl of transfection solution was then added to each well of a 24 well plate and the cells incubated for 16 hours. The medium
C.3 Optimisation of transfection

was then aspirated and the cells washed twice with 1ml PBS (0.137M NaCl, 0.0027M KCl, 0.0043M Na$_2$HPO$_4$, 0.00147M KH$_2$PO$_4$, pH 7.0) and if required exposed to a glycerol shock (10% glycerol in PBS), followed by incubation for a further 24 hours in complete medium.

Table C.1: Preparation of calcium phosphate transfection solutions.

<table>
<thead>
<tr>
<th>Solution A</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ (2.5M)</td>
<td>1.7µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1.73µg</td>
</tr>
<tr>
<td>MQ-H$_2$O</td>
<td>(make final volume up to 17µl)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2× HEPES buffered saline</td>
<td>17µl</td>
</tr>
<tr>
<td>100× phosphate buffer</td>
<td>0.34µl</td>
</tr>
<tr>
<td>(70mM Na$_2$HPO$_4$, 70mM NaH$_2$PO$_4$)</td>
<td></td>
</tr>
<tr>
<td>MQ-H$_2$O</td>
<td>(make final volume up to 17µl)</td>
</tr>
</tbody>
</table>

Table C.2: Composition of the DNA solution used in optimisation of calcium phosphate transfection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-galactosidase</td>
<td>0.12</td>
<td>0</td>
</tr>
<tr>
<td>pGL2-Basic</td>
<td>1.61</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1.73</td>
<td>0</td>
</tr>
</tbody>
</table>
C.3 Optimisation of transfection

Table C.3: Variation of the composition of DNA content during transactivation experiments.

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td></td>
</tr>
<tr>
<td>ER-α</td>
<td>59%</td>
</tr>
<tr>
<td>ERE</td>
<td>34%</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>7%</td>
</tr>
<tr>
<td>Stoica [202]</td>
<td></td>
</tr>
<tr>
<td>ER-α</td>
<td>12.49%</td>
</tr>
<tr>
<td>ERE</td>
<td>62.42%</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>4.62%</td>
</tr>
<tr>
<td>pGL2-basic</td>
<td>20.47%</td>
</tr>
<tr>
<td>Green [203]</td>
<td></td>
</tr>
<tr>
<td>ER-α</td>
<td>15%</td>
</tr>
<tr>
<td>ERE</td>
<td>49.71%</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>34.68%</td>
</tr>
</tbody>
</table>

Table C.4: Various controls that were included in transactivation experiments.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>ER-α</th>
<th>ERE.tk-luc</th>
<th>β-galactosidase</th>
<th>β-galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control 2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EtOH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
C.3 Optimisation of transfection

C.3.2 Optimisation of transfection using DEAE-Dextran as transfection method

The transfection method used in this study was based on a standard DEAE-Dextran transfection protocol [182]. This protocol was, however, optimised for this study by changing the following conditions: Number of COS-1 cells plated, amount of DNA and DEAE-Dextran, Table C.5.

Table C.5: Conditions for optimisation of DEAE-Dextran transfection.

<table>
<thead>
<tr>
<th>DNA</th>
<th>0.34, 150 or 300ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Dextran</td>
<td>3.45, 6.91 or 13.82µg/ml</td>
</tr>
<tr>
<td>Cell number</td>
<td>5×10^4, 7×10^4 or 1×10^5 cells per well in a 24 well plate</td>
</tr>
</tbody>
</table>

Transfection in 24 well plates

COS-1 cells were plated in 24 well plates, at a varying densities per well, and grown for 24 hours in DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin. The transfection solution was prepared by adding chloroquine (final concentration 100µM) to DMEM without fetal calf serum, after which the DNA (final amount between 34.45 and 150ng/µl per 250µl transfection solution) was added and finally DEAE-Dextran (final concentration 13.82µg/ml) was added to the solution. The transfection solution was mixed by vortexing, after which 250µl was placed on the cells in each well (of which the medium had been removed) and the cells incubated for 1 hour at 37°C, 90% humidity and 5% CO₂. The transfection solution was then removed and DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin was added after which the cells were then incubated for a further 24 hours.
Transfection in 10cm$^3$ plates

COS-1 cells were plated at a density of $2 \times 10^6$ cells per plate and incubated for 24 hours. COS-1 cells were transfected using the DEAE-Dextran transfection method as described above. The protocol was, however, modified by increasing amount of DNA to 4.34µg per 7.14ml of transfection solution, which was used per 10cm$^3$ plate.

C.4 Optimisation of transactivation

COS-1 cells were plated in 24 well plates (7$\times$10$^4$ cells per well) or 10cm$^3$ plates (2$\times$10$^6$ cells per plate) in DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin and incubated for 24 hours at 37°C, 90% humidity and 5% CO$_2$. Cells were then transfected using either the calcium phosphate, section C.3.1 or DEAE-Dextran, section C.3.2, transfection method and grown for a further 24 hours.

When COS-1 cells were plated in a 24 well plate then the medium was changed to DMEM without phenol red and grown for a further 24 hours at 37°C, 90% humidity and 5% CO$_2$. The growth medium was then changed to DMEM containing no phenol red with stripped fetal calf serum after which varying concentration of E$_2$ (between $1 \times 10^{-5}$ and $1 \times 10^{-12}$M or EtOH (final amount of EtOH 0.1% in both scenarios) was added. After 24 hours the medium was removed and the cells washed three times with 1ml PBS (pH 7.0) after which the PBS was removed and 50µl Galactostar lysis buffer was added and the samples frozen overnight at -20°C. If COS-1 cells were plated in 10cm$^3$ plates then the cells were replated at a density of 7$\times$10$^4$ cells per well in a 24 well plate and incubated for 24 hours at 37°C, 90% humidity and 5% CO$_2$. The protocol was the same from here on as that of the cells that were plated at 7$\times$10$^4$ cells per well.
C.4.1 Conditions for optimisation

There were a number of conditions that were considered when trying to optimize the experimental conditions for transactivation experiments, they are as follows:

The relative composition of the DNA was altered, Table C.3, type of medium (DMEM either containing phenol red or without phenol red), supplements (fetal calf serum, stripped fetal calf serum (prepared according to the protocol of Hibbert [204]), no fetal calf serum), type of ERE reporter gene used (ERE.tk-Luc vs ERE-vit), and finally the times at which medium was changed during the experiment.

C.4.2 Optimisation of β-galactosidase reporter

To analyse the transactivation results the luciferase values had to be normalized using β-galactosidase. The use of various β-galactosidase reporter genes were examined, as well as their sensitivity to the presence of estrogenic compounds. The method was the same as above, except that the cells would be transfected with only a β-galactosidase reporter (Neogal, pSV-β-galactosidase or pCMV-β-galactosidase, no other DNA was used).

C.5 Whole cell binding of 17-β-estradiol to the human ER-α

C.5.1 General method

C.5.2 COS-1 cells

COS-1 cells were plated in 10cm$^3$ plates at a density of 2×10$^6$ cells/plate in DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin. Cells were grown for 24 hours at 37°C, 90% humidity and 5% CO$_2$, after
which they were transfected using the DEAE-Dextran transfection method as described in section C.3.2. The total amount of DNA used during transfection was 4.34µg per plate. After transfection the cells were grown for 24 hours at 37°C, 90% humidity and 5% CO₂. COS-1 cells were then replated at a density of 1×10⁵ cells per well in 12 well plates and grown for 24 hours after which the medium was changed to DMEM (without phenol red) and 1% Penicillin-Streptomycin.

**Competitive binding assay**

After a further 24 hours growth the medium was removed and replaced with 1ml DMEM containing no phenol red and 0.5nM ³H-E₂ (2,4,6,7 ³H-17-β-estradiol, SA 83Ci/mmol supplied by AEC-Amersham cat. No TRK 322) and increasing amounts of unlabelled E₂ (final concentration between 1×10⁻⁵ and 1×10⁻¹³, Sigma Cat. No. E-2758) made up in EtOH (final concentration 0.01%). Following a 10 hour incubation, at 37°C, 5% CO₂ and 90% humidity, the medium was removed and the cells washed three times with ice-cold PBS-(0.2%) BSA, at 4°C for 15 minutes per wash, after each wash the PBS was removed. After the wash step had been finished 100µl of lysis buffer was added to each well and the samples frozen overnight at -20°C. The samples were then thawed and scraped after which 80µl of the sample was added to 3ml scintillation fluid (Zinsser analytic quick safe flow 2 supplied by Bioteknik cat. no. 1004000) and the remaining 20µl was kept for protein determinations, using the Bradford technique. The wells were then washed out with a further 100µl of lysis buffer, which was then also added to the scintillation fluid. The samples were then counted in a Beckman Scintillation Counter, which was set up to detect the presence of tritium.

\[^{1}0.5208\mu g \text{ER-}\alpha, \ 0.102\mu g \text{ERE-vit,} \ 2.34\mu g \text{pGL2-Basic and} \ 0.17\mu g \text{pCMV-}\beta-\text{galactosidase}\]
C.6 Human pregnancy plasma

C.5.3 Hep89 cells

Competitive binding assay

The protocol used for competitive binding assays in Hep89 cells was based on the protocol used for COS-1 cells, however the cells were not transfected as they had been stably transfected with ER-α and 1nM of $^3$H-E$_2$ was used.

Saturation binding assay

The same method was followed as for the Hep89 competitive binding assay except that cells were incubated in the presence of varying amounts of $^3$H-E$_2$ in the presence/absence of $1\times 10^{-5}$M unlabelled E$_2$. After a further 24 hours of growth the medium was removed and replaced with medium that contained varying amounts of $^3$H-E$_2$ (between 0.248 and 75nM in the presence/absence of $1\times 10^{-5}$M E$_2$. Cells were then incubated for a further 10 hours, washed, lysed and frozen. The rest of the method follows as above.

C.6 Human pregnancy plasma

C.6.1 Optimisation of system

The method used in this study was based on that of Hammond [181]. The protocol was, however, optimised by changing the following conditions: In the protocol no excess of testosterone was used. Human pregnancy plasma stored at -80°C until used.

Human pregnancy plasma was stripped of endogenous steroids by incubating 20µl in the presence of 1980µl of dextran coated charcoal (DCC) for 30 minutes at room temperature on the “the belly dancer” at a moderate speed (supplied by Tovall life sciences, SA), after which it was concentrated by centrifuging at 13000g for 10 minutes at room temperature and the supernatant removed for use in experiments.
Total binding was determined by adding 100µl supernatant (stripped human pregnancy plasma diluted 1:100 using 0.01M PBS), 100µl 0.01M PBS containing the required amount of $^3$H-E$_2$ and 0.1% EtOH, diluted in 100µl PBS, together in an 2ml eppi, the final volume being 300µl.

Non specific binding was determined in the same way except that $1 \times 10^{-5}$M E$_2$, diluted in EtOH (0.1% final volume), was added.

Samples, defined as the plasma steroid solution in an eppindorf, were then vortexed and incubated on a “belly dancer” for 10 hours at room temperature. The samples were then placed in an ice/EtOH sludge (0°C) for 15 minutes. A total of 750µl of ice cold DCC was then added to each sample with a repette, as this had to be done in less than a minute the samples were handled in batches of 12 samples per batch. After addition of DCC, the samples were incubated for a further 10 minutes at 0°C. The DCC separated from the supernatant by spinning down the samples at 13000g, 0°C for 15 minutes. A total of 850µl of the supernatant was removed and added to 3ml scintillation fluid and assayed for the presence of $^3$H-E$_2$in a Beckman Scintillation counter.

Controls used in the experiment were as follows:

- No SHBG
- No SHBG + DCC (control for DCC stripping)
- SHBG + EtOH (control for E$_2$)

### C.6.2 Competitive binding assay

The method used for these experiments was the same as above, however the samples were exposed to either E$_2$, $1 \times 10^{-5}$ to $1 \times 10^{-12}$ or EtOH and a further control, the absence of $^3$H-E$_2$, was included.
C.6.3 Saturation binding assay

Optimisation

The method used was based on that described above, however, certain modifications to the protocol were performed as described below. The amount of SHBG in the samples was varied by changing the dilution factor between 100, 50 and 25× in the initial dilution step, and the amount of DCC added was changed according to the dilution (final volume remained constant) thus is the serum was diluted 25× then the amount of DCC was increased by a factor of four. The protocol used in these experiments was based on the method used in the competitive binding assay using HPP, however, certain modifications were made: The samples were incubated, at room temperature, in the presence of varying amounts of $^3$H-E$_2$ (between 0.25nM 30nM) and presence/absence of unlabelled E$_2$ (1×10$^{-5}$M).

C.7 Thin layer chromatography

C.7.1 COS-1 cells

COS-1 cells were plated in 10cm$^2$ plates at a density of 2×10$^6$ cells per plate in DMEM containing 10% fetal calf serum, 1% Penicillin-Streptomycin and grown for two days at 37°C, 90% humidity and 5% CO$_2$, then medium being changed to DMEM without phenol red containing 1% Pen-strep on the second day.

C.7.2 Hep89 cells

Hep89 cells were plated in 10cm$^2$ plates at a density of 2×10$^6$ cells per plate in DMEM containing 10% fetal calf serum, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate and grown for two days at 37°C, 90% humidity and 5% CO$_2$, then medium being changed to DMEM without phenol red containing 10% fetal calf serum, on the second day.
C.7 Thin layer chromatography

C.7.3 Addition of test compounds

On the day of the experiment the medium was changed to DMEM without phenol red and a total of 9µl of 10^{-5} M E_2 and 1µl of ^3H-17-β-estradiol was added to 8ml of medium, which was then added to the cells. The cells were then incubated for a total of 10 hours at 37°C, 90% humidity and 5% CO₂ with samples of 1ml being taken each hour.

C.7.4 Separation of of steroids from medium

A total of 1ml of the cellular medium was added to 5ml of CH₂Cl₂, which was placed at 4°C the night before, after which 1µl of a 10 times dilution of ^3H-dexamethasone was added and the samples vortexed for 2 minutes. After vortexing the supernatant was removed and added to a 5ml glass tube and the samples dried under nitrogen flow.

C.7.5 Separation of steroids by thin layer chromatography

Thin layer chromatography (TLC) plates were supplied by Merck (Cat. no. 1.05554). Steroid preparation of ^3H-17-β-estradiol and ^3H-dexamethasone (1µl of each labelled steroid was added to the samples) prepared in 100µl absolute EtOH as well as the samples were spotted onto various lanes, separated by 1cm, on the TLC plates.

The TLC plate was then placed in a chamber which contained 70% chloroform and 30% Acetone, pre-equilibrated for 1 hour, and run for one and a half hours. After this the plates were removed and allowed to dry in the air. The steroids were then cut from the plate in 1cm sections and placed in 3ml scintillation fluid after which they were left overnight in a cupboard. The samples were then counted with a Beckman scintillation counter for 10 minutes on a program used to detect tritium.
C.8 Determination of amount of SHBG in Hep89 cells

The results were analysed by correcting for counting efficiency, this was done by comparing the amount of $^3$H-dexamethasone in the sample to the amount initially added. The ratio of final over initial amount of $^3$H-dexamethasone was used to correct the samples for extraction efficiency of the steroids from the medium.

C.8 Determination of amount of SHBG in Hep89 cells

Hep89 cells were plated in 10cm$^3$ plates at a density of $2\times10^6$ cells per plate in DMEM containing 10% fetal calf serum, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate and grown for two days at 37°C, 90% humidity and 5% CO$_2$, then medium being changed to DMEM without phenol red containing 10% fetal calf serum, on the second day.

On the third day the medium was removed and the samples were exposed to DMEM ± E$_2$ ($10^{-5}$M) for a period of 24 hours. The medium was then removed and stored at -20°C after which the cells were lysed by adding 1ml lysis solution and freezing overnight. The lysate (intracellular SHBG) and the medium (extracellular SHBG) was then sent off for testing at the University of Cape Towns chemical pathology unit, located in Groote Schuur. The samples were analysed using a SHBG immunoradiometric assay (IRMSA) supplied by Orion diagnostics (Cat. No. 68563) for the presence of SHBG.

C.9 Determination of cell volumes

COS-1 cells were seeded at $2\times10^6$ cells per well in 10cm$^3$ plates and grown for 4 days in DMEM containing 10% fetal calf serum and 1% Penicillin-Streptomycin as described in Appendix C.2.1. The cells were then removed from the plate as described in Appendix C.2.1 and the extracellular volume determined. The intracellular volume was determined by drawing up a small
C.9 Determination of cell volumes

sample, of the lysed cells, into a capillary tube. The samples were then spun for 5 minutes in a haematocrit centrifuge and the ratio of cells to medium determined.
Appendix D

Materials

D.1 Plasmids

The ERE.tk-Luc reporter promoter construct was a kind gift from Dr D.C. Harnish (Womens Health Research Institute, Wyeth-Ayerst Research, Radnor, PA USA). It contains two copies of the vitellogenin ERE element upstream of a tk promoter and is cloned into the pGL2-basic vector (Promega).

The ERE-vitellogenin reporter promoter construct plasmid contains one copy of the vitellogenin gene in the pGL2-TATA-lnr plasmid, and was a kind gift from Dr. K. Korach (Receptor Biology Section, National Institute of Environmental Health Sciences, Durham, North Carolina).

The pcDNA3-ER-α was a kind gift from Dr. D.C. Harnish. It contains the cDNA of the human ER-α, which is constitutively expressed, sub-cloned into the pcDNA3 vector (Invitrogen, San Diego, CA).

The pSV-β-galactosidase was bought from Promega (Cat. no. E1081). It is used as a positive control for transfection and constitutively expresses the β-galactosidase gene.
The pCMV-β-galactosidase reporter gene was obtained from Professor J. Hapgood. The plasmid is a mammalian reporter vector which expresses the β-galactosidase gene.

The pGL2-Basic vector was purchased from Promega (Cat. no. E1641). The vector lacks any eukaryotic promoter and enhancer sequences and contains a luc gene, expression of which is dependent on the insertion of a promoter in the correct orientation.

D.2 Chemicals

### Table D.1: Chemicals and reagents

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### D.2 Chemicals

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Bibliography


**BIBLIOGRAPHY**


male sexual behaviours in mice lacking estrogen receptor alpha and beta (alpha beta ERKO). *Proceedings of the National Academy of Science USA* 97, 14737–14741.


BIBLIOGRAPHY


The putative cofactor TIF1alpha is a protein kinase that is hyperphosphorylated upon interaction with liganded nuclear receptors. *Journal of Biological Chemistry* 273, 16199–16204.


adenosine monophosphate and insulin-like growth factor 1. *Molecular Endocrinology* 7, 743–752.


BIBLIOGRAPHY


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