

**CONSTRUCTION AND ANALYSIS OF A
KINETIC MODEL OF THE OVINE ADRENAL
STEROIDOGENIC SUBSYSTEM
COMPRISING OF CYP17 AND 3 β -HSD**

By

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Declaration

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Abstract

This study describes:

- how time-dependent experimental data, describing the reactions catalyzed by ovine cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17), was obtained using pregnenolone and progesterone as substrates
- the construction and validation of a kinetic model and determining the K_m and V_{max} - values for each reaction catalyzed by ovine CYP17
- how the time-dependent metabolism of pregnenolone, 17 α -hydroxypregnenolone and dehydroepiandrosterone by ovine 3 β -hydroxysteroid dehydrogenase (3 β -HSD), was obtained
- the construction and validation of a kinetic model and determining the K_m and V_{max} - values for each reaction catalyzed by ovine 3 β -HSD

Opsomming

Hierdie studie beskryf:

- hoe tyd-afhanklike eksperimentele data van die reaksies wat gekataliseer word deur skaap sitochroom P450 17 α -hidroksielase/17,20-liase (CYP17) met pregnenoloon en progesteron as substraat, verkry is
- die konstruksie en validasie van die kinetiese model asook die bepaling van die K_m - en V_{max} - waardes vir elke reaksie wat deur skaap CYP17 gekataliseer word.
- hoe die tyd-afhanklike metabolisme van pregnenoloon, progesteron en dehidroepiandrosteron wat gekataliseer word deur skaap 3 β -hidroksiesteroïd dehidrogenase (3 β -HSD), verkry is
- die konstruksie en validasie van die kinetiese model asook die bepaling van die K_m - en V_{max} - waardes vir elke reaksie wat deur skaap 3 β -HSD gekataliseer word

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Alphabetical List of Abbreviations

11 β HSD 11 β -hydroxysteroid dehydrogenase
DOC 11 Deoxycorticosterone
20 α -HSD 20 α -hydroxysteroid dehydrogenase
16-OHPROG 16-hydroxyprogesterone
17 β -HSD 17 β -hydroxysteroid dehydrogenase
17-OHPROG 17-hydroxyprogesterone
17-OHPREG 17-hydroxypregnenolone
3 α -HSD 3 α -hydroxysteroid dehydrogenase
3 β -HSD 3 β -hydroxysteroid dehydrogenase
3 β -HSD I 3 β -hydroxysteroid dehydrogenase type 1
3 β -HSD II 3 β -hydroxysteroid dehydrogenase type 2
3 β -HSD IV 3 β -hydroxysteroid dehydrogenase type 4
3 β -HSD VI 3 β -hydroxysteroid dehydrogenase type 6
AII Angiotensin II
A4 Androstenedione
ACAT Acyl-coenzyme A: cholesterol acyltransferase
ACTH Cyclic adenosine monophosphate
Ad^o Adrenodoxin
Ad^f Adrenodoxin reductase
AKR Aldo-keto reductase
ALD Adrenoleukodystrophy
AMP Adenosine monophosphate
ANP Atrial natriuretic peptide
ANS Autonomic Nervous System
ATPase Adenosine triphosphatase
CAH Congenital adrenal hyperplasia
cAMP cyclic Adenosine monophosphate
CD36 Cluster of Differentiation
CLA-1 CD36 and LIMPII Analogous-1
CNS Central Nervous System
CREM cAMP response element modulator

CRH Corticotrophin releasing hormone
CYP Cytochrome P450
CYP11A Cytochrome P450 11 α -hydroxylase
CYP11A1 Cholesterol side chain cleavage
CYP11B Cytochrome P450 11 β -hydroxylase
CYP11B1 Cytochrome P450 11 β -hydroxylase
CYP11B2 Aldosterone synthase
CYP17 Cytochrome P450 17 α -hydroxylase/17,20-lyase
CYP19 Cytochrome P450 19-hydroxylase
CYP21 Cytochrome P450 21-hydroxylase
DEVS Discrete-event system specification
DHEA Dehydroepiandrosterone
DMEM Dulbecco's modified Eagle's medium
DNA Deoxyribonucleic acid
DNA-PK DNA-dependent protein kinase
EAC Endocrine Active Compounds
ER Endoplasmic reticulum
FAD Flavinadenine dinucleotide
FBA Flux balance analysis
FMN Flavinmononucleotide
FSH Follicle-stimulating hormone
GH Growth hormone
GHIH Growth hormone-inhibiting hormone
GHRH Growth hormone-inhibiting hormone
GnRH Gonadotropin-releasing hormone
H295 cells Human adrenocortical tumor cell lines
HDL High-density lipoprotein
HMG-CoA 3-hydroxy-3-methylglutaryl coenzyme A
HPA axis Hypothalamic-pituitary-adrenal axis
HPLC High performance liquid chromatography
HSD Hydroxysteroid Dehydrogenase
hsp Heat shock protein
IGFs Insulin-like growth factors
IGF-I Insulin-like growth factor I

IMM Inner mitochondrial membrane
JAK/STAT Janus kinase/signal transducers and activators of transcription
JEG-3 cells Human choriocarcinoma cell lines
LDL Low-density lipoprotein
LH Luteinizing hormone
LIMP2 Lysosome membrane protein 2
MAP Mitogen-activated protein
MCA Metabolic control analysis
MCP Methyl-accepting chemotaxis protein
MDM2 Mouse double minute 2 homolog
mRNA messenger Ribonucleic acid
MSH Melanocyte-stimulating hormone
NADH Nicotinamide adenine dinucleotide
NADPH Nicotinamide adenine dinucleotide phosphate
ODE Ordinary differential equations
OMM Outer mitochondrial membrane
PROG Progesterone
PREG Pregnenolone
PIH Prolactin-inhibiting hormone
PKA cAMP-dependent protein kinase
POMC Pro-opiomelanocortin
POR P450 oxidoreductase
PRL Prolactin
RNA Ribonucleic acid
RNAi RNA interference
RNAP RNA polymerase
S1P Sphingosine-1-phosphate
SC-B1 scavenger receptor B1
SDR Short-chain alcohol dehydrogenase reductase
SF-1 Steroidogenic factor-1
SPH Sphingosine
SREBP Sterol response element binding protein
SREBP1c Sterol regulatory element-binding protein 1c
StAR Steroid acute regulatory protein

START StAR-related lipid transfer

SW13 Adrenal cells

TF transfer functions

TGF β Transforming growth factor- β

TIM Triosephosphate isomerase

TPA Tetradecanoyphorbol 13-acetate

TRH Thyrotropin-releasing hormone

TSH Thyrotropin

TSPO Peripheral benzodiazepine receptor

UPLC Ultra performance liquid chromatography

UPLC-APCI-MS UPLC-Atmospheric pressure chemical ionization-masspectometry

UPLC-MS UPLC-Masspectometry

ZPA Zone of Polarizing Activity

Chapter 1

Introduction

Conley and Bird (1997) suggested that cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) levels influence the efficiency of the rate of steroid synthesis in steroidogenic organs by imposing certain constraints. These constraints imply that the levels of 3 β -HSD, and tissue-specific distribution relative to CYP17, play an important role in the balance between androgen and estrogen synthesis. They also stated that understanding the regulatory points that have important influences on the flux of substrates converted by CYP17 and 3 β -HSD, may help us predict the consequences of increasing or decreasing levels of these enzymes or their activities. One hypothesised regulatory point is the junction at the differential metabolism of pregnenolone which can be catalyzed by CYP17 and 3 β -HSD in competing reactions (Fevold 1978). The knowledge of these regulatory points can potentially aid in the development of therapeutic approached treatments of steroidogenic endocrinological diseases (Conley and Bird 1997). In addition, Breen (2007) showed that a computational model could successfully predict biochemical responses of ovarian steroidogenesis to endocrine active compounds (EAC) and that its capabilities could aid the pharmaceutical development and environmental health assessments with EAC. This opens the door for Systems Biology to be used as a tool for the investigation of systems such as steroidogenesis.



An example of deficient steroidogenic activity can be found in the South African Angora goat (*Capra hircus*), the most efficient fibre producing small stock breed in South Africa. A significant problem facing Angora farmers in South Africa is the loss of newly shorn Angora goats during cold spells (Storbeck 2008a). While investigating the steroidogenic enzyme activity, Engelbrecht and Swart (2000) identified the enzymes, CYP17 and 3 β -HSD, as potential contributors to hypocortisolism in the Angora goat. It was found that CYP17 contributes to hypocortisolism by shifting the flux towards the Δ^5 -pathway (Engelbrecht & Swart 2000; Engelbrecht 2000). In subsequent studies, two isoforms of Angora CYP17, ACS- and ACS+, were cloned and expressed by Storbeck et al (2007). It was shown that increased 17,20 lyase activity towards 17OHPREG, by the CYP17 ACS- isoform was the cause of the

increased CYP17 lyase activity (Storbeck 2007). Storbeck (2007) identified an allele of the CYP17 enzyme that has an absence of the ACS1 restriction site at position 634-640 in the Angora goat, and thus named it ACS-. The presence of this allele was shown to have an increased effect on the 17,20 lyase activity towards 17OHPREG. Goosen (2010) subsequently showed that CYP17 was not the only contributor towards hypocortisolism in the Angora goat, but that 3 β -HSD also contributes to this disease. By comparing Angora 3 β -HSD to ovine 3 β -HSD, which is considered a hardy species, he showed that, in the presence of CYP17 ACS-, 17OHPREG production (a cortisol precursor) was significantly higher in ovine compared to production in Angora. However, the kinetic influence that CYP17 and 3 β -HSD has on each other to cause the shift in flux in the subsystem comprising of these two enzymes has not been addressed.

In this study the two fields of enzymology and systems biology will be combined in an attempt to answer the latter question. This will be done by constructing a computational model that can be used to predict the influence that CYP17 and 3 β -HSD has on the flux of steroids production in the subsystem comprising of these two enzymes.

In Chapter 2 an overview of adrenal steroidogenesis is given, starting from the HPA axis to steroid production as well as the enzymes involved in steroidogenesis. A detailed focus on enzymes CYP17 and 3 β -HSD will be discussed in Chapter 3. An overview of systems biology, followed by computational modeling, enzyme kinetics, and analysis thereof in steroidogenesis specifically, will be discussed in Chapter 4.

Following the literature reviews in Chapters 2 to 4, the experimental procedures will be described, followed by the results and discussion thereof in Chapter 5.

In Chapter 6 a general discussion of the thesis will be given.

Chapter 2

Adrenal Steroidogenesis

2.1. Introduction to steroids

Mammalian steroids are hormones that are derived from cholesterol as a common precursor within the adrenal gland, gonads (Marieb 2003) and placenta during pregnancy. Steroid hormones are not stored within these cells, but rather produced on demand from stimuli (Marieb 2003). Thus the regulation of steroid hormones depends on the activity of the steroidogenic enzymes in accordance to the availability of their substrates. The steroidogenic enzymes, expressed in the adrenal gland, give rise to three groups of steroid hormones: Mineralocorticoids (aldosterone), Glucocorticoids (cortisol and corticosterone) and Gonadocorticoids (dehydroepiandrosterone and androstenedione) (Marieb 2003; Silverthorn 2007). Aldosterone, regulated by the angiotensin II pathway, stimulates the reabsorption of sodium from urine, saliva, gastric juices and sweat (Cho 1998; Kim 1998; Palmer 2001) and thus maintains the electrolyte concentrations in the extracellular fluids. Cortisol and corticosterone, regulated by the HPA axis, effects a wide range of physiological processes such as metabolism, inflammatory responses and the immune system. Dehydroepiandrosterone and androstenedione are involved with sexual differentiation and protein anabolism as well as being precursors for the reproductive steroid hormones testosterone and estradiol (Miller & Auchus 2011).

2.2. The hypothalamic-pituitary-adrenal axis

The endocrine system, together with the nervous and circulatory systems, forms a critical integration of systems responsible for the homeostatic maintenance and coordination of various bodily functions. The circulatory system plays an indirect role in the integration with the endocrine function as blood transports released hormones to its target tissue. The CNS plays a more direct role in endocrine function in that it also synthesises and releases some hormones (e.g. oxytocin) while it also assists in the regulation of the function of most of the endocrine glands. The most prominent connection between the CNS and the endocrine system is the hypothalamus, which is part of the CNS while being an endocrine gland at the same time. The

hypothalamus controls other endocrine glands such as the thyroid and the adrenal glands through the pituitary gland. The connection between the hypothalamus, the anterior pituitary gland and the adrenal gland is called the hypothalamic-pituitary-adrenal (HPA) axis and plays a vital role in the regulation of adrenocorticoids alongside other mechanisms such as the renin-angiotensin and intra-adrenal mechanisms (Marieb 2003; Silverthorn 2007). The hypothalamus and pituitary gland will be discussed further while the adrenal part of the HPA axis will be discussed later in this chapter.

2.2.1. Hypothalamus

The hypothalamus synthesizes peptides and catecholamines using neurosecretory neurons which are released into the circulatory system and act as hormones (Marieb 2003). These hypothalamic hormones can either be released into the systemic circulation, to be transported to its distant target tissues, or can be released into the portal circulation to be transported to the anterior pituitary where they can inhibit or stimulate the release of the anterior pituitary hormones (Marieb 2003). Stimuli that originate within the CNS (interoceptive) or outside the CNS (exteroceptive) are both interpreted by the hypothalamus, serving as an integrating centre for stimuli resulting in appropriate responses. These responses are mostly mediated by the neuroendocrine and endocrine signals that are released by the hypothalamic-pituitary axis. A schematic presentation of the human hypothalamus and the pituitary gland can be seen in Figure 2.1.

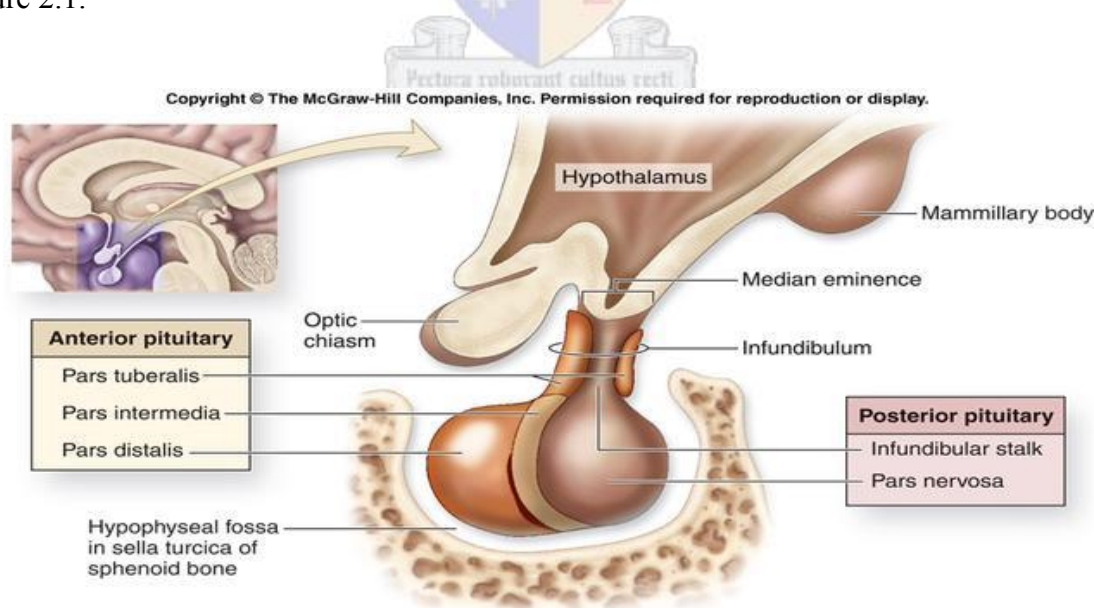


Figure 2.1: Schematic representation of the human hypothalamus and pituitary. Reproduced from (Mescher 2010).

2.2.2. Pituitary gland

The pituitary, also known as the hypophysis, is situated in the pocket of the sphenoid bone called the sella turcica just below the hypothalamus. The pituitary is connected to the hypothalamus via a stalk of nervous tissue known as the infundibulum. The pituitary consists of two different tissue types that divide the pituitary into two lobes known as the posterior and anterior pituitary (Marieb 2003; Young 2006; Silverthorn 2007).

The posterior pituitary is an extension of the downgrowth of the nervous tissue that springs from the hypothalamus. The posterior pituitary together with the infundibulum forms the neurohypophysis, or pars nervosa, which is responsible for the storage and release of the neurohormones produced by the hypothalamus (Marieb 2003). The anterior pituitary springs from the epithelial upgrowth of the roof of the primitive oral cavity, known as Rathke's pouch. This specialised glandular epithelium is wrapped around the anterior aspect of the posterior pituitary and is also known as the adenohypophysis. A vestigial cleft divides the major part of the anterior pituitary from a thin zone of tissue lying against the posterior pituitary, known as the pars intermedia. An extension of the adenohypophysis surrounds the infundibulum and is known as the pars tuberalis (Young 2006).

There is no direct neural connection between the anterior pituitary and the hypothalamus, but there is a vascular connection. The primary capillary plexus in the infundibulum communicates inferiorly via the hypophyseal portal veins with a secondary capillary plexus in the anterior pituitary. The primary and secondary capillary plexuses and the intervening hypophyseal portal veins make up the hypophyseal portal system. Via this portal system the hormones are released or inhibited by the neurons in the ventral hypothalamus to circulate to the anterior pituitary where they regulate secretion of its hormones (Marieb 2003).

The posterior pituitary secretes two hormones namely antidiuretic hormone, also known as vasopressin, and oxytocin. Vasopressin is synthesised in the cell bodies of the supraoptic nucleus, and oxytocin is synthesised in the cell bodies of the paraventricular nucleus of the hypothalamus. These hormones pass down the axons of the infundibulum to the posterior pituitary where they are stored in small vesicles of the axons. The release of the posterior pituitary hormones is controlled by nervous impulses passing down the axons from the hypothalamus through a process known as neurosecretion (Young 2006).

The hypothalamic control of the anterior pituitary hormone secretion is mediated by six hypothysiotrophic hormones. These hormones are secreted by various neurons from the

hypothalamus, which terminate in the median eminence, into the primary capillary plexus. From there the hormones are transported through the pituitary portal system to the anterior pituitary as previously discussed. These hormones are: corticotrophin-releasing hormone (CRH); growth hormone-releasing hormone (GHRH); growth hormone-inhibiting hormone (GHIH); gonadotropin-releasing hormone (GnRH); thyrotropin-releasing hormone (TRH); and prolactin-inhibiting hormone (PIH), also known as dopamine. These six hypophysiotrophic hormones regulate the production and release of the anterior pituitary trophic hormones into the main blood system (Young 2006; Silverthorn 2007).

There are five different secretory cell types in the anterior pituitary that are responsible for the production of the hormones the gland releases. They are somatotrophs, mammotrophs, corticotrophs, thyrotrophs and gonadotrophs. Somatotrophs are responsible for growth hormone (GH) secretion and make up 50% of the anterior pituitary. GH helps in the regulation of protein, lipid and carbohydrate metabolism while GH in turn is regulated by GHRH and GHIH for stimulation and inhibition respectively. Mammotrophs comprise up to 20% of the anterior pituitary and are responsible for the secretion of prolactin. These cells increase in number during pregnancy while prolactin regulates milk production during lactation. Prolactin is also known to play a role in the immune system regulation of both sexes. The mammotrophs are inhibited by PIH. Corticotrophs constitute 20% of the anterior pituitary and are responsible for the secretion of adrenocorticotrophic hormone (ACTH). ACTH is a polypeptide derived from a larger peptide called pro-opiomelanocortin (POMC). Also derived from POMC are lipotropins that are involved in the regulation of lipid metabolism; the endogenous opioids endorphins; and various species of melanocyte-stimulating hormone (MSH) that are responsible for melanin pigment synthesis in amphibians, reptiles and other animals. Although, in humans, MSH serves more as a neurotransmitter than a hormone. ACTH stimulates the release of glucocorticoids as well as mineralocorticoids and androgens in lesser concentrations in the adrenal cortex. The production of ACTH is stimulated by CRH. Thyrotrophs make up about 5% of the anterior pituitary and is responsible for the secretion of thyrotrophin (TSH). TSH production is stimulated by TRH and in turn stimulates the production and release of thyroid hormones. The last 5% of the anterior pituitary consists of gonadotrophs, responsible for the secretion of the gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH promotes ovarian follicle maturation and estrogen production in females while in males it promotes sperm production. LH works together with FSH in promoting

ovulation as well as estrogen and progesterone production in females while in males it promotes testosterone production. Gonadotrophins are produced and released via the stimulation from GnRH (Marieb 2003; Young 2006; Silverthorn 2007).

2.3. Adrenal gland

2.3.1. Anatomy and histology

The adrenal glands are situated on the cranial end of each kidney in most mammals and are only 3-5 cm in length and weigh 1.5-2.5 g (Hardy 1981). The gland consists of inner medullary cells which form part of the sympathetic nervous system which secretes catecholamines (epinephrine and norepinephrine) and outer cortical cells which secrete the steroid hormones previously discussed. The inner medullary cells only make up 28% of the gland while the outer cortical cells make up the other 72% of the gland (Ganong 1995). The outer cortical cells further consists of three layers: the outer *zona glomerulosa*; the middle *zona fasciculata* and the inner *zona reticularis* (Marieb 2003; Silverthorn 2007). The glomerulosa tissue consists of parenchymatous cells arranged in small irregular clusters, separated by delicate trabeculae containing capillaries. It makes up 15% of the cortical tissue and is also the region which secretes aldosterone (Hardy 1981; Ganong 1995; Young 2006). Glomerulosa tissue is also capable of regenerating new cortical cells including the other layers if it is removed (Tebken & Scheumann 2000). Fasciculata tissue consists of narrow columns and cords of cells separated by fine strands of collagen and wide bore capillaries. This region secretes cortisol, corticosterone and small amounts of androgens and occupy 50% of the cortical tissue (Ganong 1995; Young 2006). The reticularis consists of an irregular network of branching cords and clusters of glandular cells separated by wide diameter capillaries. This tissue region secretes small amounts of androgens and glucocorticoids and occupy 7% of the cortical tissue (Ganong 1995; Young 2006).

As previously mentioned the adrenal medulla tissue release epinephrine and norepinephrine regulated by the sympathetic nervous system. This tissue consists of clusters of cells with granular cytoplasm with numerous capillaries in their supporting stroma (Young 2006).

2.3.2. Blood supply to the adrenal gland

The adrenal tissue is richly supplied with blood from the superior, middle and suprarenal arteries that forms a plexus surrounding the gland, just below the capsule. The cortex and the medulla are supplied of blood from this subcapsular plexus through the short cortical arteries and the long cortical arteries, respectively. From the short cortical arteries a network of thin – walled capillary sinusoids extend through the *zona fasciculata* ending in a plexus in the *zona reticularis* to supply the cortex of blood. The blood then drains form this plexus into venules which converge to the central medullary vein. This central vein contains smooth muscle that regulates the blood flow by contracting the smooth muscles causing cortical blood retention. The capillaries surrounding the medullary secretory cells also drain into the central medullary vein and is thus exposed to arterial blood as well as adrenocorticosteroid rich blood. This is believed to have a major influence on the synthesis of adrenaline in the medulla (Young 2006). A schematic presentation of the blood supply to and in the adrenal gland can be seen in Figure 2.2.

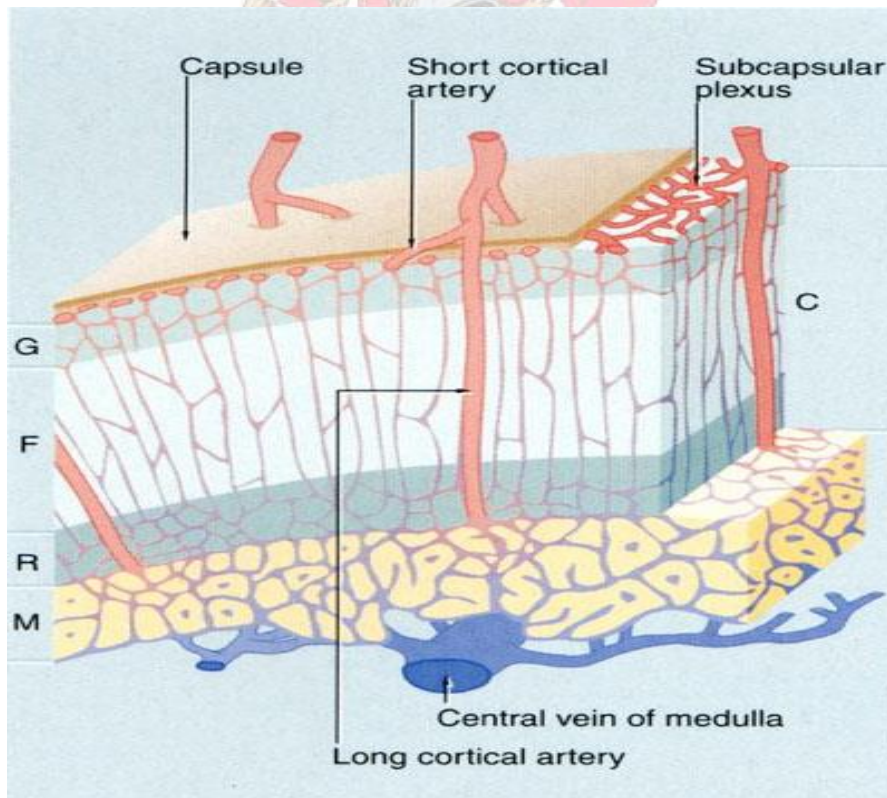


Figure 2.2: Schematic representation of the blood supply to and in the adrenal gland. C -cortex; G - *zona glomerulosa*; F - *zona fasciculata*; R - *zona reticularis*; M - medulla. Reproduced from (Young 2006).

2.4. Hormones of the adrenal cortex

As previously stated, the adrenal cortex produces three types of steroids namely mineralocorticoids, glucocorticoids and adrenal androgens. All of these steroids are derived from cholesterol and thus lipophilic in nature. Being lipophilic, these steroid hormones penetrate the plasma membranes of tissue cells without using second-messenger systems as in the case of the amino acid-based hormones (Marieb 2003).

2.4.1. Control of hormone release

The synthesis and release of most hormones are regulated by negative feedback systems. In these systems, secretion of hormones gets triggered by the stimuli of other hormones produced by other endocrine organs. This mechanism is called hormonal stimuli. The production and inhibition of hypothalamic hormones regulate the release of hormones produced by the anterior pituitary. In turn many of the hormones produced by the anterior pituitary stimulate the release of hormones from other endocrine organs. To regulate this cascade and the release of hormones from the target endocrine organs, the hypothalamic-pituitary-target endocrine organ feedback loop comes in to play as illustrated in Figure 2.3. As the blood levels of the hormones released by the final target endocrine organs increase, they inhibit the release of the anterior pituitary hormones and in turn inhibit their own stimuli (Marieb 2003).

2.4.2. Glucocorticoids

Glucocorticoids are essential for life in that they influence energy metabolism and resist stressors. Under normal circumstances the glucocorticoids help the body to adapt to different types of food intake by keeping the blood sugar levels fairly constant while maintaining the blood volume to prevent the diffusion of water into the tissue cells. Under severe stress, caused by hemorrhage, infections, or physical or emotional trauma, however, a much higher release of glucocorticoids is evoked which helps the body cope with the situation. The glucocorticoid group consists of cortisol, cortisone and corticosterone and its mechanism of action on target cells is to modify gene activity. The secretion of glucocorticoids is regulated by negative feedback loops. CRH is released by the hypothalamus which triggers the release of ACTH by the anterior pituitary which in turn promotes the release of cortisol from the *zona fasciculata* in the adrenal cortex. The rising cortisol levels feedback to inhibit the

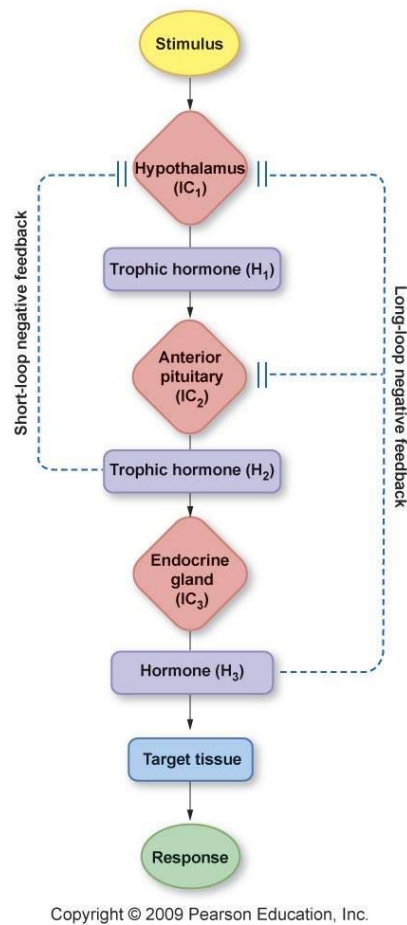


Figure 2.3: Graphical representation of the negative feedback loops in the hypothalamic anterior pituitary pathway. Reproduced from (Silverthorn 2007).

release of CRH from the hypothalamus as well as the release of ACTH from the anterior pituitary (Marieb 2003).

There are two main stimuli that promote the release of cortisol in different amounts namely the circadian rhythm and stress. The circadian rhythm promotes a normal pattern release of cortisol throughout the day and night as can be seen in Figure 2.4. In contrast, acute stress caused by exposure to a cold environment, will override the inhibitory effects of elevated cortisol levels and triggers CRH release. This is the case for the newly shorn Angora goats whose fibres are the primary source of mohair production in South Africa. In such conditions, the increased release of cortisol leads to increased levels of glucose, fatty acid and amino acids in the bloodstream to help the body cope with increased energy usage and heat production. Failing in doing so will lead to dire consequences, such is the case for the South African Angora goat which is unable to metabolize glucose during prolonged periods of cold

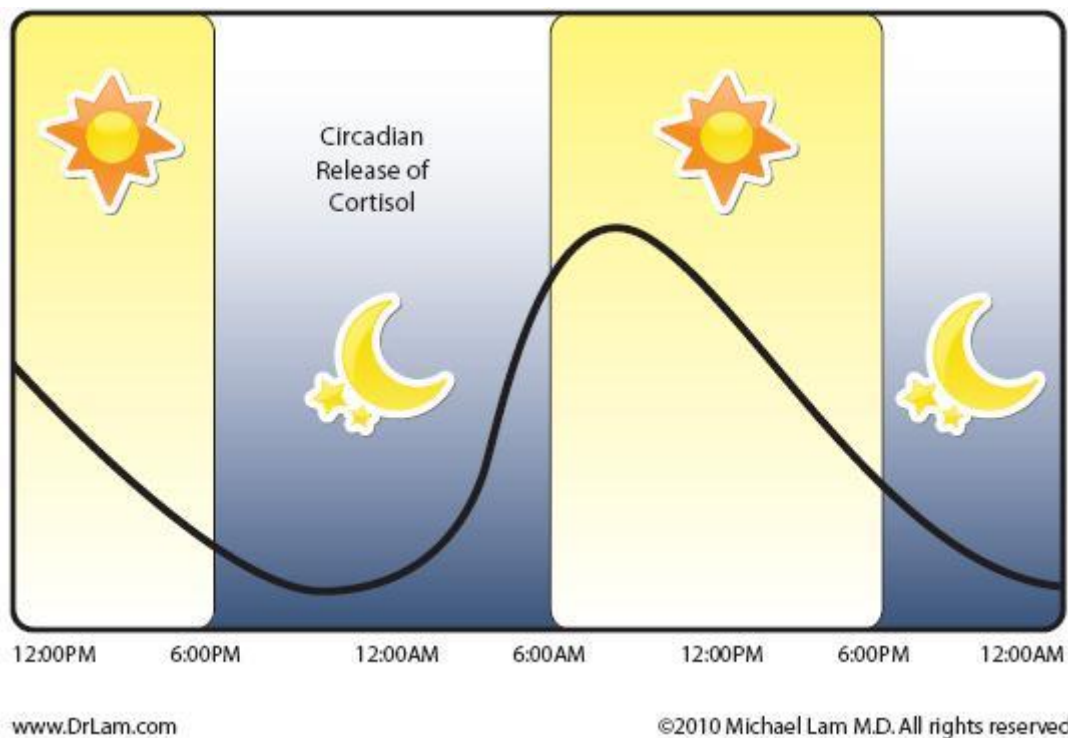


Figure 2.4: Schematic representation of the cortisol levels in the blood during the circadian rhythm. Reproduced from (Lam 2010).

exposure, thereby reducing its ability to produce sufficient metabolic heat (Wentzel 1974, 1979). This condition has been shown to be caused by hypocortisolism, stemming from a CYP17 isoform found in the Angora goat that alters the flux of steroid intermediate away from cortisol production (Engelbrecht 2000; Storbeck 2007). The prime metabolic effect of cortisol is to provoke gluconeogenesis and the use of fatty acids as energy for the brain. Cortisol assist in mobilizing stored proteins to be broken down to provide building blocks for repair or for making enzymes to be used in metabolic processes. Cortisol also enhances the vasoconstrictive effects of epinephrine which leads to the rise in blood pressure and circulatory efficiency, helping to ensure the quick distribution of the nutrients to the cells (Marieb 2003).

Some significant anti-inflammatory and immunosuppressive effects are associated with excess cortisol production (Coutinho & Chapman 2011). Some of these effects include a) the decrease of cartilage and bone formation; b) the inhibition of inflammation by stabilizing lysosomal membranes as well as preventing vasodilation; c) the immunosuppressive actions of repressing key immunomodulatory transcription factors and; d) promoting changes in

cardiovascular, neural and gastrointestinal function. Taking advantage of these effects of glucocorticoid hypersecretion led to a widespread development of glucocorticoid drugs to control symptoms of many inflammatory disorders as well as treatment for immunosuppressive regimes (Coutinho & Chapman 2011). However, the excess levels of glucocorticoids can also lead to their negative effects, including osteoporosis, metabolic disease and increased risk of cardiovascular disease, paradoxically caused by inflammatory conditions (Souverein 2004; Wei 2004; de Vries 2007; Vegiopoulos & Herzig 2007).

2.4.3. Mineralocorticoids

As previously stated, the primary function of mineralocorticoids is to regulate the mineral salt concentrations such as sodium and potassium in the extracellular fluids. The most abundant of the mineral salts is sodium and although it is vital to homeostasis, excessive sodium levels may promote hypertension in some individuals. Maintaining the sodium balance in the body is the mineralocorticoid, aldosterone which is the most potent and accounts for more than 95% of the mineralocorticoids produced. Aldosterone reduces the excretion of sodium from the body by stimulating transcription of the sodium and potassium ATPase, the sodium pump that exchanges potassium for sodium. The regulation of sodium is coupled to the regulation of other ions, including potassium, hydrogen, bicarbonate and chloride due to their coupling to sodium. Hence, where sodium goes the water follows which leads to changes in blood volume and blood pressure. The primary target for aldosterone is the distal parts of the kidney tubules, where sodium is reabsorbed from the forming urine, returning the sodium to the blood stream. Secondary targets for sodium reabsorption include perspiration, saliva and gastric juice. In short, the effects of aldosterone on the renal tubules cause sodium and water retention and excretion of potassium and in some cases alterations in the acid-base balance of the blood due to hydrogen excretion. The regulatory effects of aldosterone to continuously balance the mineral salts in the plasma takes approximately 20 minutes (Marieb 2003).

Aldosterone secretion is stimulated by the rising of potassium levels in the blood; low levels of sodium in the blood; and the decrease of blood volume and blood pressure. The inhibition of aldosterone is just the reverse of the previous mentioned conditions. The four mechanisms that cause the above mentioned conditions are 1) the renin-angiotensin mechanism; 2) the plasma concentration of sodium and potassium ions; 3) ACTH; and 4) atrial natriuretic peptide (ANP) as can be seen in Figure 2.5.

1. The renin-angiotensin mechanism is the main regulator of aldosterone secretion and influences the balance between the water and the mineral salts in the blood as well as the blood pressure. The specialized cells of the juxtaglomerular apparatus in the kidneys are stimulated when either of the blood pressure or the plasma osmolarity declines. The cells respond by releasing renin into the blood which cleaves off part of the plasma protein angiotensinogen, resulting in an enzymatic cascade to produce angiotensin II. The latter is a potent stimulator of aldosterone secretion by the glomerulosa cells. Renin-angiotensin mechanism is also responsible for ultimately raising the blood pressure and in so creating a negative feedback response to inhibit the secretion of aldosterone.
2. Fluctuating concentrations of sodium and/or potassium influence the *zona glomerulosa* cells directly. The stimulation of aldosterone secretion is produced by an increase in potassium concentration and/or a decrease in the sodium concentration, while the opposite fluctuations inhibit aldosterone secretion.
3. Although ACTH, released by anterior pituitary, has little or no effect on aldosterone secretion under normal circumstances, under severe stressful circumstances the increased release of ACTH plays a partial role in the increase of aldosterone secretion. This is due to the increased release of CRH from the hypothalamus which leads to an increase in ACTH release from the anterior pituitary. The result is an increase in blood volume and blood pressure that helps in the adequate transport of nutrients and respiratory gases during stressful periods.
4. ANP is a hormone that is secreted by the heart when the blood pressure increases. It fine-tunes the blood pressure and sodium-water balance by inhibiting the renin-angiotensin mechanism. ANP does this by blocking renin and aldosterone secretion as well as inhibits other angiotensin-induced mechanisms that increase water and sodium reabsorption. The result is that ANP decreases the blood pressure by allowing sodium and water to leave the body through means of urine (Marieb 2003).

Hypersecretion of aldosterone, also known as *aldosteronism*, results from adrenal neoplasms. Aldosteronism causes two major problems: 1) Hypertention and edema due to excessive sodium and water retention; and 2) the accelerated excretion of potassium due to the latter. In the case of extreme potassium loss the neurons become nonresponsive and muscle weakness occurs which eventually leads to paralysis (Marieb 2003).

As previously discussed, Addisons's disease (hyposecretory disease of the adrenal cortex) involves a deficient release of mineralocorticoids (aldosterone) and glucocorticoids (Marieb 2003).

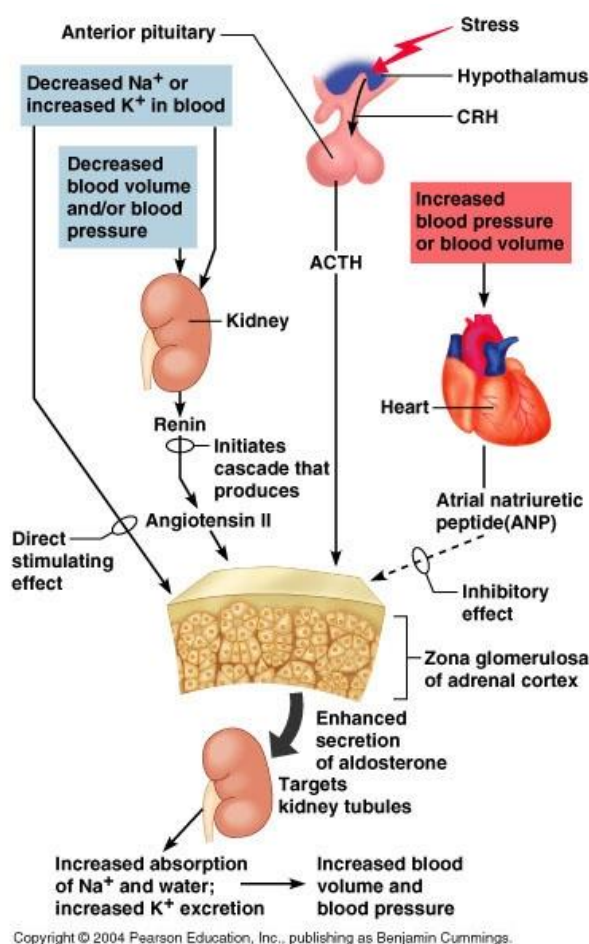


Figure 2.4: Schematic representation of the mechanisms effecting aldosterone release. Reproduced from (Marieb 2003).

2.4.4. Gonadocorticoids

Most of the gonadocorticoids secreted by the adrenal cortex are androgens, also known as male sex hormones, such as androstenedione and dehydroepiandrosterone (DHEA). In the male these hormones are converted to testosterone, while converted to estrogens in the female. The adrenal cortex also produces female sex hormones such as estradiol and other estrogens, but

only in small amounts. Although the adrenal cortex produces gonadocorticoids, the amounts are insignificant compared to those produced by the gonads during late puberty and adulthood. The role of the adrenal gonadocorticoids seems to come in play during the ages of 7 to 13 in boys and girls. During these ages the hormones contribute to the onset of puberty and the appearance of axillary and pubic hair due to the continuous rise of the adrenal gonadocorticoid levels. In females the adrenal sex hormones may be responsible for the sex drive as well as the production of estrogens after menopause when ovarian estrogens are no longer produced (Marieb 2003).

Hypersecretion of gonadocorticoids causes androgenital syndrome (masculinisation) due to the dominant secretion of androgens. In the adult male the effects of elevated gonadocorticoids may be obscured due to the fact that testicular testosterone already produces virilisation. In contrast, the effects may be dramatic in prepubertal males and females. In prepubertal males the maturation of the reproductive organs and appearance of the secondary sex characteristics occur rapidly and the sex drive emerges vigorously. In females the development of a beard and a masculine pattern of body hair distribution occurs while the clitoris grows to resemble a small penis (Marieb 2003).

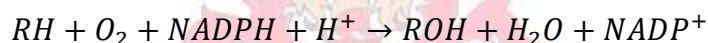
2.5. Enzymes involved in adrenal steroidogenesis

All three major groups of steroids (mineralocorticoids, glucocorticoids and gonadocorticoids) produced by the adrenal gland are biosynthesized by enzymes from cholesterol as common precursor. These enzymes fall into two major groups of proteins: the cytochrome P450-dependent enzymes (P450s) and the hydroxysteroid dehydrogenases. The two groups of enzymes will be discussed in the following sections.

2.5.1. The cytochrome P450-dependent enzymes

All of the P450s involved in adrenal steroidogenesis are membrane-bound proteins, either associated with the mitochondrial membrane or the endoplasmic reticulum (microsomal) (Payne & Hales, 2004). These enzymes form part of a superfamily of haem-containing proteins that can be found in bacteria, fungi, plants and animals (Nelson 1993). The first evidence of

experiments done regarding P450s were done by Garfinkel (1958) and Klingenberg (1958) when both isolated the P450s from mammalian liver microsomes in 1958. However, the first evidence of the spectral properties of cytochrome P450 was identified by Omura and Sato in 1962. They found that, when the reduced cytochrome enzyme is complexed *in vitro* with exogenous carbon monoxide, the enzyme absorbs light maximally at 450 nm, hence the name P450 (Payne & Hales 2004; Miller & Auchus 2011). When microsomes containing P450s are subjected to detergents, the cytochrome is quantitatively converted to a soluble form that gives a peak at 420nm rather than 450 nm. This soluble form is known as P420 (Omura & Sato, 1964). The function of the P450s in the biosynthesis of adrenal steroids from cholesterol is to catalyze the hydroxylation and cleavage of the steroid substrate (RH). The P450s utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor for the reduction of molecular oxygen, thus functioning as a monooxygenases. The general reaction is as follows:



In this reaction the oxygen is activated by a P450. One oxygen atom is incorporated into the substrate RH as a hydroxyl group while the second oxygen atom is reduced to H₂O. There are two distinct electron transport systems through which the electrons, obtained from NADPH are transferred during P450-catalysed reactions: the mitochondrial and the microsomal electron transfer systems. The mitochondrial electron transfer involves the transfer of the high potential electron from flavin adenine dinucleotide (FAD) to a flavoprotein, adrenodoxin reductase (Ad^r). From Ad^r the electron is transported to the mitochondrial cytochrome P450 through means of adrenodoxin (Ad^o), a nonhaem iron-sulfur protein; and sequentially to the substrate. Ad^o serves as a shuttle between Ad^r and the mitochondrial cytochrome P450. The cascade described can be seen in Figure 2.5A (Payne & Hales, 2004).

Microsomal electron transfer only involves one protein, called cytochrome P450 oxidoreductase, which contains two flavins. The transfer of the electrons is initiated in the same manner as for the mitochondrial system, where electrons are transferred from NADPH to FAD; and then sequentially to flavinmononucleotide (FMN), P450s and lastly the substrate as can be seen in Figure 2.5B (Payne & Hales, 2004).

The nomenclature for P450 genes differ between species. The nomenclature for the human genes are CYP while Cyp is used for the mouse genes, followed by an Arabic numeral that

represents the family and a letter indicating the subfamily (Nelson 1993). If there is more than one subfamily, the letter is followed by an Arabic numeral which indicates the individual gene. Examples of the nomenclature can be seen in the P450s associated with the mitochondrial membrane CYP11A, CYP11B1 and CYP11B2, or in the microsomal membrane CYP17, CYP19 and CYP21 (Payne & Hales, 2004). For the purpose of this thesis, further discussion on CYP17 will be in the next chapter.

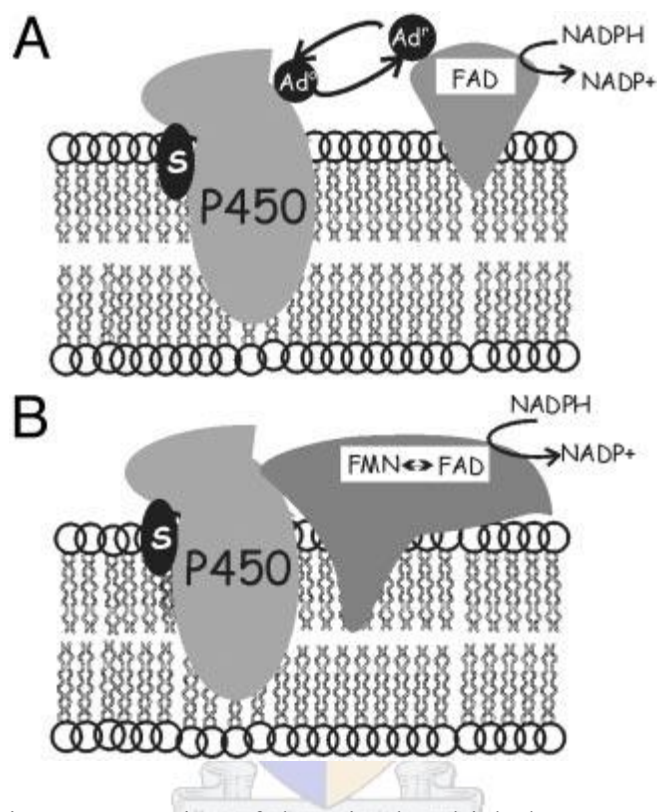


Figure 2.5: Schematic representation of the mitochondrial electron transfer system (A) and microsomal electron transfer system (B) for P450s. FAD, Flavin adenine dinucleotide; FMN, Flavinmononucleotide; Ad^f, Adrenodoxin reductase; Ad^o, Adrenodoxin; S, Substrate. Produced from (Payne & Hales 2004).

2.5.2. Hydroxysteroid dehydrogenases

Hydroxysteroid dehydrogenases (HSDs) are very important to the production and inactivation of all steroid hormones. In steroidogenic tissues they are responsible for the catalysis in the final steps of androgen, estrogen and progesterone production. In peripheral and steroid hormone target tissues, they are responsible for the inactivation of potent steroid hormones and the regulation of the amount of hormones that bind to their respective nuclear receptors; in all regulating gene expression (Penning 1997). HSDs are non-metallic enzymes; have molecular masses in the range of 35-kDa to 45-kDa; and use NADH/NAD⁺ or NADPH/NADP⁺ as

cofactors to reduce or oxidize a steroid by two electrons through a hydride transfer mechanism (Agarwal & Auchus 2005). There is a difference between P450s and HSDs when it comes to the steroidogenic reaction catalysis. A single form of a P450 enzyme catalyzes most of the reactions in steroidogenesis whereas each of the reactions catalyzed by HSDs can be catalyzed by at least two isozymes (Miller & Auchus 2011). The difference lies in that each P450 enzyme is a product of a single gene, whereas the HSDs consists of several isoforms and isozymes, each being a product of a distinct gene. The different isoforms and isozymes varies in terms of species, tissue distribution, catalytic activity, substrate and cofactor specificity, and subcellular localization (Payne & Hales 2004).

The HSDs fall into two groups of proteins based on their structures: the short-chain alcohol dehydrogenase reductase superfamily (SDR) consisting of 3 β -HSD/ketosteroid isomerase (3 β -HSD/KSI), 11 β -HSD and 17 β -HSD; and the aldo-keto reductase superfamily (AKR) consisting of 3 α -HSD and 20 α -HSD (Pawlowski et al. 1991; Lacy 1993; Miura et al. 1994; Deyashiki 1995; Jornvall 1995; Krozowski 1995). The SDR enzymes are β - α - β proteins where up to seven parallel β -strands fan across the centre of the molecule, forming so-called “Rossmann fold”, which is characteristic of oxidation and reduction enzymes that use nicotinamide cofactors. The AKR enzymes are soluble proteins that contain a β -barrel or triosephosphate isomerase (TIM)-barrel motif in which eight parallel β -strands lie in a slanted circular distribution like the staves of a barrel. In both cases, the active site contains a critical pair of tyrosine and lysine residues that participate in proton transfer from or to the steroid alcohol during catalysis. When looking at the quaternary structures, the AKR enzymes are monomeric whereas the SDR are dimeric or tetrameric (Miller & Auchus 2011).

When inspecting their activities, HSDs are divided into two groups namely dehydrogenases or reductases. The dehydrogenases use NAD⁺ as their cofactor to oxidize hydroxysteroids to ketosteroids. The reductases on the other hand use NADPH to reduce ketosteroids to hydroxysteroids. Although *in vitro* these enzymes catalyze bidirectional reactions, based on pH and cofactor concentrations, *in vivo* they tend to function in one direction determined by the availability of the cofactor(s) (Penning 1997; Agarwal & Auchus 2005). The preferences for the reaction directions derive from the relative amounts of the oxidized and reduced form of cofactors and the relative affinity of each enzyme for each cofactor, because of the orders of magnitude that the cofactor concentrations exceed that of the steroid concentrations (Agarwal

& Auchus 2005; Sherbet 2007). For the purpose of this thesis only 3 β -HSD will be discussed in terms of HSD enzymes in the next chapter.

2.5.3. Overview of the adrenal steroidogenesis pathway

All steroid hormones produced has a common precursor derived from cholesterol. The nucleus of cholesterol, cycloperhydropentanophenanthrene, shown in Figure 2.6 as part of the pregnenolone structure, is common to all steroid hormones. Cholesterol, synthesized or mobilized from storage depots, is transported to the mitochondria of the adrenal cortex cells where it is shuttled between the membranes via the steroidogenic acute regulatory (StAR) protein. This step is rate-limiting to the production of steroid hormones. After cholesterol is shuttled into the inner membrane a series of reactions, catalyzed by five P450s and one hydroxysteroid dehydrogenase, occurs. The enzymes involved are the mitochondrial enzymes: cytochrome P450 side-chain cleavage (CYP11A1); cytochrome P450 11 β -hydroxylase (CYP11B1); aldosterone synthase (CYP11B2); and the microsomal enzymes: cytochrome P450 17 α -hydroxylase/17, 20-lyase (CYP17); cytochrome P450 21-hydroxylase (CYP21) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (New & Wilson 1999; Fail 2005; Storbeck 2008a).

The first reaction, after cholesterol enters the inner mitochondrial membrane, is the catalysis of cholesterol to pregnenolone by CYP11A1 after which pregnenolone moves to the endoplasmic reticulum (ER). Pregnenolone serves as a pivotal steroid differential point in adrenal steroidogenesis due to it being the precursor steroid from which all the other steroids originate. Pregnenolone can be converted by the CYP17 hydroxylase reaction to produce 17 α -hydroxypregnenolone which in turn can serve as a substrate for the CYP17 lyase reaction to produce DHEA. Pregnenolone, 17 α -hydroxypregnenolone and DHEA form the Δ^5 steroids and each of them can be dehydrogenated by 3 β -HSD to produce their Δ^4 steroid isoforms progesterone, 17 α -hydroxyprogesterone and androstenedione respectively. An alternative way to form the Δ^4 -steroids 17 α -hydroxyprogesterone and androstenedione, is through the same CYP17 enzyme that catalyses the conversion of the Δ^5 -steroids.

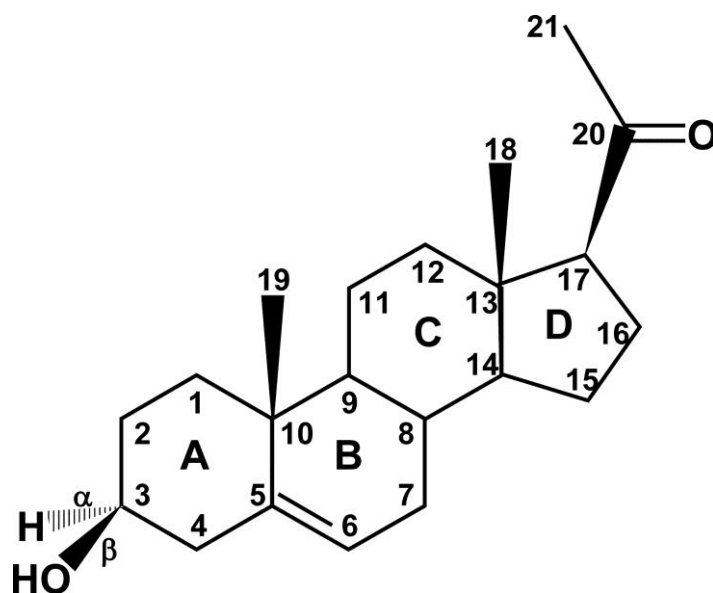


Figure 2.6: Structure of Pregnenolone, illustrating the cycloperhydropentanophenanthrene structure common to all steroids. The carbon atoms are indicated by *numbers*, and the rings are designated by *letters* according to standard convention. Substituents and hydrogens are labeled as α or β if they are positioned behind or in front of the plane of the page, respectively. Reproduced from (Miller & Auchus 2011).

Progesterone can be converted by the CYP17 hydroxylase reaction to produce 17α -hydroxyprogesterone, which in turn can serve as a substrate for the CYP17 lyase reaction to produce androstenedione. In most mammals the latter reaction tends to be very slow and can be considered insignificant. In the ongoing process to produce aldosterone and cortisol, progesterone and 17α -hydroxyprogesterone are both converted by CYP21 to produce deoxycorticosterone and deoxycortisol respectively. These two intermediates then move to the mitochondria where they are converted by CYP11B1 to produce corticosterone and cortisol respectively. Deoxycorticosterone can also be converted by CYP11B2 to produce aldosterone (Payne & Hales 2004). The zonation of the adrenal cortex reflects the spatial distribution and expression of the steroidogenic function (Conley & Bird 1997). The *zona glomerulosa*, the major site for aldosterone production, expresses CYP11A1; 3β -HSD; CYP21; CYP11B1 and CYP11B2, but no CYP17. CYP11B2 is not expressed in the other two zones, while CYP17 expression is required. The *zona fasciculata* expresses CYP11A1, CYP17, 3β -HSD, CYP21 and CYP11B1, producing cortisol and small amounts of DHEA. Lastly the *zona reticularis* expresses CYP11A1, 3β -HSD, higher levels of CYP17 and very low levels of CYP21 and CYP11B1 to mainly produce DHEA and androstenedione (Conley & Bird 1997; Payne & Hales 2004). A schematic of the zonation of the adrenal steroidogenic pathway and its enzymes is shown in Figure 2.7.

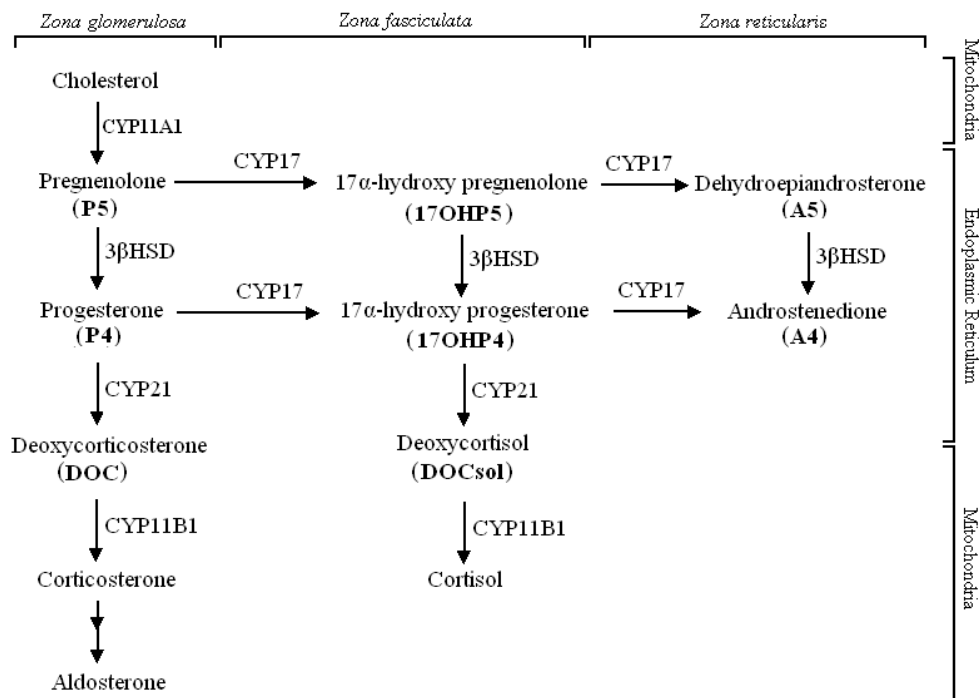


Figure 2.7: Schematic representation of the adrenal steroidogenic pathway. Corticosterone is further converted by CYP11B2 to produce 18-hydroxycorticosterone which in turn serves as a substrate to produce aldosterone by CYP11B2 again. Deoxycorticosterone can also be converted by CYP11B2 to produce corticosterone.

2.6. Cholesterol in adrenalcortical cells

All steroid hormones are derived from the precursor cholesterol as previously mentioned. Obtaining most of the cholesterol for steroid synthesis requires an uptake of circulating lipoproteins through receptor-mediated mechanisms although the cells can produce cholesterol *de novo* from acetate (Mason & Rainey 1987). From the circulating lipoproteins, adrenocortical cells obtain cholesterol through receptor-mediated low density lipoproteins (LDL) and high density lipoproteins (HDL). LDL is obtained through binding to the LDL receptor after which endocytosis takes place and is further degraded to cholesterol by lysosomal vesicles which in turn transport the cholesterol to the ER (Liu et al. 2000; Mason 2002). When the LDL levels are adequate, it may suppress 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme for endogenous cholesterol synthesis (Mason & Rainey 1987). ACTH stimulates the activity of HMG-CoA reductase but at the same time

stimulates LDL receptor-mediated uptake to regulate the HMG-CoA reductase activities (Miller & Auchus 2011).

HDL is obtained through the scavenger receptor BI (SC-BI) in mice and through CLA-1 (CD36 and LIMPII Analogous-1) in humans (Liu et al. 2000). Between the circulating lipoproteins LDL and HDL, LDL serves as the major source of cholesterol in humans with HDL as an alternative source of cholesterol, while in rodents HDL serves as the major source of cholesterol (Liu et al. 2000; Miller & Auchus 2011).

Cholesterol exists in two forms in cells and plasma lipoproteins, namely free cholesterol and cholesterol esters. Steroidogenesis uses free cholesterol as precursor substrate, while the cholesterol esters, consisting of cholesterol esterified to polyunsaturated fatty-acids, or to sulphate, are converted by the microsomal acyl coenzyme A:cholesterol acyltransferase (ACAT). The cholesterol esters that are synthesized accumulate within the rough ER and form cytoplasmic lipid droplets that bud off from the ER. These cytoplasmic lipid droplets can be activated by hormone-sensitive lipase (HSL) to release free cholesterol. ACTH stimulates HSL and inhibits ACAT, thus increasing the availability of free cholesterol for steroidogenesis (Miller & Auchus 2011). The fatty acid esters present in the cytoplasmic lipid droplets and lipoprotein particles cannot replace the free cholesterol that serves as a structural ingredient of the plasma membrane or as substrate for steroidogenesis (Christenson & Devoto 2003). The cellular cholesterol economy is represented in Figure 2.8.

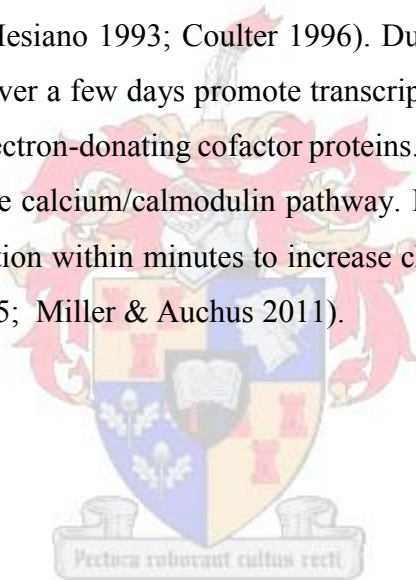
As previously mentioned, steroidogenesis starts in the mitochondria. Cholesterol is transported from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) through the protein StAR. StAR forms part of a family of proteins that contain StAR-related lipid transfer (START) domains (Ponting & Aravind 1999). Fifteen of these START domain (StarD) proteins play a role in binding and mediating intracellular transfer lipids in mammals (Soccio & Breslow 2003). Through sterol response element binding protein (SREBP)-responsive analysis, a group of transcription factors that regulate genes involved in the biosynthesis of cholesterol and fatty acids (Horton et al. 2002), it is believed that a family of proteins related to StarD4 are responsible for the transport of free cholesterol to the OMM from everywhere inside the cell, including lipid droplets and the ER (Miller & Auchus 2011).

There are several genetic disorders effecting the early steps of steroidogenesis and the steps leading up to it. Some of these disorders include adrenoleukodystrophy (ALD), also known as Schilder disease, and disorders of cholesterol synthesis and metabolism such as Wolman

disease, cholesterol ester storage disease, and Smith-Lemli-Optiz syndrome. These diseases primarily cause adrenal insufficiency (Miller & Auchus 2011).

2.8. The role of StAR in steroidogenic regulation

Steroid hormones are not stored in the steroidogenic cells in large amounts as in the case of cells with hormone storage that produce polypeptide hormones. Thus the production of new steroid hormones must be rapid in response to stimuli such as stress. ACTH promotes adrenal steroidogenic cell growth and maintain the steroidogenic enzymes and cofactors in three distinct time periods. At first, long-term exposure, weeks or months, of ACTH promotes adrenal cell growth through the stimulation of cAMP, which in turn promotes the synthesis of IGF-II, basic fibroblast growth factor, and epidermal growth factor (Voutilainen & Miller 1987; Mesiano et al. 1991; Mesiano 1993; Coulter 1996). During the second period, ACTH and angiotensin II exposure over a few days promote transcription of genes encoding various steroidogenic enzymes and electron-donating cofactor proteins. ACTH acts through cAMP and angiotensin II acts through the calcium/calmodulin pathway. During the third period, ACTH promote StAR gene transcription within minutes to increase cholesterol flow from the OMM to the IMM (Stocco et al. 2005; Miller & Auchus 2011).



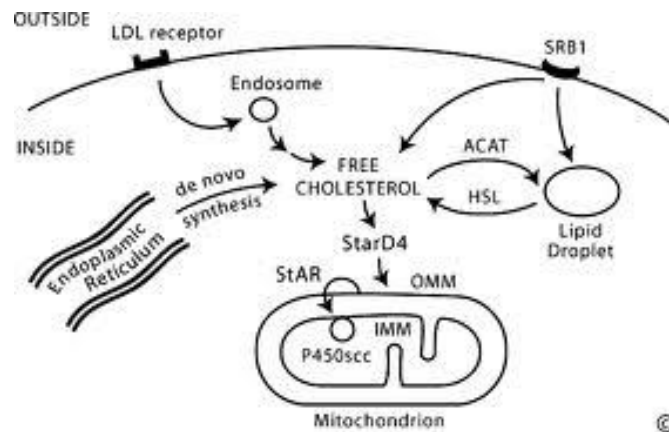


Figure 2.8: Schematic representation of the principal features of the cellular cholesterol economy. Human cells typically pick up circulating LDLs through receptor-mediated endocytosis, directing the cholesterol to endosomes. Rodent cells pick up high-density lipoproteins via scavenger receptor B1 (SRB1) and direct it to lipid droplets. Cholesterol can also be synthesized *de novo* from acetate in the endoplasmic reticulum. Irrespective of source, cholesterol can be esterified by ACAT and stored in lipid droplets as cholesterol esters. Free cholesterol, produced by the action of HSL, may be bound by StarD4 for transcytoplasmic transport to membrane destinations, including the OMM. In the adrenals and gonads, StAR is responsible for the rapid movement of cholesterol from the OMM to the IMM, where it can be taken up by CYP11A1 (P450_{scc}) and converted to pregnenolone. Reproduced from (Miller & Auchus 2011).

2.8.1. The mechanism of action of StAR

StAR is a 37-kDa cytoplasmic protein that gets cleaved upon entering the mitochondria where it yields a 30-kDa intramitochondrial protein. The half-life of the 37-kDa cytoplasmic protein is short compared to the longer half-life of the 30-kDa intramitochondrial protein. This would suggest that the 30-kDa protein is most likely the biological active form of StAR, but it has been shown that both 37-kDa and 30-kDa forms are equally active (Arakane et al. 1996). Studies have shown that StAR is only active when it is immobilized on the OMM and not when localized to the mitochondrial intramembranous space or the matrix (Arakane et al. 1996; Bose & Lingappa 2002). It has been suggested that the time StAR is bound to the OMM is proportional to the activity in promoting steroidogenesis (Bose & Lingappa 2002). The interaction of StAR with the OMM promotes conformational changes that are needed for StAR to accept and release cholesterol molecules (Bose et al. 1999; Baker et al. 2005). StAR can only accommodate a single cholesterol molecule in its sterol-binding pocket (Tsujishita 2000). Several proteins on the OMM, including TSPO (also known as the peripheral benzodiazepine receptor), voltage-dependent anion channel 1, and phosphate carrier protein, are needed to

interact with StAR (Papadopoulos 1993; Hauet et al. 2005; Jun Liu et al. 2006; Bose et al. 2008). The StAR proteins are recycled after transporting cholesterol over the OMM and so moves hundreds of cholesterol molecules over the OMM before it is cleaved or inactivated (Artemenko 2001). Biophysical and partial proteolysis studies have shown that the reason why StAR can be recycled numerous times before cleavage is that it contains a region that is protease-resistant. This region causes a “pause-transfer” sequence which enables the bioactive carboxy-terminal domain to have repeated interactions with the OMM (Bose et al. 1999). As in the name, StAR is required for the acute steroidogenesis response, but in tissues like the placenta and the brain where StAR is absent, steroidogenesis still produces steroids at 14% of the StAR-induced rate (Bose et al. 1996; Miller 1999).

The StAR protein contain a α -helix and adjacent Ω -loops in the exterior surface of the C-terminal that interacts with the OMM. The Ω -loops form hydrogen bonds with the C-terminal helix, prohibiting access of cholesterol to StAR's hydrophobic cholesterol binding pocket. The interaction with the charged phospholipids head groups on the OMM disrupts these hydrogen bonds, permitting the C-helix to swing open and closed, governing access of cholesterol to the sterol-binding pocket. Immobilizing the C-helix by forming disulfide bonds with the adjacent loops ablates activity, and disrupting such artificial disulfide bonds restores activity (Baker et al. 2005).

2.9. Significance of this study

As the evidence discussed in this chapter shows, the HPA axis plays a critical role in mediating adrenocortical synthesis and regulation in mammals. Understanding adrenal steroidogenesis and its regulation may help us in the development of therapeutic approached treatments of steroidogenic or even endocrinological diseases (Conley & Bird 1997). Systems biology tools, such as computer modelling can assist us in understanding the adrenal steroidogenic system as can be seen in the development of a kinetic model that was used to predict biochemical responses of ovarian steroidogenesis to Endocrine Active Compounds (Breen 2007).

As mentioned in chapter 1, enzymes CYP17 and 3β -HSD were shown to be contributors to hypocortisolism in the Angora goat by shifting the flux of steroid intermediate production towards the Δ^5 -pathway.

The expression and activity of CYP17 and 3 β -HSD thus plays an important role in the biosynthesis of everyday needed steroids in mammals. The next chapter will focus on CYP17 and 3 β -HSD, addressing both enzymes in more detail.



Chapter 3

Cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD)

3.1 Cytochrome P450 17 α -hydroxylase/17,20-lyase

3.1.1 Catalytic activity

Microsomal CYP17 catalyzes multiple reactions in the adrenal gland through both 17 α -hydroxylase and 17,20-lyase activities as shown in Figure 3.1 (Payne & Hales 2004). These reactions include the hydroxylation activity of the C21 steroids pregnenolone (Δ^5 -steroid) and progesterone (Δ^4 -steroid) to produce 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone as respective intermediates, followed by the lyase activity to produce C19 steroids, DHEA and androstenedione, respectively. Each of the reactions requires molecular oxygen and one molecule of NADPH. It was initially believed that the reactions were catalyzed by two distinct enzymes. This notion was overturned by the cloning and expression of bovine CYP17 cDNA in nonsteroidogenic COS-1 cells, which showed a single protein catalyzing both the hydroxylation and lyase activities (Nakajin 1981; Nakajin & Hall 1981; Zuber 1986). These experiments indicated that 17 α -hydroxylase and 17,20-lyase differed functionally but not genetically or structurally (Miller & Auchus 2011). For all species the hydroxylation activity catalyses the conversion of pregnenolone and progesterone to their 17-hydroxylated derivatives with approximately equal efficiency (Lee-Robichaud 1995; Auchus 1998), but the lyase activity for the Δ^5 - and Δ^4 -substrates is species-dependent. Within the human adrenal the lyase activity, converting 17 α -hydroxypregnenolone to DHEA, is about 50 times more efficient than the conversion of 17 α -hydroxyprogesterone to androstenedione (Lee-Robichaud 1995; Auchus 1998), while in the rodent the lyase activity prefers 17 α -hydroxyprogesterone as substrate (Nakajin 1981; Zuber 1986a; Swart 1993; Katagiri 1995; Brock 1999). The rate of the human lyase activity can be increased 10-fold by cytochrome b₅, but still does not reach the rate of the hydroxylase activity. Cytochrome b₅ also stimulates the rat lyase activity for 17 α -hydroxyprogesterone, but the fold increase is small relative to the increase observed with

the human enzyme (Brock 1999). It has been shown that human CYP17 can also 16 α -hydroxylate progesterone but not pregnenolone (Swart 1993)

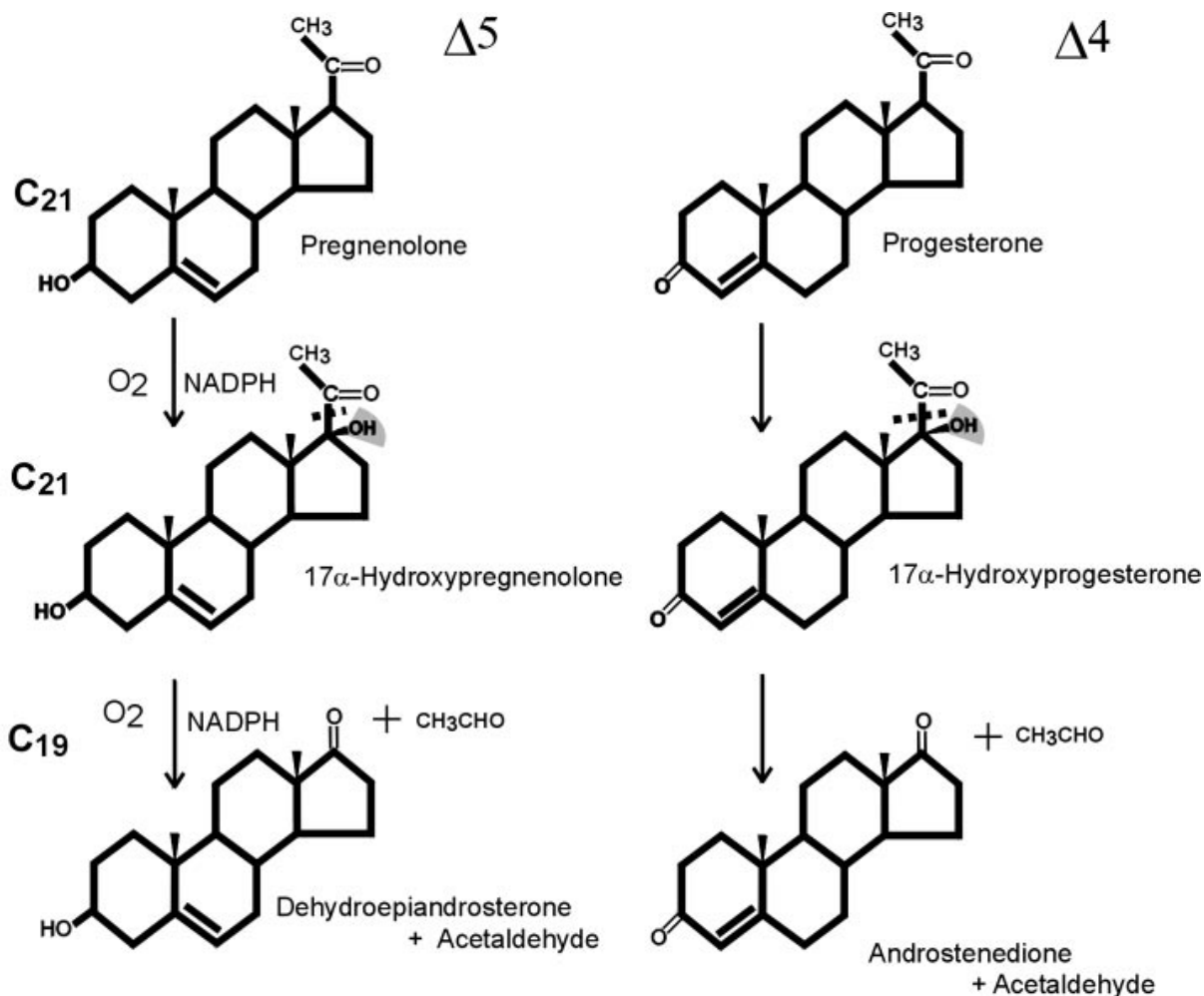


Figure 3.1: Schematic representation of the enzymatic reactions catalyzed by CYP17. CYP17 catalyzes two mixed-function oxidase reactions, 17 α -hydroxylation and C17–C20 cleavage. Each reaction requires molecular oxygen and one molecule of NADPH and uses the microsomal electron transfer system. The use of the Δ^5 or the Δ^4 steroid as substrate is species-dependent. Reproduced from (Payne & Hales 2004).

3.1.2. Molecular structure

In numerous species a single gene encodes for the CYP17 enzyme (Picado-Leonard 1987; Bhasker 1989; Fevold 1989; Kagimoto 1989; Youngblood 1991; Zhang 1992; Nelson 1996). This enzyme is a 57-kDa protein that yields form a single 2.1-kb mRNA (Miller & Auchus 2011). The human gene, *CYP17*, has been mapped to chromosome 10q24.3 (Matteson 1986; Fan 1992), while the mouse gene, *Cyp17*, has been mapped to chromosome 19 at 46 cM (Youngblood 1991). The *CYP17* gene shares high homology at the 5' upstream region of the

human (Matteson 1986; Chung 1987; Yanase 1991; Fan 1992), mouse (Youngblood 1991), and rat (Fevold 1989) in the first 550 bp including the nonconsensus TATA box. The human CYP17 enzyme contains 508 amino acids (aa) compared to the 507 aa (Chung 1987) found in mouse (Youngblood 1991) and rat (Fevold 1989) enzymes respectively, supporting 66% identity when compared. This shows that there are regions of high homology within the CYP enzyme among different species (Youngblood 1991). These regions have been identified as the putative binding regions that play a role in substrate specificity (Zuber 1986a) for mouse aa 434-454 (Youngblood 1991), human aa 435-455 (Yanase 1991), and the ozols tridecapeptide sequence (343-372 aa) (Ozols 1981). Other regions among species of CYP17 (296-319 aa) are specifically conserved to function in catalysis for example, the arginine³⁴⁶ in the rat (Kitamura 1991) enzyme and the arginine³⁴⁷ in the human enzyme (Lin 1994) were found to be critical for catalyzing the lyase activity (Ono 1988; Payne & Hales 2004).

3.1.3. CYP17 expression is species-dependent

Expression of CYP17 is seen in all steroidogenic tissues, namely the adrenal cortex, ovary, placenta and testis; however, there are species-related differences in the expression of CYP17 in the adrenal cortex and the placenta (Payne & Hales 2004). CYP17 expression in the testis of all species are restricted to the Leydig cells (Saez 1994; Payne 1996; Pelletier 2001), while in the ovary, CYP17 expression is restricted to thecal cells that are the site of androgen production (Sasano 1989; Tamura 1992; Lauber 1993; Conley 1995). It was generally accepted that the granulosa and luteal cells do not express CYP17 (Voutilainen 1986; Sasano 1989; Tamura 1992; Lauber 1993; Conley 1995), but a recent report suggested that human luteinized granulosa cells in culture do express the enzyme (Moran 2003).

In the human, macaque, and guinea pig adrenal gland, CYP17 is expressed in the *zona reticularis* and the *zona fasciculata* for androgen and glucocorticoid production, respectively (Hyatt 1983a; Hyatt 1983b; Endoh 1996). However, CYP17 is not expressed in the *zona glomerulosa* which is the site of mineralocorticoid production (Harkins 1974; Crivello 1983). In bovine and ovine adrenal glands, CYP17 is only expressed in the *zona fasciculata*, lacking a well-defined *zona reticularis* (Conley and Bird 1997). In contrast, mouse (Perkins 1988) or rat (Brock 1999; Pelletier 2001) do not express CYP17 in the adrenal glands at all resulting in the adrenals of these animals producing corticosterone as the major glucocorticoid, rather than cortisol as is the case in most mammals (Payne & Hales 2004).

In humans, CYP17 is not expressed in the placenta. Instead the C19 substrates for placental androgens and estrogens are derived from fetal adrenal glands as DHEA sulphate that is converted to 16 α -hydroxydehydroepiandrosterone sulphate in the fetal liver and transported to the placenta, where it is acted on by the placental steroid sulfatase (Miller 1998). In the mouse and rat placenta, however, CYP17 expression starts at midpregnancy, declining just before parturition (Durkee 1992; Arensburg & Payne 1999).

CYP17 mRNA and protein has been detected in the transitional zone and fetal zone of human (Mesiano 1993; Narasaka 2001) and monkey (Mesiano 1993) fetal adrenal glands throughout gestation, but not in the definitive zone. As stated earlier, CYP17 is not expressed postnatally in mouse or rat, but it has been reported that CYP17 mRNA is detected in mouse fetal adrenal cells between embryonic days 12.5 and 14.5, after which expression disappeared (Keeney 1995).

In mouse fetal testes, CYP17 expression is observed from embryonic day 13, which was the earliest time examined, and throughout pregnancy. Postnatally, CYP17 expression is low between birth and day 20, rising between day 20 and day 25, and reaching maximum expression after postnatal day 40 (O'Shaughnessy & Willerton 2002). In mouse fetal ovary, no CYP17 expression is observed until birth (Greco 1994; Payne & Hales 2004).

3.1.4. Regulation of CYP17 expression

The regulation of steroidogenesis is governed by peptide hormones secreted by the anterior pituitary gland in response to signalling from the hypothalamus. These peptide hormones augment steroid hormone output by acting as ligands for G protein-coupled receptors (Sewer 2008). There are numerous signalling molecules that are known as regulators of steroidogenic gene transcription in the adrenal cortex (Ehrhart-bornstein 1998) and of all the signalling molecules ACTH and in a lesser sense AII, are known as the most important factors in CYP17 regulation (Conley & Bird 1997; Gallo-Payet & Payet 2003; Sewer 2003; Foster 2004; Spät & Hunyady 2004; Enyeart 2005; Sewer 2007).

ACTH induces steroidogenesis by activating intracellular signalling pathways that facilitate cholesterol uptake, transport, and delivery (Miller 2007; Stocco 2005) as well as promoting steroidogenic gene transcription (Miller 1988; Moore & Miller 1991; Waterman & Keeney 1996; Sewer & Waterman 2001; Sewer & Waterman 2002; Sewer & Waterman 2003). These intracellular signalling pathways promote rapid production of steroid hormones by increasing

substrate availability and sustaining enzyme activity by inducing gene expression (Sewer 2008).

One of the most familiar intracellular signalling pathways is the cyclic AMP (cAMP) cascade. ACTH acts via G protein-coupled receptors to activate adenylyl cyclase, increasing intracellular cAMP which leads to the activation of cAMP-dependent protein kinase (PKA). Downstream targets, such as cAMP response element modulator (CREM), are phosphorylated by PKA which leads to the induction of StAR transcription (Sugawara 2006) and hormone sensitive lipase (Jefcoate 1992) for the production of free cholesterol. This in turn leads to an increase in steroidogenic enzyme synthesis and in essence CYP17 (Waterman 1994; Waterman & Keeney 1996b; Payne & Hales 2004; Sewer 2008).

Although additional factors are involved in maintaining steroidogenic enzyme expression, CYP17 expression only appears to be dependent on cAMP stimulation (Anakwe & Payne 1987; Waterman 1994; Payne & Hales 2004; Sewer 2008). ACTH, via cAMP/PKA, rapidly activates the synthesis and secretion of a sphingolipid, sphingosine-1-phosphate (S1P), which binds to its specific G protein-coupled receptors, ultimately resulting in CYP17 transcription (Ozbay 2004; Ozbay 2006). This is done through activating the sterol regulatory element-binding protein 1c (SREBP1c), a downstream transcription factor of the S1P cascade (Figure 3.2), which leads to the coordination of cholesterol and other sterols (Shea-Eaton 2001; Ozbay 2006), precursors for steroidogenesis. It has been found that another sphingolipid, sphingosine (SPH), is an antagonist of the nuclear receptor steroidogenic factor-1 (SF-1) (Urs 2006). SF-1 and several other transcription factors are required for adrenal expression of CYP17 (Rodriguez 1997; Lin 2001; Sewer 2002; Jimenez 2003; Fluck & Miller 2004; Sewer & Jagarlapudi 2009). In light of the previous statements, ACTH may increase the transactivation potential of SF-1 by promoting dissociation of SPH and converting SPH to S1P. In contrast, recent studies indicate that SF1 may activate human CYP17A1 expression in response to SPH (Urs 2006; Li 2007; Sewer 2008; Miller & Auchus 2011).

In contrast to adrenal CYP17 expression being consistent across species in response to ACTH, the response to AII are notable different. AII stimulated adrenal CYP17 expression is only marginal in human (Lebrethon 1994; Rainey 1994; Bird 1995; Bird 1996) and bovine (Bird 1993). The best observation of inconsistent adrenal CYP17 expression across different species is in response to AII, combined with ACTH or other factors that activate the cAMP signalling cascade. However, the combined treatment mostly attenuates adrenal CYP17 expression in

human (Bird 1993; Lebrethon 1994), ovine (Rainey 1991; Bird 1992) and bovine (Rainey 1991), leading to reduced production in 17α -hydroxysteroids and subsequently the C19 steroids DHEA and androstenedione (Bird 1993). The porcine enzyme, however, differs in combined treatment of AII and ACTH in that the adrenal CYP17 expression is similar to that of ACTH alone with respect to 17α -hydroxylase activity and steroid production (Conley 1994; Conley & Bird 1997).

Another difference between AII and ACTH, in accordance to adrenal CYP17 expression, is the second messenger pathways used. While ACTH acts primarily through cAMP and its cascades, AII mediates CYP17 expression through phosphoinositidase C, which in turn mediates the Ca^{2+} and protein kinase C signalling cascades. Tetradecanoylphorbol 13-acetate (TPA) also activates protein kinase C in cattle (Rainey 1991) and humans (Lebrethon 1994; Rainey 1994; Bird 1996). However, while both TPA and AII can attenuate ACTH-induced adrenal CYP17 expression, treatment with TPA alone reduces basal adrenal CYP17 expression in the human (Lebrethon 1994; Rainey 1994; Bird 1995; Bird 1996) in contrast to marginal adrenal CYP17 expression stimulated by AII alone. Since TPA and AII use the protein kinase C signalling cascade to effect adrenal CYP17 expression, and AII also uses the Ca^{2+} signalling cascade to increase adrenal CYP17 expression in human (Lebrethon 1994; Rainey 1994; Bird 1995; Bird 1996), it is likely that the majority of AII stimulated adrenal CYP17 expression is mediated through the Ca^{2+} signalling cascade. These alternative signalling cascades may explain how adrenal CYP17 expression is maintained in the tissues of different species, although more studies into the mechanism of action and even more alternative signalling cascades are needed (Conley & Bird 1997).

The expression of CYP17 in the gonads have many similarities as well as notable differences with that seen in the adrenal cortex. Although the cAMP/PKA cascades are still used to activate gonadal CYP17 expression, it appears that LH, instead of ACTH, is the primary activator of these cascades (Leung & Steele 1992; Richards 1994). In human (Lacroix 1974), cattle (Demeter-Arlotto 1993; Voss & Fortune 1993), rat (Hedin 1987; Fournet 1996) and pig (Chu 1996) thecal cells, CYP17 expression is dependent on cAMP, while the same is true in Leydig cells of mice (Anakwe & Payne 1987; Payne & Youngblood 1995) and pigs (Saez 1994). When we look at the feedback of androgens on CYP17 expression

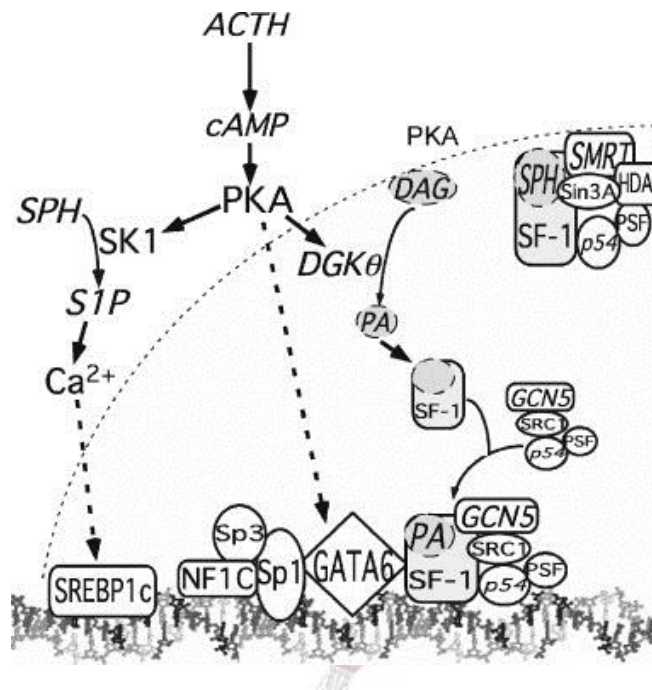


Figure 3.2: Model for transcriptional regulation of human CYP17. In response to ACTH, PKA activation stimulates multiple proteins, including SK1 (sphingosine kinase 1), DGKθ (diacylglycerol kinase theta). It is likely that PKA modulates the activity of several other proteins in adrenocortical cells. Activated DGKθ phosphorylates diacylglycerol (DAG) to form phosphatidic acid (PA). Increased nuclear concentrations of PA facilitate dissociation of sphingosine (SPH) and corepressor proteins [Sin3A, histone deacetylase (HDAC), silencing mediator of retinoid and thyroid receptors (SMRT)]. Binding of PA to steroidogenic factor-1 (SF-1) promotes the assembly of a transcription activator complex containing SRC1 (steroid receptor coactivator 1), the histone acetyltransferase GCN5 (general control nonderepressed 5), p54, and polypyrimidine-tract binding protein-associated splicing factor (PSF). PKA also activates SK1, which converts SPH to sphingosine-1-phosphate (S1P). S1P activates the cleavage and nuclear import of sterol regulatory element binding protein 1c (SREBP1c) via a Ca²⁺-dependent pathway. Constitutive CYP17 expression is mediated by a complex containing specificity protein (Sp) 1, Sp3, and nuclear factor 1C (NF1C). Basal transcription is also mediated by GATA-6, which binds to Sp1. In response to cAMP GATA-6 synergizes with SF-1 to activate CYP17 transcription. Reproduced from (Sewer & Jagarlapudi 2009).

(Hales & Payne 1987) in mouse Leydig cells and rat theca cells (Fournet 1996), the direct effects of cAMP on transcription are in question. Some factors, using alternative second messenger pathways in gonads (Leung & Steele 1992; Gore-Langton & Armstrong 1994; Saez 1994), may modify cAMP responses and provide a unique input into steroidogenesis. One such a factor is the insulin-like growth factor I (IGF-I) which stimulates CYP17 expression in rat theca cells (Magoffin & Weitsman 1993) as well as augments cAMP effects on CYP17 expression in human theca cells (McAllister 1994). Another factor is the transforming growth factor beta (TGFβ) which inhibits CYP17 expression in theca cells of the human and rat

(McAllister 1994) as well as in pig Leydig cells (Saez 1994). In human primary thecal cultures, cAMP induction of CYP17 expression is inhibited by phorbol ester (McAllister 1994). Many additional factors such as activin, inhibin, IGFs, and prostaglandins appear to contribute to gonadal CYP17 expression regulation (McGee 1995; McGee 1996; Sawetawan 1996). The regulation of CYP17 expression in granulosa cells in response to AII is inconsistent. Some suggest that AII stimulated progesterone and estradiol secretion in human (Pepperell 1994) while others find no steroidogenic or second messenger response (Rainey 1993). However, results of studies conducted in vivo are consistent with an effect of AII on human gonadal steroidogenesis (Conley & Bird 1997).

3.1.5. Physiological importance of CYP17

As mentioned earlier, CYP17 has both 17α -hydroxylase and $17,20$ -lyase activity; acting as a key branch point in adrenal steroidogenesis. In the *zona glomerulosa* where neither CYP17 activities are present, pregnenolone is converted to mineralocorticoid aldosterone which helps in maintaining the water and mineral balance. In the *zona fasciculata* only the 17α -hydroxylase activity is present, producing the glucocorticoid cortisol from pregnenolone. Cortisol helps in the regulation of lipid, carbohydrate and protein metabolism (Dallman 2004). In the *zona reticularis* as well as in the gonads both activities are present, leading to the production of androgens DHEA and androstenedione from pregnenolone (Miller & Auchus 2011). In males androgens are responsible for the sexual differentiation before birth; sexual maturation during puberty; and maintaining the male reproductive function in adults (Heinlein & Chang 2002). In females androgens serve as precursors for estrogen biosynthesis (Simpson 2000); as well as playing a crucial role in normal and pathological ovarian development (Ito 1993; Hillier & Testsuka 1997; De Leo 1998; Speiser 2001; Hu 2004; Storbeck 2008).

As can be gleaned from the discussion above, the deficiency of 17α -hydroxylase activity or $17,20$ -lyase activity or both, may lead to several problems in maintenance, regulation and maturation of several biological systems. The deficiency of 17α -hydroxylase activity leads to a decrease in cortisol synthesis, overproduction of ACTH, and stimulation of the steps proximal to CYP17 (Miller & Auchus 2011), showing mild symptoms of glucocorticoid deficiency. In humans who alternatively produce corticosterone in the absence of CYP17 activity, this is not life-threatening due to corticosterone also having glucocorticoid activity. This is similar to rodents producing corticosterone as its primary and only glucocorticoid due to a lack of adrenal

CYP17 enzymes (Voutilainen 1986). 17α -hydroxylase deficiency in humans also leads to overproduction of 11 deoxycorticosterone (DOC) in the *zona fasciculata*, causing sodium retention, hypertension and hypokalemia with suppressed plasma renin activity and suppressed aldosterone secretion from the *zona glomerulosa* (Miller & Auchus 2011). When these patients are treated with glucocorticoids it suppresses DOC secretion while plasma levels of renin activity and aldosterone concentration rise to normal (Scaroni 1986).

Deficiency of 17,20-lyase activity in the *zona reticularis* and the gonads lead to insufficient DHEA and androstenedione production which in turn leads to immature sexual development, just one of many systems needing androgens. Deficiency in 17,20-lyase activity has been reported in many cases (Yanase 1991) which initially lead to the incorrect conclusion that 17α -hydroxylase and 17,20-lyase are separate enzymes (Miller & Auchus 2011). It was not clear if a syndrome of 17,20-lyase deficiency could exist due to both 17α -hydroxylase and 17,20-lyase activities being catalyzed on the same active site. This was confirmed when two patients with genital ambiguity, normal excretion of 17-hydroxycorticosteroids, and reduced production of DHEA and androstenedione were studied (Geller 1997). The patients had different CYP17 mutations, but both the mutations changed the distribution of surface charges in the redox-partner binding site of CYP17 (Geller 1997). In an *in vitro* assay, both mutants retained almost normal 17α -hydroxylase activity but had no 17,20-lyase activity (Geller 1997; Geller 1999), and the substrate binding site remained normal in enzymatic competition experiments (Geller 1999). With an excess addition of P450 oxidoreductase (POR) and cytochrome b_5 , some 17,20-lyase activity was restored, showing that the loss in lyase activity was caused by impaired electron transfer (Geller 1999). Some girls have been described with similar mutations where they fail to manifest adrenarche (Van Den Akker 2002). By contrast, another CYP17 mutation, E305G, causes 17,20-lyase deficiency by selectively disrupting binding of 17α -hydroxypregnenolone and preventing DHEA synthesis despite increased conversion of 17α -hydroxyprogesterone to androstenedione (Sherbet 2003). However, this unusual variant of 17,20-lyase deficiency is not sufficient to form normal male external genitalia (Miller & Auchus 2011).

The deficiency of both 17α -hydroxylase and 17,20-lyase activities prevents the synthesis of adrenal and gonadal androgens. This would not affect females phenotypically but they will fail to undergo adrenarche and puberty (Biglieri 1966), while males will have absent or incomplete development of external genitalia, also termed “46,XY disorder of sex development” (New &

Suvannakul 1970). Humans with 17 α -hydroxylase and 17,20-lyase deficiency will represent a teenage female with sexual infantilism and hypertension (Biglieri 1966; New & Suvannakul 1970). The diagnosis is made by finding low or absent 17-hydroxylated C₂₁ and C₁₉ plasma steroids, which respond poorly to stimulation with cosyntropin. Serum levels of DOC, cortocosterone, 18-hydroxycorticosterone, and 18-hydroxy DOC are elevated with cosyntropin and suppressed with glucocorticoid administration (Miller & Auchus 2011).

Computational modeling of CYP17 predicts the effects of all known mutations, including those with partial retention of both activities and those causing selective 17,20-lyase deficiency (Auchus & Miller 1999). The model identifies Arg 347 and Arg 358 and several other arginine and lysine residues in the redox-partner binding site; mutations of these residues all cause varying degrees of selective loss of 17,20-lyase activity (Geller 1997; Lee-Robichaud 1998; Auchus & Miller 1999; Geller 1999). Another example of the critical nature of redox-partner interactions comes from the rare syndrome of cytochrome *b*₅ deficiency. The first patient was a male pseudohermaphrodite with severe methemoglobinemia, but he was not evaluated hormonally (Giordano 1994). A recent, well-studied patient homozygous for the cytochrome *b*₅ mutation W27X had hormonal findings indicative of isolated 17,20-lyase deficiency and clinically in apparent but elevated concentrations of methemoglobin, as expected from the known role of cytochrome *b*₅ in the reduction of methemoglobin (Kok 2010). Electron transfer for the 17,20-lyase reaction is promoted by the action of cytochrome *b*₅ as an allosteric factor rather than as an alternate electron donor (Auchus 1998). The 17,20-lyase activity is also favored by the phosphorylation of serine residues on CYP17 by a cAMP-dependent protein kinase (Zhang 1995; Biason-Lauber 2000; Pandey 2003; Pandey & Miller 2005). The availability of electrons determines whether CYP17 performs only 17 α -hydroxylation or also performs 17,20-lyase bond scission; increasing the ratio of POR or cytochrome *b*₅ to CYP17 *in vitro* or *in vivo* increases the ratio of 17,20-lyase activity to 17 α -hydroxylase activity. Competition between CYP17 and CYP21 for available 17 α -hydroxyprogesterone does not appear to be important in determining whether 17 α -hydroxyprogesterone undergoes 21-hydroxylation or 17,20-lyase bond scission (Yanagibashi & Hall 1986). Thus, the regulation of 17,20-lyase activity, and consequently of DHEA production, depends on factors that facilitate the flow of electrons to CYP17: high concentrations of POR, the presence of cytochrome *b*₅, and serine phosphorylation of CYP17 (Miller 1997; Miller & Auchus 2011).

3.2. 3 β -Hydroxysteroid Dehydrogenase

3.2.1. Catalytic activity

The microsomal enzyme 3 β -HSD catalyzes the conversions of the three Δ^5 -3 β -hydroxysteroids, pregnenolone, 17 α -hydroxypregnenolone and DHEA, to their Δ^4 -3-ketosteroids, progesterone, 17 α -hydroxyprogesterone and androstenedione, respectively. The conversion of the Δ^5 -3 β -hydroxysteroid to a Δ^4 -3-ketosteroid consists of two sequential reactions. The first reaction is the dehydrogenation of the 3 β -equatorial hydroxysteroid requiring the coenzyme NAD⁺ yielding a Δ^5 -3-keto intermediate and reduced NADH. The reduced coenzyme, NADH, then activates the isomerization of the Δ^5 -3-ketosteroid to yield the Δ^4 -3-ketosteroid as shown in figure 3.3 (Thomas 1989; Thomas 2003; Thomas 1995). This reaction is catalyzed by a single dimeric protein without the release of the intermediate or coenzyme (Thomas 2003). Comparing the K_m values of the human and mouse isoforms showed that the mouse 3 β -HSD VI has a higher affinity for pregnenolone than its 3 β -HSD I isoform (Abbaszade 1997). The human 3 β -HSD I was shown to have a higher affinity for pregnenolone than 3 β -HSD II (Rheaume 1991; Thomas 2002). Coinciding with the latter, Rheaume (1991) showed that the human catalytic efficiency (V_{max}/K_m) of isoform 3 β -HSD I is 5.9- and 4.5-fold higher than isoform 3 β -HSD II using pregnenolone and DHEA as substrates, respectively (Penning 1997; Payne & Hales 2004; Miller & Auchus 2011). In a comparison between Angora and ovine 3 β -HSD enzyme activity, Goosen (2010) showed that DHEA has a higher affinity for Angora 3 β -HSD than ovine 3 β -HSD. In addition Goosen (2010) also showed that 17 α -hydroxypregnenolone has a higher affinity for ovine 3 β -HSD than Angora 3 β -HSD. Contrarily to belief that cytochrome b₅ only stimulates the 17,20-lyase activity of CYP17 enzyme, Goosen (2011) showed that cytochrome b₅ also augments 3 β -HSD activity. Goosen (2011) showed that the coexpression of cytochrome b₅ with Angora 3 β -HSD, in nonsteroidogenic COS-1 cells, increases Δ^4 production from their respective Δ^5 substrates compared to Angora 3 β -HSD expression alone. Coexpression of cytochrome b₅ with ovine 3 β -HSD in nonsteroidogenic COS-1 cells only show a significant increase of progesterone and androstenedione production from their respective substrates, pregnolone and DHEA. This implies that cytochrome b₅ augmentation of 3 β -HSD occurs in a species and substrate specific manner (Goosen 2011).

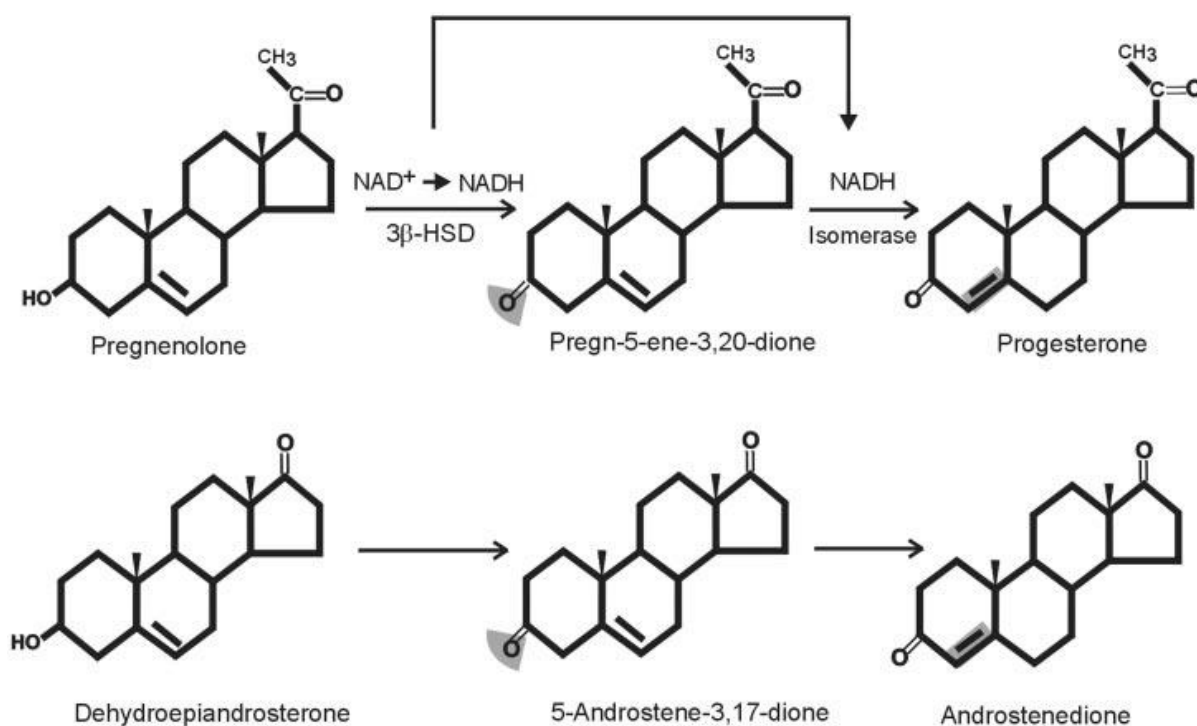


Figure 3.3. Enzymatic reaction catalyzed by human 3βHSD I and II and mouse 3βHSD I and VI. The enzyme catalyzes the dehydrogenation of the 3β-hydroxyl group yielding a Δ⁵-3-keto intermediate and reduced NADH that activates isomerisation of the Δ⁵-3-keto to yield the Δ⁴-ketosteroid. Reproduced from (Payne & Hales 2004).

3.2.2. Molecular structure

The microsomal 3β-HSD enzyme, with a molecular mass of 42-kDa, is a product of a distinct gene (Abbaszade 1997; Miller & Auchus 2011). These enzymes are primarily membrane-bound in microsomes and mitochondria (Thomas 1989). The mouse has multiple isoforms of the 3β-HSD enzyme located on chromosome 3, while only two human isoforms exist, located on chromosome 1p13.1 (Payne & Hales 2004; Miller & Auchus 2011). The human type 1 and type 2 enzymes share 93.5% amino acid identity and are biochemically and enzymatically very similar (Miller & Auchus 2011). Stopped-flow spectroscopy studies show that NADH activates the isomerase activity by inducing a time-dependant conformational change in the enzyme, suggesting that the 3β-HSD and isomerase domains of the enzyme are linked by a shared coenzyme domain that functions both as the binding site for NAD⁺ during the 3β-HSD reaction and as the coenzyme domain for the allosteric activation of the isomerase reaction (Thomas 1995). The 3β-HSD isoenzymes belong to the short-chain alcohol dehydrogenase superfamily, mainly determined by the nucleotide-binding site sequence located at the amino terminus. It

consists of a β -strand, α -helix, β -strand in a fold that provides a hydrophobic pocket for the AMP part of the nucleotide factor. The turn between the first β -strand and the α -helix is a glycine-rich segment, Gly-X-X-Gly-X-X-Gly, similar to the common Rossmann fold sequence Gly-X-Gly-X-X-Gly conserved among most NAD(H)-binding enzymes (Scrutton 1990). This well-conserved glycine-rich fragment forms a hydrophobic pocket that allows close association of the AMP part of the cofactor. A preliminary study of rat type III enzyme has targeted Asp (Tapia Freses 1965) as the amino acid that may be responsible for the strict NAD⁺ specificity of the enzyme (Simard 1996). More recent mutagenesis studies in human type I enzyme demonstrate that the D36A/K37R mutant shifts cofactor preferences of both 3 β -HSD and isomerase activities from NAD(H) to NADP(H), thus showing that the two activities utilize a common coenzyme domain (Thomas 2003). Affinity labelling of purified human type I identified two tryptic peptides, comprising amino acids Asn176 to Arg186 and Gly251 to Lys274 that contain residues involved in the putative substrate-binding domain (Thomas 1993). These studies have shown that the Gly251 to Lys274 peptide was associated with the site of isomerase activity, whereas Tyr253 appears to function as the general proton donor in the isomerase reaction (Mason 1998). His261 also appears to be a critical residue for the 3 β -HSD activity (Thomas 1998). Additional kinetic analyses of D257L and D258L mutants suggest that this region is part of the isomerase substrate domain (Thomas 2003). In contrast to other short chain dehydrogenases with a single catalytic Y-X-X-X-K motif (Chen 1993; Penning 1997; Tanabe 1998), there are two potential catalytic motifs (Y154-X-X-X-K158 and Y269-X-X-XK273) in the primary structure of all 3 β -HSDs. Human type I and type II only differ at position 156 in this motif, type I having a tyrosine whereas type II has a histidine residue. The H156Y mutant form of the type I enzyme shifts the substrate kinetics for DHEA and pregnenolone to the same K_m and V_{max} values exhibited by the type II enzyme; thus, H156 in the type I vs. Y156 in type II 3 β -HSD accounts for the substantially higher affinity of the type I 3 β -HSD activity for these substrates and inhibitor epostane relative to the type II enzyme (Thomas 2002). There are two membrane-binding domains lying between residues 72 and 89 in the NH₂-terminal region and between residues 283 and 310 in the COOH-terminal region. Confirming the latter, the deletion of the 283–310 region causes the enzyme to localize in the cytosol without affecting its activities (Thomas 1999). The region is therefore a membrane-binding domain of 3 β -HSD that can be deleted without compromising enzyme function (Thomas 1999; Thomas 2001). Deletion of residues 72–89 in the NH₂-terminal region produces

a mutant protein that is distributed among the microsomes, mitochondria, and cytosol (Thomas 1999). Because 28% of the 3 β -HSD and isomerase activities remain in the membranes of microsomes and mitochondria, the presence of the 283–310 domain in this mutant allows the protein to retain significant hydrophobicity. However, a majority (72%) of the protein is shifted into the cytosol, so the 72–89 region does contribute to membrane association. The 8-fold loss of both 3 β -HSD and isomerase activity that results from the 72–89 deletion underscores the importance of this region to enzyme function (Thomas 1999). The data obtained by Thomas' group (Thomas 1999) with the human type I enzyme are consistent with previous studies in which the increased polarity of the domain between residues 75 and 91 in the rat type II 3 β -HSD/isomerase was responsible for it having much lower activity than the rat type I enzyme (Simard 1991). Thus, the presence of this highly conserved hydrophobic domain may be crucial to activity in the entire 3 β -HSD gene family (Simard 2005).

3.2.3. Regulation of 3 β -HSD expression

Most *in vivo* regulation studies on the 3 β -HSD isoforms have been performed in the rat and may be relevant to their human orthologs. Interest in the regulation of the adrenal 3 β -HSD exists because of its pivotal role in adrenal steroidogenesis and the knowledge that deficiencies in this enzyme can result in congenital adrenal hyperplasia. Studies on the rat adrenal 3 β -HSD revealed that its mRNA was up-regulated by ACTH in intact male rats and down-regulated by corticosterone. Hypophysectomy reduced mRNA levels markedly, and these could be elevated by the administration of ACTH (Trudel 1991). These data indicate that the gene is regulated by the adrenal-pituitary axis in a manner consistent with controlling circulating glucocorticoid levels. The stimulating effect of ACTH on 3 β -HSD expression has also been observed in bovine adrenocortical cells (Naville 1991). The protein kinase A- dependent signaling mechanism that results in altered transcription of the 3 β -HSD gene, however, has not been fully elucidated. Interest in the regulation of the ovarian 3 β -HSD exists because it catalyzes the final step in progesterone biosynthesis. Down-regulation of this enzyme may contribute to declining progesterone levels, which is a sign of luteolysis. Collectively, the studies on the regulation of rat tissue specific isoforms of 3 β -HSD indicate that expression is under the control of trophic hormones and that alterations in expression occur in response to the need to change circulating steroid hormone levels. The ability of the appropriate trophic hormone to increase 3 β -HSD mRNA levels and protein and enzyme activities on demand in the adrenal, ovary, and testis

points to common mechanisms of regulation that involve cAMP. Gene regulation studies on human 3β -HSD type I and type II have been performed in human choriocarcinoma (JEG-3) cells and human adrenocortical tumor cell lines H295 cells, respectively. This was due to type I being expressed at high levels in syncytial trophoblasts and type II being expressed at high levels in the adrenal cortex. In human JEG-3 cells, 3β -HSD is up-regulated by a cAMP-dependent mechanism and by phorbol esters. The phorbol ester response is not mediated by a diacylglycerol-dependent kinase (Tremblay & Beaudoin 1993). The increases in steady state levels observed in 3β -HSD mRNA after treatment with cAMP or phorbol esters can be observed in the presence of cycloheximide and occur independently of new protein synthesis. In human adrenal cells, activators of the protein kinase A signaling pathway [*e.g.* forskolin and; (Bu)₂cAMP] enhance the production of dehydroepiandrosterone and androstenedione with a concomitant increase in steady state levels of type II 3β -HSD and 17α -hydroxylase (CYP17). In contrast, phorbol ester treatment dramatically elevated 3β -HSD mRNA but attenuated 17α -hydroxylase mRNA. The 59-flanking region of the 3β -HSD type II gene was found to contain a consensus sequence for the orphan nuclear receptor steroidogenic factor 1 (SF-1) (Penning 1997). The functionality of the SF-1 site was tested in promoter-chloramphenicol acetyl transferase constructs in which the SF-1 site was either present or deleted. It was found that the phorbol ester response had an absolute requirement for the SF-1 element, and that the response was further enhanced if the cDNA encoding for SF-1 was cotransfected (Bird 1996). In an attempt to identify *cis*-regulating elements that may control tissue-specific expression of the type II 3β -HSD genes in the adrenal, it was found that a strong positive regulatory element exists in the first intron of the type II gene. This 3b 1-A element was able to drive transcription of a reporter gene in adrenal cells (SW13) and was found to bind four proteins including SP-1 (Guerin 1995). Therefore, this intronic element appears to be important for gene transcription but is not responsible for the tissue-specific expression of the type II gene (Guerin 1995; Penning 1997).

Along with ACTH, PRL, hCG and phorbol esters, AII also plays a role in 3β -HSD regulation. The effect of AII differs from that of ACTH with respect to adrenal enzyme expression, and species differences are notable. Specifically, the expression of 3β -HSD is potently increased by AII stimulation alone in human (McAllister & Hornsby 1988; Lebrethon 1994; Rainey 1994; Bird 1995; Bird 1996) and bovine (Rainey 1991) adrenal cortical cells. Species differences in adrenal steroidogenic response are most apparent when AII is combined with

ACTH or other factors that activate the cAMP signaling pathway, particularly with respect to 3β -HSD expression. In ovine (Mason 1992) and bovine (Rainey 1991) adrenal cells exposed to ACTH and AII, there is some attenuation of 3β -HSD expression, while in human adrenal cells, there is a small increase in 3β -HSD expression (Lebrethon 1994; Bird 1996) over the effect of ACTH alone. The differential regulation of CYP17 and 3β -HSD expression can be considered in light of the second messenger pathways invoked after adrenal cortical exposure to ACTH or AII. While the actions of ACTH are clearly mediated through cAMP, the actions of AII are known to be coupled to activation of phosphoinositidase C and thus the Ca^{2+} and protein kinase C signaling cascades. Accordingly, the effects of AII on bovine and human 3β -HSD expression (Rainey 1991), (Lebrethon 1994; Rainey 1994; Bird 1996) can be fully reproduced by tetradecanoylphorbol 13-acetate (TPA), the phorbol ester activator of protein kinase C (Conley & Bird 1997).

3.2.4. Physiological importance of 3β -HSD

Steroid hormones play a crucial role in the differentiation, development, growth, and physiological function of most vertebrate tissues. The major pathways of steroid hormone synthesis are well established, and the sequence of the responsible steroidogenic enzymes has been elucidated (Labrie 1996; Penning 1997; Miller 1998b; Grumbach & Auchus 1999; Mahendroo & Russel, 1999; Labrie 2003; Payne & Hales 2004). For example, in the human, after the conversion of cholesterol to pregnenolone by the mitochondrial side-chain cleavage system, the adrenal cortex may direct pregnenolone toward one of three different pathways. First, pregnenolone may remain as a C₂₁,17-deoxysteroid and proceed down the pathway to produce the mineralocorticoid, aldosterone. Second, it may undergo 17 α -hydroxylation and proceed down the C₂₁,17-hydroxy pathway to form the principal glucocorticoid, cortisol. The third option is that, after 17 α -hydroxylation, it may undergo cleavage of the C₁₇–20 bond to become a C₁₉–17- ketosteroid, leading to the formation of androgens and estrogens. Whichever pathway is followed, the subsequent formation of all classes of steroid hormones relies upon the action of the enzyme 3β -HSD (Simard 1996; Mason 1997; Payne 1997; Simard 2005).

Human 3β -HSD II deficiency can cause a rare form of congenital adrenal hyperplasia (CAH) but is only responsible for 10% of patients with CAH. The remainder 90% of patients have defects in either CYP21 or CYP17 enzymes. In its most severe form, 3β -HSD deficiency results

in the blockade of steroidogenesis in both the adrenal and gonads, and as a result there is an elevated Δ^5 - to Δ^4 -steroid ratio (Penning 1997). In its classic form, genetic females may have clitoromegaly and mild virilization because the fetal adrenal overproduces large amounts of DHEA, a small portion of which is converted to testosterone via extra adrenal 3β -HSD I. In males some androgens are synthesized by peripheral conversion of adrenal and testicular DHEA, but the concentrations are insufficient for complete male genital development so that these males have a small phallus and severe hypospadias (Miller & Auchus 2011).

Since the human 3β -HSD II gene is the predominant form in the adrenal and gonads, it is not surprising that the defects occur in this gene product. Simard and colleagues (Thomas 1994; Thomas 1995; Thomas 1997) analyzed 12 male and four female patients who had defects in this gene. It was found that there were 14 unique point mutations in the 3β -HSD II gene. The combination of these point mutations can lead to two different diseases causing either a salt-wasting defect or a non-salt-wasting defect. Kinetic analysis of the mutants revealed that of the point mutations present in the salt-wasting form, the G15D and the G15A mutants showed a 4-fold decrease in catalytic efficiency for the conversion of pregnenolone to progesterone, and the L108W and P186L mutants resulted in a decrease in catalytic efficiency of 40-fold. Interestingly, the N100S mutant found in the non-salt-wasting form of the disease resulted in a 30-fold decrease in catalytic efficiency. The similar decreases in catalytic efficiency observed with mutations found in the salt and non-salt wasting forms of the disease indicate that they alone cannot explain the genetic lesions responsible for the differences in the two diseases (Penning 1997).

3.3. Conclusion

The catalytic activity of CYP17 appears to vary, being highly characteristic for a species. It is apparent that it is accompanied by other changes in steroidogenic processes that are also species-specific. The levels of 3β -HSD, and the tissue-specific distribution of the enzyme and its isoforms relative to those of CYP17, seem to be important components in the evolution of adrenal and gonadal steroidogenesis. These corresponding physiological adjustments allow a balance between all steroid synthesis by the gonads and the adrenal glands. They also impose constraints on the rate of steroid synthesis by each organ and influence the efficiency of the process. Understanding the points in these pathways that have important effects on the flux of substrates may allow us to predict the consequences of increasing or decreasing the levels of

expression of the enzymes themselves or of inhibiting their activities. This knowledge holds the potential for enabling the development of novel, targeted, therapeutic approaches to treat endocrinological diseases. Thus, a detailed knowledge and understanding of adreno-gonadal steroidogenesis will lead to advances of both basic and applied significance (Conley & Bird 1997). One approach to understanding and obtaining this knowledge is to use the tools that the field of Systems Biology can offer us. Tools such as computational modelling can be used to predict the effects of inhibition and enhancement on enzyme activity, while metabolic control analysis (MCA) can be used to predict the flux of steroids in the system due to specific enzyme activity. In the next chapters I will discuss how Systems Biology was used to form an integral part of this project.



Chapter 4

Systems Biology in steroidogenesis

In this chapter I will discuss a quick overview on Systems Biology; what methods and tools in Systems Biology are needed to further analyse and study a biological system; and where Systems Biology has been used in the field of steroids.

4.1. Introduction

The ultimate goal of Molecular Biology is to understand how regulation, mechanism of action, and maintenance of a system works and how the system interacts and integrates with other systems. The field of Molecular Biology got underway when Watson and Crick (1953) identified the structure of DNA, thus revolutionizing the way biology is pursued. The relevance of their work was that they grounded biological phenomena on a molecular basis. This made it possible to describe every aspect of biology, such as heredity, development, disease, and evolution, on a solid theoretical ground. Since then, the field of Molecular Biology has emerged and enormous progress has been made. Today, we have in-depth understanding of elementary processes behind heredity, evolution, development, and disease. While an understanding of these processes continues to be important, it does not give us an understanding on how an intact system will behave. This is where the field of Systems Biology comes in. Systems Biology encompasses Molecular Biology, a discipline that investigates the complex mechanisms underlying biological systems, by treating the behaviour of genes, proteins, biochemical networks, and physiological responses as integrated parts within a whole system (Chuang, 2010). To accomplish the integration of the latter parts, a collection of large data sets from numerous sources is needed. These sources include genomic, biochemical, proteomic, and metabolomic data (McAuley 2015). This data is then used to inform computational models that are capable of examining, quantitatively and qualitatively, the behaviour of biological systems under a wide variety of conditions (Nigsch 2009; Grandjean 2011). The major advantage of this approach lies in the researcher's ability to model a multitude of complex biochemical events, many of which occur simultaneously (Wilkinson 2009). This is made possible by computational modelling, a tool used in Systems Biology to make quantitative behavioural predictions of a biological system, or in the case of this thesis, biological sub-system.

Computational modelling is the epicentre of systems biology and incorporates a wide variety of quantitative techniques that can aid steroidogenic studies. Using quantitative techniques, such as Ordinary Differential Equations (ODE), a computational model can quantitatively represent the components of a particular cellular pathway and how it responds to stimuli. Computational modelling integrates with other disciplines in Molecular Biology, as quantitative data from diverse fields including genomics, metabolomics, and proteomics can be utilized to inform model construction and refinement. To aid the accurate construction of a computational model, quantitative data sets need to be obtained from technologies with high precision measurements.

4.2. Measurement technologies and experimental methods

As mentioned before, high precision measurements are needed to obtain accurate quantitative data sets. An example in the field of steroidogenesis is the separation and quantification of adrenal steroid metabolites from heterologous expression media through use of an ultra performance liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (UPLC–APCI–MS) (Storbeck 2008b). Another example is the development of a sensitive and rapid UPLC-MS/MS method, for the detection of all the major endogenous adrenal steroids, without derivatisation, from sheep plasma and adrenal primary cells (Hough 2012). Although these studies are currently limited to understanding the components of the system and their local relationship with other components, the combination of experimental work and computational modelling provides a foundation for Systems Biology. High-throughput, comprehensive, and accurate measurement is the most essential part of biological science (Kitano 2001). The data sets obtained from these high precision techniques will be fundamental to the accuracy of a computational model simulating the biological system, sub-system, or metabolic pathway. Computational models do not rely on accurate data sets only to describe the prediction of an analyte in response to different stimuli, but also on the estimation of the parameters that describe the characteristics of an analyte within the system. While expectations are high for a computational approach to overcome limitations in the traditional approach in biology, it will never generate serious results without experimental data upon which computational studies can be grounded. For the computational and systems approach to be successful, measurement of data has to be comprehensive, quantitatively accurate, and systematic. For a systematic measurement to be accurate, the structure and components of the

biological system must first be identified. This can be accomplished through an iterative process of fitting computational model predictions on observed experimental data. Through this process, structural- and parameter identifiability of the system may be studied.

4.3. System Identifiability

Each biological system consists of components that interact with each other within a specific structure. Dependent on this structure, a component has specific characteristics which can be described by mathematical equations. Knowledge of the system structure and the experimental design allows for evaluating the identifiability of the system structure and the rate equations (Faller 2003). Although the structure of the steroidogenic sub-system, applicable to this thesis, is known, all the parameters of the components within the structure are not. This thesis will try to identify the parameters applicable to the sub-system through means of computational modelling.

4.3.1. Parameter identification

Parameter identification in Systems Biology is usually part of an iterative process to develop computational models for biological systems that should have predictive value (Anguelova 2007). The iterative process starts with hypotheses based on the knowledge of the underlying network structure of a pathway which are translated into a system of kinetic equations. Computational models are then constructed using ODEs consisting of the relevant kinetic equations. Furthermore, parameters are either obtained from literature or estimated using the constructed computational models by means of data fitting. Parameter estimation or data fitting typically starts with an educated guess about parameter values and then changes those values to minimize the discrepancy between model and data using a particular measuring method (Anguelova 2007). In this thesis the function `NMinimize` in Mathematica 8 was used where the minimum discrepancy between model and data corresponds to a minimization of the weighted residual sum of squares. Using the estimated or literature obtained parameters, model predictions are made that can be tested with further experiments. If the model predictions give deviances, it could mean that either wrong parameters were used, thus inaccurately estimated, or a wrong model structure has been identified. Therefore, model parameters are best estimated from observed experimental data. Based on these parameter estimates, the model can be

statistically validated if it is able to reproduce the observed dynamic behaviour (Cox & Hinkley 1994).

Using observed experimental data does have its drawbacks in that not all methods are optimally designed to produce continuous and error-free data. Thus, a computational model estimating its parameters on insufficient or incorrect observed data may have dire consequences in terms of structural and practical identifiability. This emphasises the need to find, develop and use optimal designed methods to obtain accurately measured observed data. Estimating computational model parameters using observed experimental data, does not only require optimal designed methods, but also metabolic reactions that are accurately described through mathematical equations. Predicting the course of analytes simultaneously over time needs accurate kinetic equations describing the different ODEs of the model. Thus, practical identifiability plays a crucial role in identifying the correct parameters of a computational model.

4.3.2. Approaches to modelling biological systems

One of the objectives of modelling biological systems is to define the genetic, protein, and biochemical reactions as integrated and interacting networks of an organism, and to characterize the flow of information that links these elements, and their networks, to an emergent biological process (Hood & Perlmutter 2004). There are two broadly described approaches used to assist the latter objective namely ‘top-down’ and ‘bottom-up’. Top-down methods start with data and fit models to them. Traditional statistics is therefore a top-down method, as are machine learning, pattern recognition and (broadly) bioinformatics. This approach can discern meaningful biological relationships and sometimes even quite complex networks; in such cases, they are often referred to as ‘reverse-engineering’. This approach is regularly used to process ‘omics’ data, especially metabolomics (Edwards & Thiele 2013). In contrast to the top-down approach, the bottom-up approach attempt to build models based on existing or acquired knowledge of network behaviour. This include traditional kinetic models, based on a system of differential equations, and stochastic methods. Top-down approaches enable discovery of new and essential components to biological processes without prior knowledge of them. The systems biology cycle begins with a hypothesis tested by perturbing the system. Molecular changes are measured at multiple levels using high-throughput technologies. The obtained data sets will lead to the development of algorithms to infer

predictive models. The system is then mathematically formulated a systems of ordinary differential equations, or stochastic differential equations, to produce a model that can be computationally simulated and tested. Key parameters of the system are derived based on estimates from other models of well-known systems. Simulations will elucidate revisions in the system to produce the desired outputs. Once these characteristics are well defined computationally, they are verified experimentally. Finally, revisions are incorporated, leading to another iteration of computational modelling to validate the dynamics of the system (Garcia-Reyero & Perkins 2011).

4.4. Computational Modelling in Steroidogenesis

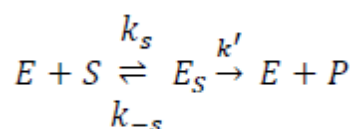
The aim of this thesis is to develop a computational model that describes the interaction of the reactions between the intermediate steroids catalyzed by enzymes CYP17 and 3 β -HSD. In the previous chapters the importance of the steroidogenic system and the purpose of using Systems Biology to better understand the behaviour of a biological system as a whole, were discussed. In this section I will discuss the role of computational modelling, a tool used in Systems Biology, in assisting the study of steroidogenesis.

4.4.1. Enzyme kinetics

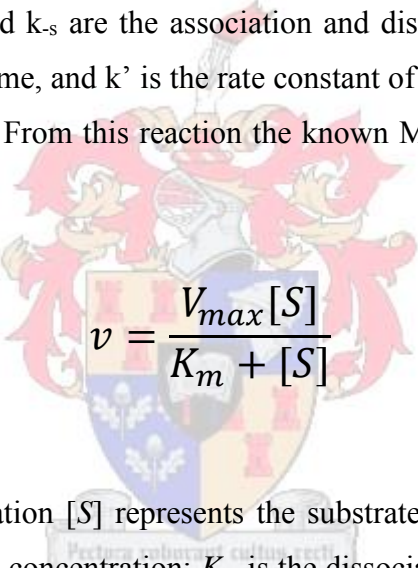
The factors that affect the rates of enzyme-catalyzed reactions include enzyme concentration; ligand concentrations (substrates, products, inhibitors and activators); pH; ionic strength; and temperature (Segel 1993). Enzyme kinetics is a powerful tool, used to study the effect of the different factors on enzyme activity. Analyzing the effect of these factors may lead to construction of a model for a specific enzyme-catalyzed reaction which in turn can be used to form a kinetic equation for a model. Having the kinetic equation, we can test a model experimentally. In this thesis a derivative of the Michaelis-Menton equation was used with the assumption that the reactions follow random order kinetics with product inhibition. How this equation was derived will be discussed in the next section.

4.4.1.1. The Michaelis-Menten equation

The Michaelis-Menten model of an enzyme-catalyzed reaction contains two elementary steps. The first step is the reversible association of an enzyme with a substrate, resulting in the formation of an enzyme-substrate complex. The second step is the conversion of the substrate into a product and then the product is released from the enzyme (Cornish-Bowden, 2001b). Thus, a whole enzyme-catalyzed reaction can be described by the following scheme.



In this reaction scheme, k_s and k_{-s} are the association and dissociation rate constants of the substrate to and from the enzyme, and k' is the rate constant of the conversion of the enzyme-substrate complex to product. From this reaction the known Michaelis-Menten rate equation was derived.

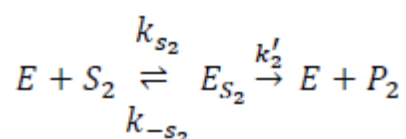
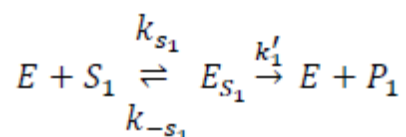


$$v = \frac{V_{max}[S]}{K_m + [S]}$$

In the Michaelis-Menten equation $[S]$ represents the substrate concentration; v is the initial velocity at any given substrate concentration; K_m is the dissociation constant concentration of S ; and V_{max} represents the catalytic rate of the enzyme when the enzyme is saturated with S . Many steroidogenic analysis have used the Michaelis-Menten equation, or derivative thereof, in calculating parameter values of the enzymes correlated to the specific pathways. One of these can be seen in the work of Goosen et al (2011) where the K_m and V_{max} values of Angora and ovine 3β -HSD towards PREG, 17OHPREG and DHEA were respectively determined.

Although the Michaelis-Menten equation is derived from a simple reaction comprising of one ligand, one catalytic site and one enzyme-substrate complex, the steroidogenic subsystem in this thesis comprises of many reactions and many ligands binding to the same enzymes. Both enzymes 3β -HSD and CYP17 can catalyze more than one reaction; therefore reactions with

different substrates catalyzed by the same enzyme must compete with each other for the same enzyme (Nguyen 2012). Here, we review the model for the case when there are two competing substrates that individually give Michaelis-Menten kinetics when studied separately (Cornish-Bowden, 2001a). The schemes of the two reactions in this simplest case are as follows.



As for the previous reactions, using the law of action of chemical kinetics, two Michaelis-Menten rate equations for an enzyme with two competing substrates, were derived.

$$v_1 = \frac{V_{max1} \left[\frac{S_1}{K_{m1}} \right]}{1 + \left[\frac{S_1}{K_{m1}} \right] + \left[\frac{S_2}{K_{m2}} \right]}$$

and

$$v_2 = \frac{V_{max2} \left[\frac{S_2}{K_{m2}} \right]}{1 + \left[\frac{S_1}{K_{m1}} \right] + \left[\frac{S_2}{K_{m2}} \right]}$$

Although these rate equations represent two substrates competing for the same enzyme, the sub-system studied in this thesis comprises of 7 steroid intermediates that compete for the same enzyme. For this reason, a derivative of the Michaelis-Menten rate equation, where numerous substrates compete for the same enzyme, is needed.

Thus, using the law of action of chemical kinetics for each reaction in the steroidogenic sub-system, the following generic rate equation for steroid intermediate conversion by an enzyme, was derived:

$$v_x = \frac{V_{max} \frac{S}{S_{0.5}}}{1 + \frac{S}{S_{0.5}} + \sum_{i=1}^n \left(\frac{P_i}{P_{0.5_i}} \right)}$$

where x = the corresponding reaction

P = is the vector of all the other steroid intermediates influencing the reaction,

S = is the substrate concentration

$S_{0.5}$ = is the K_m concentration of S

$P_{0.5}$ = is the K_m concentration of the corresponding steroid intermediate

V_{max} = is the catalytic rate of the enzyme when the enzyme is saturated with S

n = is the number of steroid intermediates influencing the reaction

i = is the iterator for the steroid intermediates.

This generic rate equation for steroid intermediate conversion was used to form ordinary differential equations (ODE's). These ODE's in turn were used to construct a computational model that can estimate kinetic parameters using progress curve analysis and thus estimate the concentration of steroid intermediates over time.

The next section will be a discussion on why progress curves were used to estimate kinetic parameters instead of using initial reaction rates.

4.4.1.2. Progress curve analysis vs initial rate analysis

The derivation of reaction rates from the changing concentrations is always an approximation with implicit error, but it is widely used in the determination of kinetic parameters because of the simplicity of its mathematical description with differential rate equations (Nikolova & Kolev 2008). There are two principal ways for determining the kinetic parameters of enzyme-catalysed reactions: (i) the method of initial velocities (initial rate analysis) and (ii) the method of full kinetic curves (progress curve analysis). Of these the method of initial rate analysis, most widely used when the parameters of the Michaelis- Menten equation are to be calculated, is generally employed to ensure more accurate results (Atkins & Nimmo 1980). However, difficulties are bound to arise during determination of the initial velocity of a reaction at low

substrate concentration (K_m approx. $10\mu\text{M}$) or solubility, high degree of product inhibition or in investigations of unstable enzymes (Dagys 1986). In these cases the determination of the initial velocity on the basis of product concentration at a fixed time of a reaction ($\Delta p/\Delta t$, where p is the product concentration and t is time) is most likely to generate errors, and therefore the use of progress curve analysis is more reliable (Atkins & Nimmo 1980).

Because progress curves contain much more information than is available in the initial slopes of reaction curves, the number of experimental manipulations, and the attendant possibility of random error, can be reduced if a few curves are recorded in place of a larger number of initial slopes (Schwert 1969). In addition, it has been demonstrated that progress curves are an excellent method for obtaining parameter estimations, with the benefit of less experimental time, even when the reaction is reversible and shows product inhibition (Canela & Franco 1986). However, the presence of relatively small errors in the data, either random or systematic, leads to relatively large errors in the estimates of kinetic parameters (Atkins & Nimmo 1980). Although progress curve analysis has experimental benefits, the analysis of initial rates still remains the first choice for enzyme characterization for many biochemists, although this methodology leads to an enormous loss of the information related to the reaction kinetics (Duggleby 2001). In addition, it has been suggested to estimate initial rates using progress curves (Atkins & Nimmo 1980). However, as stated earlier, the initial rate methods make use of only a small proportion of the information in any progress curve. Thus, attempts have also been made to obtain enzyme kinetics parameters by fitting the computed curves over the entire concentration time-courses of the Michaelis–Menten-type reactions through numerical integration (Duggleby 2001; Kuzmic 2009) or algebraic solutions to the rate equation in terms of the Lambert W function (Beal 1982; Schnell & Mendoza 1997; Goličnik 2012). However, a surprisingly large number of enzymes do not display conventional Michaelis–Menten kinetics. Many enzymes have specialized sites where a second substrate molecule can bind and act as an allosteric effector (Goličnik 2013). In these cases the conventional Michaelis–Menten equation is subject to addition of these allosteric effectors as in the case of cytochrome b_5 on CYP17 and 3β -HSD in steroidogenesis (unpublished work by Conradie & van Schalkwyk, 2010).

Although progress curve analysis has its drawbacks in modelling diagnostics, implementation on computers and longer time for computation, the latter two are fast becoming possible due to the computing advances and power available today. Thus the progress curve is a method

used among enzymologists for kinetic parameter estimations while saving valuable experimental time (Canela & Franco 1986). Likewise, this method can be used as one of the first steps in studying complex systems such as steroidogenesis. For example, in a coupled system such as steroidogenesis, each reaction can be studied by the progress curve method so as to obtain the estimated kinetic parameters and mechanism of each enzyme in a short amount of time. An example of progress curve analysis being used in steroidogenesis can be seen in the study of the characterisation of the catalytic activity of human steroid 5α -reductase towards novel C19 substrates (Quanson 2015).

Progress curve analysis was used in this study of a steroidogenic subsystem comprising of CYP17 and 3β -HSD. First the reactions catalyzed by CYP17 was studied and kinetic parameters were estimated using minimal experimental data and time. The same was done for the reactions catalyzed by 3β -HSD. After the kinetic parameters of both enzymes for each of the various steroid intermediates were obtained, a computational model was constructed containing the integrated rate equations of all the reactions in the subsystem. In addition, initial rates for pregnenolone conversion by CYP17 and 3β -HSD respectively, were estimated using progress curves. These initial rates were used to normalise each experiment during subsequent kinetic parameter determinations.

4.5 Conclusion

In the study of biological systems through Systems Biology, accurate quantitative experimental approaches were shown to be critical in determining enzyme parameters using computational models. This requires a combination of new analysis techniques, measurement technologies, experimental methods, software tools, and new concepts of looking at biological systems (Kitano, 2001). In this study a small adrenal steroidogenic subsystem was studied by using a Systems Biology approach where theory, modelling and experimentation were employed in an iterative cycle. This iterative cycle was used to construct a kinetic model that describes the steroidogenic reactions catalyzed by CYP17 and 3β -HSD simultaneously. The main aim of this study was to see whether a generic rate equation, that describes the catalysis by the adrenal steroidogenic enzymes, could be constructed. A derivative of the Michaelis-Menton equation was used with the assumption that the reactions follow random order kinetics with product inhibition. Progress curve analysis was used to estimate the parameters for the enzymes CYP17 and 3β -HSD to their respective steroid intermediate. Such a generic rate equation should not only be able to describe the competitive behaviour of the steroid intermediates for the catalytic

site of the respective enzymes, but should also give insight to the flux of steroid intermediate production. This will be the focus of the next chapter.



Chapter 5

COMPUTATIONAL MODELING OF THE OVINE ADRENAL STEROIDOGENIC SUBSYSTEM COMPRISING OF CYP17 AND 3 β HSD

As mentioned before, the goal of this thesis is to construct and validate a computational model that can be used to estimate the concentrations of the various steroid intermediates in the ovine adrenal steroidogenic sub-system comprising of enzymes CYP17 and 3 β -HSD, over time. The importance of accomplishing this goal is discussed in the literature chapters foregoing this chapter. In this chapter methods and materials used to achieve the goal stated above will be discussed, as well as the results and discussion of the results.

The accurate detection and quantification of endogenous steroid hormones from experimental subjects remains a challenging task, due to structural homology and the low concentrations at which such hormones are normally present in biological fluids (Soldin & Soldin 2009; Makin & Gower 2010; Rauh 2010). Routine quantification of endogenous steroid hormones is based predominantly on immunoassay methods, which are easily accessible to hospitals and laboratories (Soldin & Soldin, 2009; Kushnir 2011). However, immunoassays are steroid specific, hampered by cross-reactivity and prone to the overestimation of true steroid concentrations (Middle 1998; Dorgan 2002; Marks 2002; Valdes & Jortani 2002; Minutti 2004; Etter 2006; Soldin & Soldin 2009). Gas chromatography linked to mass spectrometry (GCMS) is considered the golden standard in steroid analysis and can quantify a large number of steroids at great sensitivity (65 steroids in 50 min: Ha et al. 2009). However, sample preparations involved in GC-MS can be laborious and complex, thus limiting the throughput (Ha et al. 2009; Rauh 2010; Shackleton 2010; Kushnir 2011).

The development of modern HPLC resulted in significant improvements in resolution and selectivity of steroids over the traditionally used thin layer chromatography. Expensive radiolabeled tracers and radio-active flow detection are, however, required to quantify steroids that do not absorb in the UV-region, such as the Δ^5 -steroids (PREG, 17-OHPREG and DHEA) (Makin & Gower 2010; Rauh 2010; Shackleton 2010). In addition, conventional HPLC methods require relatively long run times, in excess of 50 minutes, to achieve the desired

resolution (Miksik 1999; Nithipatikom 2005). Coupling MS to HPLC has made MS available as a universal detection alternative for liquid chromatography. Run times can be significantly reduced as a number of precursor and product ions can be detected simultaneously and selectively (Hauser 2008; Soldin & Soldin 2009; Rauh 2010; Shackleton 2010). Further developments in the field led to the incorporation of UPLC-MS/MS as an analytical tool which allows for smaller sample volumes together with higher throughput while achieving good resolution in shorter run times.

Various studies have been successful in achieving the quantification of 7 to 21 endogenous steroids in less than 18 minutes with UPLC-MS/MS (Guo 2006; Carvalho 2008; Janzen 2008; Cho 2009; Simersky 2009; Xing 2011). In this thesis the method used by Storbeck (2008b) as well as an in-house HPLC method (section 5.1.3.1) was used to separate and quantify the steroid intermediates described in this study so far.

Goosen (2010) showed that enzymes are transcribed at a similar level for co-transfections in nonsteroidogenic COS-1 cells and that differences observed in steroid metabolism are due to catalytic differences, rather than inherent differences in the level of transcription and translation. Goosen (2010) also provided evidence that it is not necessary to determine the level of enzyme expression with techniques such as Western blot analyses, which requires higher concentrations of recombinant proteins than the concentrations that are present in nonsteroidogenic COS-1 cells. Thus, for his study nonsteroidogenic COS-1 cells were used to carry the enzyme activity assay.

Furthermore, Conradie (2010, unpublished) constructed a kinetic model showing the interaction between CYP17 and the Δ^5 -steroid intermediates PREG, 17-OHPREG and DHEA. This kinetic model consists of ODE's (Equations 17-19) that can estimate the concentration of the afore mentioned steroid intermediates over time.

Nonsteroidogenic COS-1 cells transfected with CYP17 were incubated with PREG and experimental data was obtained (Table 5.3). The kinetic model mentioned above was used to describe the competitive interaction between CYP17 and all the steroid intermediates partaking in the conversion of PREG to 17-OHPREG (Equation 12); and 17-OHPREG to DHEA (Equation 13)

In the rest of this chapter, further work done on CYP17 was built on the work that Conradie (2010, unpublished) has done. This entails the construction of a kinetic model showing the

interactions between CYP17 and the Δ^4 -steroid intermediates PROG, 17-OHPROG, 16-OHPROG and A4 as well as the validation of both the kinetic models.

5.1. Materials and methods

5.1.1. Materials

Nucleobond® AX plasmid purification kits were supplied by Macherey–Nagel (Duren, Germany). Nonsteroidogenic COS-1 cells were obtained from the American Type tissue Culture Collection (Manassas, VA, USA). Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Fetal calf serum and bacterial culture medium were purchased from Highveld Biological (Lyndhurst, SA) and Difco Laboratories (Detroit, MI, USA), respectively. Penicillin–streptomycin, trypsin–EDTA were purchased from Gibco BRL (Gaithersburg, MD, USA). [7-3H]-PREG was purchased from PerkinElmer Life Sciences (Boston, MA, USA). PREG, 17-OHPREG, DHEA, Dulbecco's modified Eagle's medium (DMEM) were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

5.1.2. Ovine CYP17 and 3 β -HSD enzyme activity assay in transfected COS-1 cells

Nonsteroidogenic COS-1 cells were grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin and 0.12% NaHCO₃. Confluent cells were plated into dishes, 1.0×10^5 cells/ml, 24h prior to transfection. Nonsteroidogenic COS-1 cells were transfected with the ovine CYP17 plasmid construct (previously cloned by and obtained from Storbeck (2007)) and 3 β -HSD plasmid construct (previously cloned by and obtained from Goosen (2010)), respectively using TransIT®-LT1 transfection reagent (Mirus), according to manufacturer's instructions. After 48h transfected cells were seeded into 12-well plates, 1.0×10^5 cells/ml, and incubated a further 24h under the same conditions mentioned above. All transfections were performed using a total of 1 μ g plasmid DNA. 3 β -HSD enzyme activity was assayed by adding 2 μ M and 5 μ M of each substrate PREG and [3H]PREG respectively alongside mixtures of 2 μ M PREG/2 μ M PROG and 5 μ M PREG/5 μ M PROG respectively. The experiment was repeated using substrates 17-OHPREG/17-OHPROG and DHEA/A4, respectively. Another 3 β -HSD

enzyme activity was assayed by adding 3 μ M of each substrate (PREG; 17-OHPREG; DHEA) simultaneously alongside a mixture of 3 μ M of each substrate PREG, PROG, 17-OHPREG, 17-OHPROG, DHEA and A4. CYP17 enzyme activity was assayed by adding 2 μ M and 3 μ M of each substrate PREG and [3H]PREG respectively. The experiment was repeated by adding 1.5 μ M, 2 μ M and 3 μ M of substrate PROG. Another CYP17 enzyme activity was assayed by adding 3 μ M of each substrate PREG and PROG simultaneously.

The initial reaction rate of 5 μ M PREG and [3H]PREG conversion with the respective enzymes CYP17 and 3 β -HSD were assayed alongside each corresponding experiment to be used to standardize each experiment (see section 5.1.5). At specific time intervals over a period of time (7 h for 3 β -HSD transfected cells and 12h for CYP17 transfected cells), aliquots (500 μ l) were removed from the assay mixtures. Each time interval consisted of triplicate aliquots. The steroid metabolites were subsequently extracted by liquid–liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 20 min and centrifuged at 500 \times g for 5 min. The water phase was aspirated off, and the dichloromethane phase transferred to a clean test tube and dried under N₂. The dried steroid residue was redissolved in 200 μ l methanol prior to HPLC or UPLC–MS analysis.

5.1.2.1. Steroid separation and quantification by HPLC

Chromatography was performed on a SpectraSYSTEM P4000 high performance liquid chromatograph (Thermo Separation™ Products, San Jose, CA, USA) coupled to a SpectraSYSTEM AS3000 automatic injector (Thermo Separation™ Products, San Jose, CA, USA) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL, USA). The ratio of scintillant to column effluent was 3:1. Steroid metabolites were separated on a Phenomenex® C12 (60 mm \times 150 mm, 4 μ m) column at a flow rate of 1 ml min⁻¹. The mobile phases for PREG separation from PROG consisted of solvent A (75% methanol: 25% water) and solvent B (methanol). An isocratic flow consisting of 100 % A for 2 minutes was applied, followed by a 9 minute linear gradient to 100% B and isocratic elution with 100 % solvent B for 2 min. A 3 minute linear gradient returned the column to 100% A. The total run time per sample was 16 minutes and the injection volume was 90 μ l.

5.1.2.2. Steroid quantification by UPLC

The metabolites 17-OHPREG, 17-OHPROG, DHEA and A4 were analyzed by UPLC-coupled atmospheric pressure chemical ionization (APCI) mass spectrometry (MS). Chromatography was performed on an ACQUITY ultra performance liquid chromatograph (Waters, Milford, MA, USA). The substrates were separated from their respective metabolites on a Waters UPLC BEH C18 (2.1 mm x 50 mm, 1.7 μ m) column at 50°C flow rate of 0.4 ml min⁻¹. The UPLC was connected to an API Quattro Micro tandem massspectrometer (Waters, Milford, USA) as previously described (Storbeck 2008a)

5.1.3. Kinetic modelling of 3 β -HSD reactions

Rate equations- A kinetic model that describes the interaction between 3 β -HSD and steroid intermediates PREG, 17-OHPREG and DHEA where the reactions initialise conversion to PROG, 17-OHPROG and DHEA respectively, was constructed (See Addendum A). The kinetic model was constructed on the assumption that all the reactions follow random order kinetics as well as product inhibition. From this kinetic model a generic rate equation (Equation 1) was derived to describe the competitive interaction between 3 β -HSD and all the steroid intermediates partaking in the conversion of PREG to PROG (Equation 2), 17-OHPREG to 17-OHPROG (Equation 3) and DHEA to A4, respectively (Equation 4). These rate equations were used in Mathematica 8 (Wolfram Research Inc.) to construct a computational model consisting out of ordinary differential equations (ODE's) that can estimate the concentration of steroid intermediates over time (Equations 5-10).

$$v_x = \frac{Vmax_x \frac{S}{S_{0.5}}}{1 + \frac{S}{S_{0.5}} + \sum_{i=1}^n \left(\frac{P_i}{P_{0.5i}} \right)} \quad (\text{Equation 1})$$

Equation 1: The generic rate equation for 3 β -HSD steroid intermediate conversion where x refer to the same reaction, P is the vector of all the steroid intermediates partaking in the reaction, S is the substrate concentration, $S_{0.5}$ is the K_m concentration of S , $Vmax$ is the catalytic rate of the enzyme when S is saturated with the enzyme, n is the number of steroid intermediates partaking in the reaction and i is the iterator for the steroid intermediates.

$$v_1 = \frac{Vmax_1 \left(\frac{PREG}{PREG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}}} \quad (\text{Equation 2})$$

$$v_2 = \frac{Vmax_2 \left(\frac{17-OHPREG}{17-OHPREG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}}} \quad (\text{Equation 3})$$

$$v_3 = \frac{Vmax_3 \left(\frac{DHEA}{DHEA_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}}} \quad (\text{Equation 4})$$

Ordinary Differential Equations (ODE's)-

$$\frac{dPREG}{dx} = -v_1 \quad (\text{Equation 5})$$

$$\frac{dPROG}{dx} = v_1 \quad (\text{Equation 6})$$

$$\frac{d17-OHPREG}{dx} = -v_2 \quad (\text{Equation 7})$$

$$\frac{d17-OHPROG}{dx} = v_2 \quad (\text{Equation 8})$$

$$\frac{dDHEA}{dx} = -v_3 \quad (\text{Equation 9})$$

$$\frac{dA4}{dx} = v_3 \quad (\text{Equation 10})$$

5.1.4. Kinetic modelling for CYP17 reactions

Rate equations- A kinetic model that describes the interaction between CYP17 and steroid intermediates PROG, 17-OHPROG 16-OHPROG and A4 was constructed (See Addendum A). The kinetic model was constructed on the assumption that all the reactions follow random order kinetics as well as product inhibition. This kinetic model used the same generic rate equation (Equation 1) mentioned earlier and was used to describe the competitive interaction between CYP17 and all the steroid intermediates partaking in the conversion of PROG to 17OHPROG

(Equation 14), 17OHPROG to A4 (Equation 15) and PROG to 16OHPROG (Equation 16). These rate equations were used in Mathematica 8 (Wolfram Research Inc.) to construct a computational model consisting out of ordinary differential equations (ODE's) that can estimate the concentration of steroid intermediates over time (Equations 20-23).

$$v_4 = \frac{Vmax_4 \left(\frac{PREG}{PREG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}} + \frac{16-OHPROG}{16-OHPROG_{0.5}}} \quad (\text{Equation 12})$$

$$v_5 = \frac{Vmax_5 \left(\frac{17-OHPREG}{17-OHPREG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}} + \frac{16-OHPROG}{16-OHPROG_{0.5}}} \quad (\text{Equation 13})$$

$$v_6 = \frac{Vmax_6 \left(\frac{PROG}{PROG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}} + \frac{16-OHPROG}{16-OHPROG_{0.5}}} \quad (\text{Equation 14})$$

$$v_7 = \frac{Vmax_7 \left(\frac{17-OHPROG}{17-OHPROG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}} + \frac{16-OHPROG}{16-OHPROG_{0.5}}} \quad (\text{Equation 15})$$

$$v_8 = \frac{Vmax_8 \left(\frac{16-OHPROG}{16-OHPROG_{0.5}} \right)}{1 + \frac{PREG}{PREG_{0.5}} + \frac{PROG}{PROG_{0.5}} + \frac{17-OHPREG}{17-OHPREG_{0.5}} + \frac{17-OHPROG}{17-OHPROG_{0.5}} + \frac{DHEA}{DHEA_{0.5}} + \frac{A4}{A4_{0.5}} + \frac{16-OHPROG}{16-OHPROG_{0.5}}} \quad (\text{Equation 16})$$

Ordinary Differential Equations (ODE's)-

$$\frac{dPREG}{dx} = -v_4 \quad (\text{Equation 17})$$

$$\frac{d17-OHPREG}{dx} = v_4 - v_5 \quad (\text{Equation 18})$$

$$\frac{dDHEA}{dx} = v_5 \text{ (Equation 19)}$$

$$\frac{dPROG}{dx} = -v_6 \text{ (Equation 20)}$$

$$\frac{d17 - OHPROG}{dx} = v_6 - v_7 \text{ (Equation 21)}$$

$$\frac{d16 - OHPROG}{dx} = v_6 - v_8 \text{ (Equation 22)}$$

$$\frac{dA4}{dx} = v_7 \text{ (Equation 23)}$$

5.1.5. Experiment standardization

A problem to overcome in this study is the variability in transfection efficiency in different cell batches. To overcome this, each experiments' cell batch was standardized using a reference cell batch. The V_{max} of each reaction is subjected to the initial activity (Unit) of the batch cells used within an experiment. Alongside each experiment (obtained from the same cell batch as the experiment) 3 β -HSD transfected nonsteroidogenic COS-1 cells (PREG, 17OHPREG and DHEA) and CYP17 transfected nonsteroidogenic COS-1 cells (PREG and PROG), were respectively assayed with 5 μ M PREG as substrate for a period of 4 hours and its Unit used to standardize the difference between the activity of each experiment. For example, if the Unit of the first experiment was 2.5 μ M/h, it would be used as the reference value which all further experiments will be measured against. If the next experiment (using a different batch of cells, thus having a different initial activity) have a Unit of 1.7 μ M/h, the new experiment Unit would be dividing by the reference Unit. Through this a standardized factor, in this case 0.68, would be obtained by which the new experiment Unit would be multiplied by to be standardized to the reference Unit. In so the experiments can be compared using different cell bathes.

5.1.6. Parameter estimations

A parameter estimation routine was done by fitting the equations mentioned above, to the experimental data obtained from the UPLC-APCI-MS and HPLC analysis. First the standardization factors were obtained (see section 5.1.4) for each experiment. This will be taken into account to standardize the V_{\max} of each reaction as it is factored into the function OBJ (Equation 11) which gives the objective function value. The function of OBJ is to establish the parameter value that gives the smallest difference between the model and experimental data. Trying to establish the parameter values for the different components one at a time is difficult and time consuming. To overcome this obstacle, each unknown parameter was constrained to random values greater than zero and incorporated into the ODEs using Mathematica 8. These new parameter values were incorporated into the function OBJ which was subjected to the Mathematica 8 NMinimize function which tried to minimize the objective function value. The objective function uses kinetic constants as input values and yields a value (the OBJ function) that gives a measure of the goodness of fit. The goodness of fit is measured by how good the kinetic model calculated the steroid intermediate concentrations over time, fits the experimental data mentioned earlier. Both the experimental data and the calculated steroid intermediate concentrations are plotted on the same graph to see the goodness of fit. See Figure 5.1 for an example.

$$OBJ = \sum_{j=1}^b \left[\left(\sum_{i=0}^n (exp_{ji} - mod_{ji}) \right)^2 \right] \quad (Equation 11)$$

Equation 11: The OBJ function is the summation of the smallest difference between experimental and model data of each steroid intermediate where j is the iterator for the steroid intermediates, b is the number of different steroid intermediates, i is the iterator for the square of the different experimental data at a certain time.

5.2. Results for 3 β -HSD reactions

5.2.1. Ovine 3 β -HSD activity in COS-1 cells

The 3 β -HSD enzyme converts Δ^5 -steroid intermediates PREG, 17-OHPREG and DHEA to their Δ^4 -steroid intermediates PROG, 17-OHPROG and A4, respectively. Nonsteroidogenic COS-1 cells were transfected with 3 β -HSD plasmid constructs previously mentioned (Goosen

2010) and individually assayed with the Δ^5 -steroid intermediates mentioned above. Each reaction was assayed over a period of 7 hours.

Four different enzyme activity assays were done with the same batch of 3β -HSD transfected nonsteroidogenic COS-1 cells. The first assay had an addition of $2\mu\text{M}$ substrate (not shown) with the second assay an addition of $5\mu\text{M}$ substrate. The third, a mixture of $2\mu\text{M}$ substrate and $2\mu\text{M}$ immediate product, and the fourth a mixture of $5\mu\text{M}$ substrate and $5\mu\text{M}$ immediate product (not shown). Graphs showing conversion of PREG to PROG (Figure 5.1, Figure 5.4), 17-OHPREG to 17-OHPROG (Figure 5.2, Figure 5.5) and DHEA to A4 (Figure 5.3, Figure 5.6) concentrations over time, were plotted as dots with their respective standard error (SE) bars. The concentrations of each steroid intermediate were calculated from $500\mu\text{l}$ aliquots, obtained from 1.0×10^5 3β -HSD transfected cells in a 1ml volume (see section 5.1.2). The continuous lines on the same graphs represent the steroid intermediate concentrations calculated by the kinetic model over time (see section 5.1.6). Method 1 (see section 5.2.3.2) was used to calculate the steroid intermediates in Figures 5.1 to 5.6.

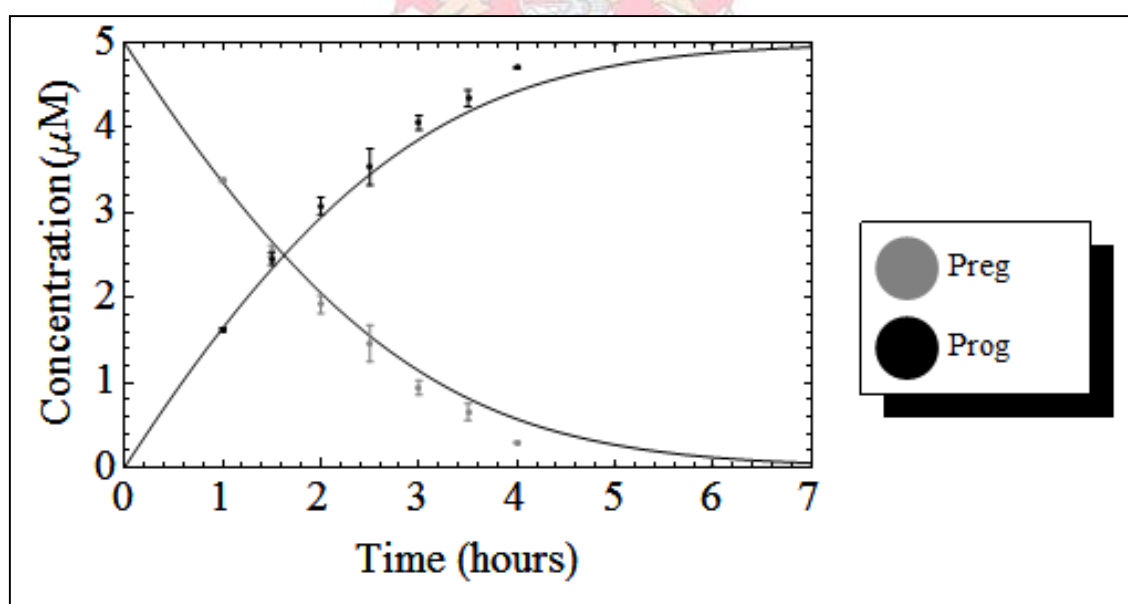


Figure 5.1: Conversion of $5\mu\text{M}$ pregnenolone to progesterone over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from $500\mu\text{l}$ aliquots of each time interval. The initial activity used for V_{max} standardization is $1.254\mu\text{M/h}$. Preg: pregnenolone; Prog: progesterone.

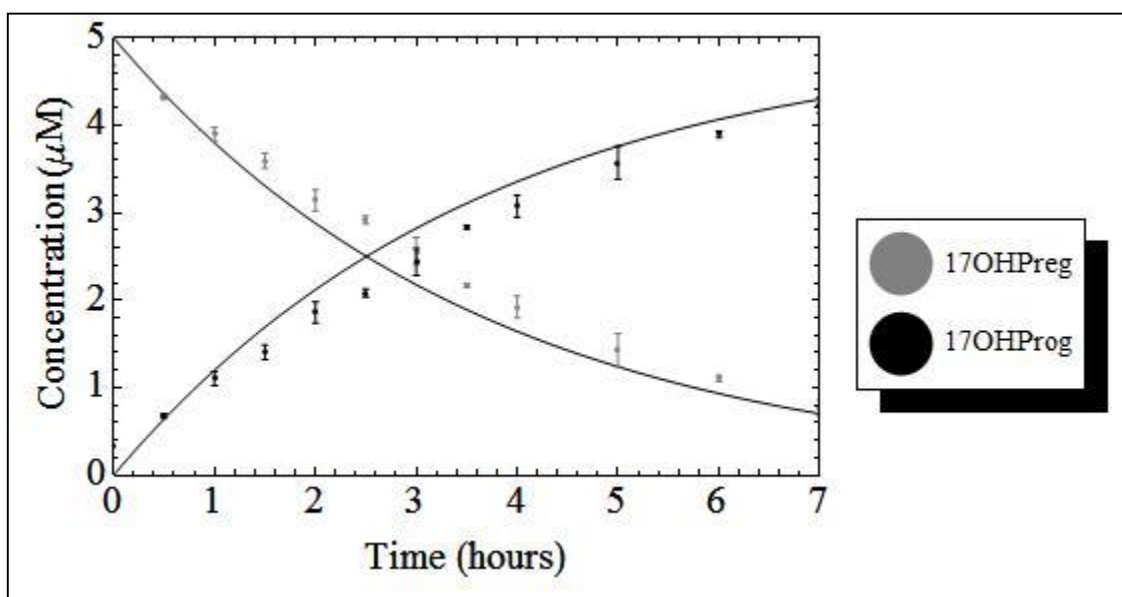


Figure 5.2: Conversion of 5 μM 17-hydroxyprogrenolone to 17-hydroxyprogesterone over 7 in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μl aliquots of each time interval. The initial activity used for V_{max} standardization is $0.662 \mu\text{M/h}$. 17OHPreg: 17-hydroxyprogrenolone; 17OHProg: 17-hydroxyprogesterone.

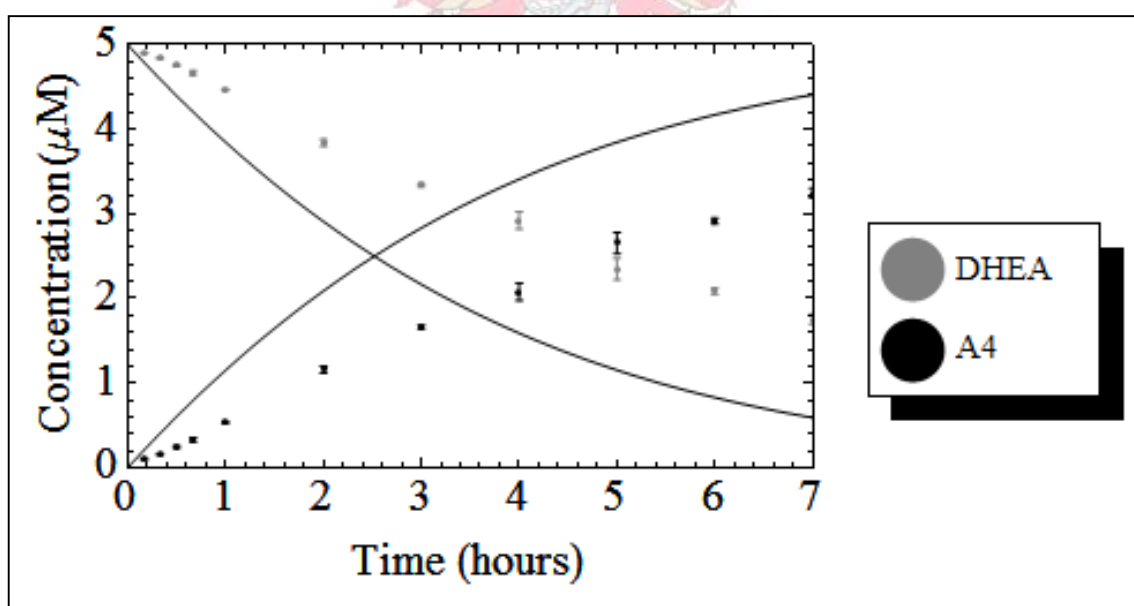


Figure 5.3: Conversion of 5 μM dehydroepiandrosterone to androstenedione over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μl aliquots of each time interval. The initial activity used for V_{max} standardization is $0.628 \mu\text{M/h}$. DHEA: dehydroepiandrosterone; A4: androstenedione.

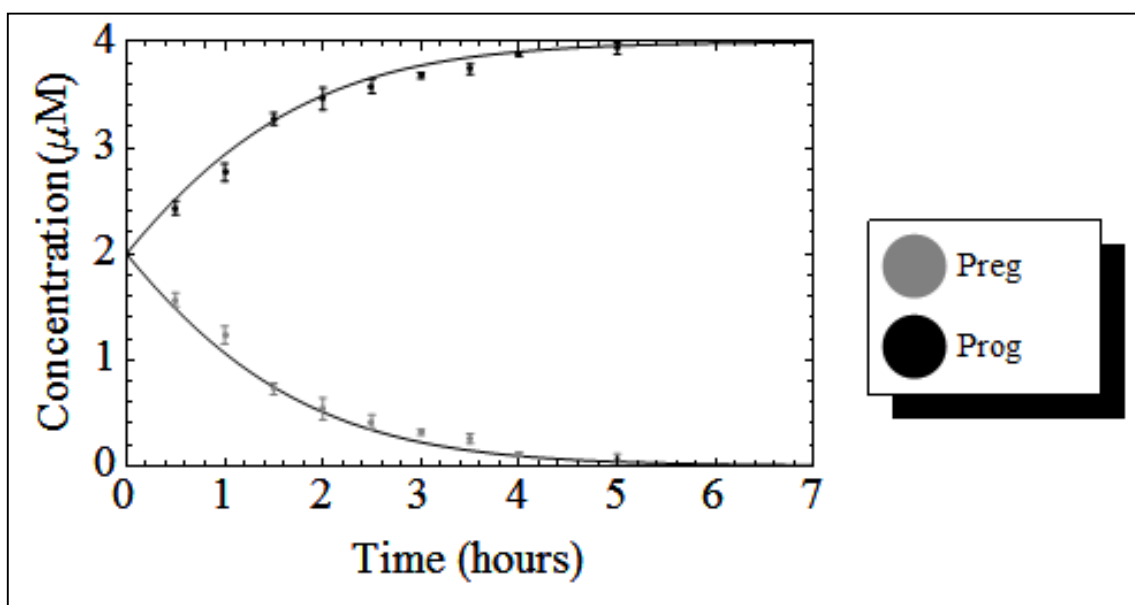


Figure 5.4: Conversion of 2 μM pregnenolone and 2 μM progesterone over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μl aliquots of each time interval. The initial activity used for V_{max} standardization is 1.254 $\mu\text{M}/\text{h}$.

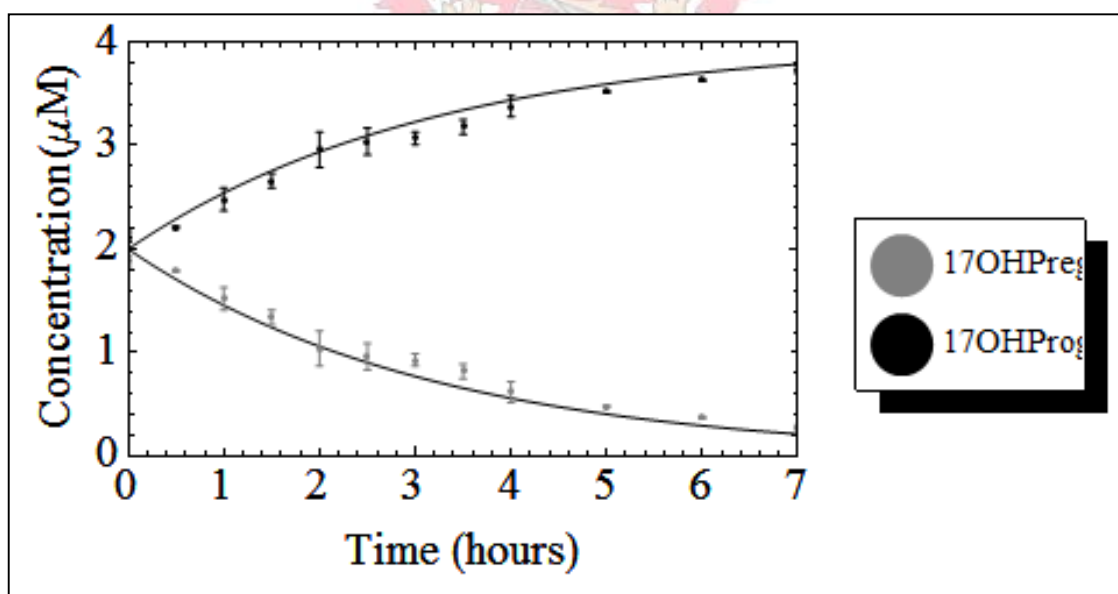


Figure 5.5: Conversion of 2 μM 17-hydroypregnenolone and 2 μM 17-hydroxyprogesterone over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μl aliquots of each time interval. The initial activity used for V_{max} standardization is 0.662 $\mu\text{M}/\text{h}$.

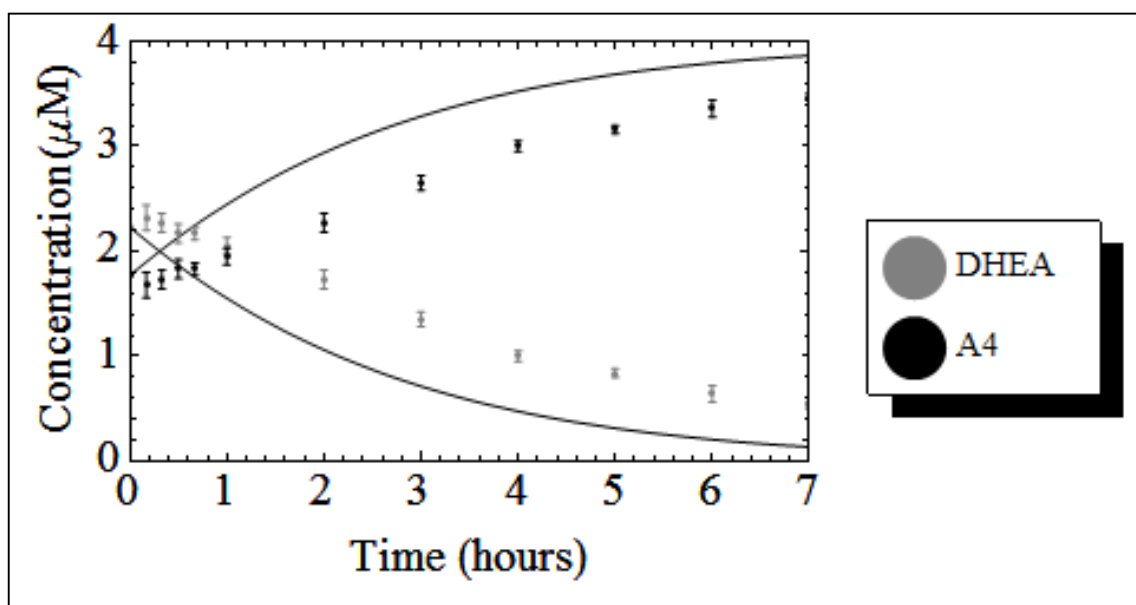


Figure 5.6: Conversion of 2µM dehydroepiandrosterone and 2µM androstenedione over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.628µM/h.

5.2.2. Kinetic analysis of 3β -HSD

A computer model consisting of ODEs was constructed to estimate the parameters V_{\max} and K_m of 3β -HSD for its respective substrates PREG, 17OHPREG and DHEA and respective products PROG, 17OHPROG and A4. Previous work done by Goosen (2010) measured the apparent K_m values for PREG, 17OHPREG and DHEA through Michaelis-Menten plots.

For this reason the model was used to estimate parameters using two methods:

Method 1.) Constrain (fix) the K_m values for the substrates (K_s) to the values obtained from Goosen (2010) while constraining the V_{\max} and K_m values for the products (K_p) to be greater than zero.

Method 2.) Constrain all the parameters (V_{\max} , K_s and K_p) to be greater than zero.

5.2.2.1. Standardization of 3β -HSD experiments

The 17OHPREG and DHEA experiments were standardized by dividing its Unit by the reference Unit, respectively (See section 5.1.5). The 17OHPREG experiment was repeated with two different batches of nonsteroidogenic COS-1 cells. The Unit for the first batch was 0.662µM/h and the second batch 1.066µM/h. These two batches were standardized by a factor of 0.52 and 0.85 respectively. The DHEA experiment has a Unit of 0.628µM/h and was standardized by a factor of 0.50.

5.2.2.2. Estimated parameter values for 3 β -HSD reactions

The apparent K_m values obtained from Goosen (2010) are 4.7 μ M (PREG), 0.9 μ M (17OHPREG) and 1.8 μ M (DHEA). For method 1 the model was subjected to these fixed apparent K_m (K_s) parameters and the $V_{max}/Unit$ and K_p for each reaction, constrained to be greater than zero, were estimated. This was done by fitting the model with the obtained experimental data. The results are shown in Table 5.1.

For method 2 the model was set to estimate the parameters $V_{max}/Unit$, K_s and K_p for each reaction and was constrained to be greater than zero. As with model one, parameters were estimated by fitting the model with the obtained experimental data. The results are shown in Table 5.2.

Interestingly the parameters estimated using the two different methods produced different results. Looking at the K_s values in Tables 5.1 and 5.2, 3 β -HSD shows the highest affinity towards DHEA (0.6 μ M) using method 2 while in method 1 the highest affinity is towards 17OHPREG (0.9 μ M). In contrast to method 1, the K_s value of 3 β -HSD in method 2 show the lowest affinity towards 17OHPREG (3.64 μ M) but has a higher $V_{max}/Unit$ (2.35) than that of method 1 (1.44). Although the estimated parameters differ between methods, a pattern does emerge. When looking at PREG to PROG conversion, the $V_{max}/Unit$ for method 1 (3.98) is approximately two times faster than in method 2 (2.5). Comparing the affinities between the methods gives an insightful revelation. The affinity of 3 β -HSD for PREG in method 1 (4.7 μ M) is also approximately two times higher than for method 2. We could argue that, although the estimated parameters differ between methods, the relationship between the $V_{max}/Unit$ and the K_m of the PREG to PROG reaction stays the same, thus, at the same time interval, PREG and PROG will display approximately the same concentration. Thus, estimating the accurate individual parameters is difficult, but estimating the relationship between them is a better method for comparison.

Using this comparison method, we see a difference in the 17OHPREG to 17OHPROG reaction. The $V_{max}/Unit$ for 17OHPREG in method 1 (1.44) is approximately 1.6 times slower than method 2 (2.35), while the affinity of 3 β -HSD for 17OHPREG in method 1 (0.9 μ M) is 4 times higher than in method 2 (3.64 μ M). This indicates that although the $V_{max}/Unit$ for method 2 is faster, the overall conversion of 17OHPREG to 17OHPROG will be faster using the estimated parameters in method 1. While the $V_{max}/Unit$ for DHEA does not significantly differ between the two methods, the affinity of 3 β -HSD for DHEA in method 1 (1.8 μ M) is three times lower

than for method 2 (0.6 μ M). This indicates that the conversion of DHEA to A4 will be much faster using estimated parameters from method 2, than method 1. In both methods 3 β -HSD show a low affinity towards PROG in the reaction of PREG to PROG conversion. This stands in contrast with both the 17OHPREG to 17OGPROG and DHEA to A4 conversions where the 3 β -HSD affinity towards the substrate and product are similar in the respective reactions. This might indicate that product inhibition does not have a great effect on the reaction for PREG to PROG conversion, but does play a role in both the reactions of 17OHPREG to 17OGPROG and DHEA to A4 conversions.

Table 5.1: The estimated parameter values (V_{\max}/Unit and K_p) of 3 β -HSD for its respective $\Delta 5$ -reactions (PREG, 17OHPREG and DHEA) producing their respective $\Delta 4$ -products (PROG, 17OHPROG and A4). The K_s parameter values were fixed as obtained from Goosen (2010) while V_{\max}/Unit and K_p were constrained only to be positive. Preg: pregnenolone; Prog: progesterone; 17OHPreg: 17-hydroxypregnenolone; 17OHProg: 17-hydroxyprogesterone; DHEA: dehydroepiandrosterone; A4: androstenedione; K_s : K_m of 3 β -HSD for the substrate; K_p : K_m of 3 β -HSD for the product.

| Reaction | PREG | 17OHPREG | DHEA |
|--------------------------|------------------|------------------|------------------|
| V_{\max}/Unit^* | 3.98 | 1.44 | 1.89 |
| K_s (μM) | 4.7 ^γ | 0.9 ^γ | 1.8 ^γ |
| K_p (μM) | 14.53 | 1.38 | 1.96 |

* (1.254 $\mu\text{M}/\text{h}$); ^γ(values obtained from Goosen (2010))

Table 5.2: The estimated parameter values (V_{\max}/Unit , K_s and K_p) of 3 β -HSD for its respective $\Delta 5$ -reactions (PREG, 17OHPREG and DHEA) producing their respective $\Delta 4$ -products (PROG, 17OHPROG and A4). The parameter values were constrained only to be positive.

| Reaction | PREG | 17OHPREG | DHEA |
|--------------------------|-------|----------|------|
| V_{\max}/Unit^* | 2.5 | 2.35 | 1.41 |
| K_s (μM) | 1.98 | 3.64 | 0.6 |
| K_p (μM) | 10.06 | 3.97 | 0.89 |

* (1.254 $\mu\text{M}/\text{h}$)

5.2.3. Validation of 3 β -HSD subsystem

To ensure that the estimated parameters were correct, a validation needed to be done. 3 β -HSD transfected nonsteroidogenic COS-1 cells were assayed with 3 μM of each substrate PREG,

17OHPREG and DHEA simultaneously (Figures 5.7, 5.8 and 5.9 respectively) alongside another assay with $3\mu\text{M}$ of each substrate PREG, PROG, 17OHPREG, 17OHPROG, DHEA and A4 simultaneously (Figures 5.10, 5.11 and 5.12 respectively).

The estimated parameters and the apparent K_s values obtained from method 1 and method 2, were integrated into the model as fixed parameters. The model will give a prediction on how the steroid intermediates change over time according to the fixed parameters. Each of these predicted reactions were compared to the obtained experimental data and a goodness of the fit was analysed (See section 5.1.6).

For the first parameter validation, the parameters from method 1 were integrated into the model and a goodness of the fit of each reaction was plotted. This was repeated for the parameters obtained from method 2.

5.2.3.1. Method 1 parameters

The parameters used in this validation can be obtained from Table 5.1. Using these parameters an assay prediction of a simultaneous addition of $3\mu\text{M}$ of each substrate PREG, 17OHPREG and DHEA was plotted over 7 hours. This assay prediction was tested for the goodness of the fit (Figures 5.7, 5.8 and 5.9). The assay prediction was repeated with a simultaneous addition of $3\mu\text{M}$ of each substrate PREG, PROG, 17OHPREG, 17OHPROG, DHEA and A4 and constructed over 7 hours (Figures 5.10, 5.11 and 5.12).

Graphs showing conversion of PREG to PROG (Figure 5.7, Figure 5.10), 17-OHPREG to 17-OHPROG (Figure 5.8, Figure 5.11) and DHEA to A4 (Figure 5.9, Figure 5.12) concentrations over time, were plotted as dots with their respective standard error (SE) bars. The concentrations of each steroid intermediate were calculated from $500\mu\text{l}$ aliquots, obtained from 1.0×10^5 3β -HSD transfected cells in a 1ml volume (see section 5.1.2). The continuous lines on the same graphs represent the steroid intermediate concentrations calculated by the kinetic model over time (see section 5.1.6).

Looking at the results, the model predicted the PREG to PROG reaction quite well. In contrast, the model predictions for reactions 17OHPREG to 17OHPROG and DHEA to A4 are not good. The model over-predicts the conversion of 17OHPREG to 17OHPROG and under-predicts the conversion of DHEA to A4.

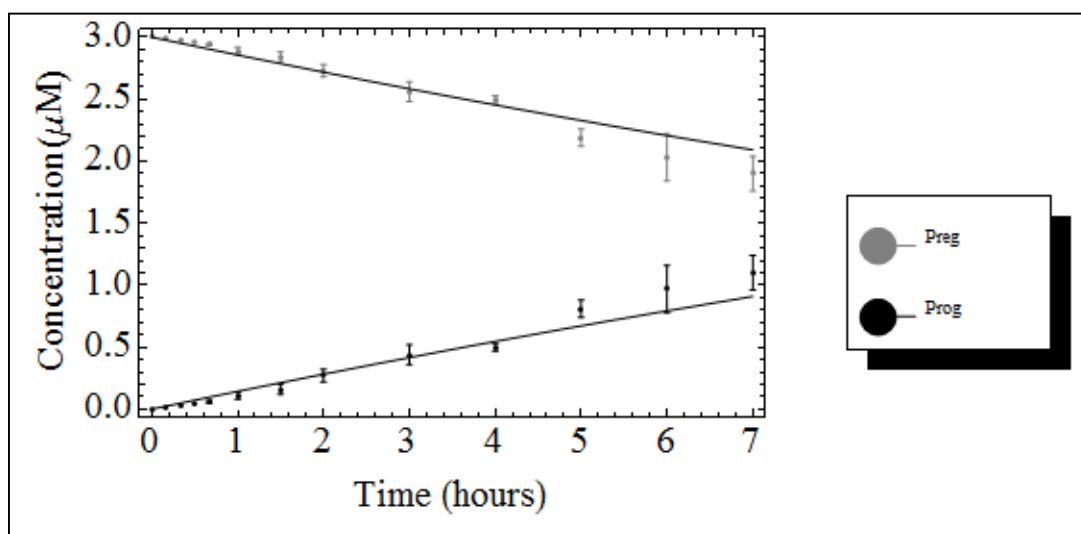


Figure 5.7: Conversion of 3 μ M pregnenolone to progesterone in the presence of 3 μ M 17-hydroypregnenolone and 3 μ M dehydroepiandrosterone over 7 hours in 1.0×10^5 cells/ml 3 β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μ l aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.475 μ M/h.

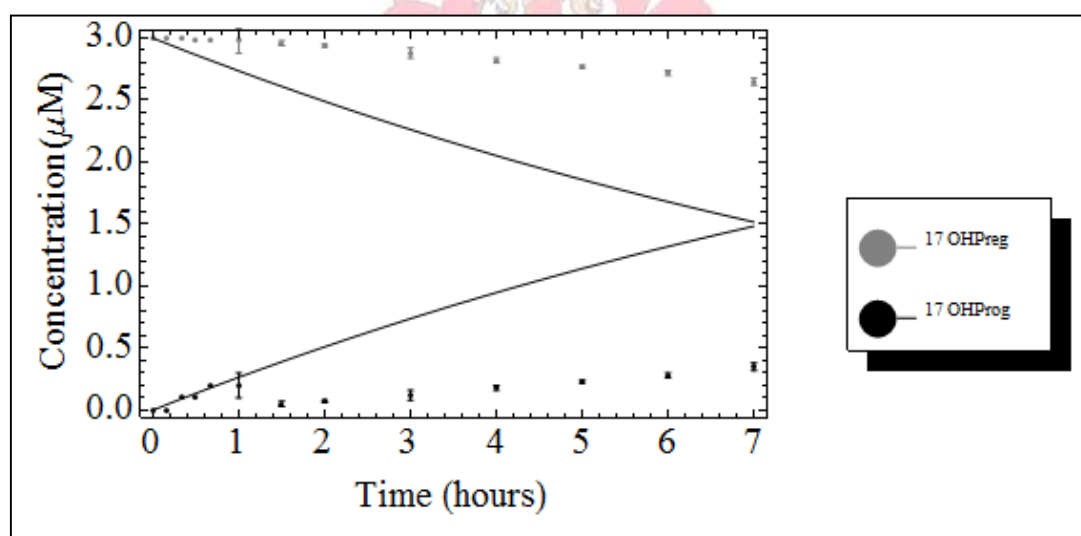


Figure 5.8: Conversion of 3 μ M 17-hydroypregnenolone to 17-hydroxy progesterone in the presence of 3 μ M pregnenolone and 3 μ M dehydroepiandrosterone over 7 hours in 1.0×10^5 cells/ml 3 β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μ l aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.475 μ M/h.

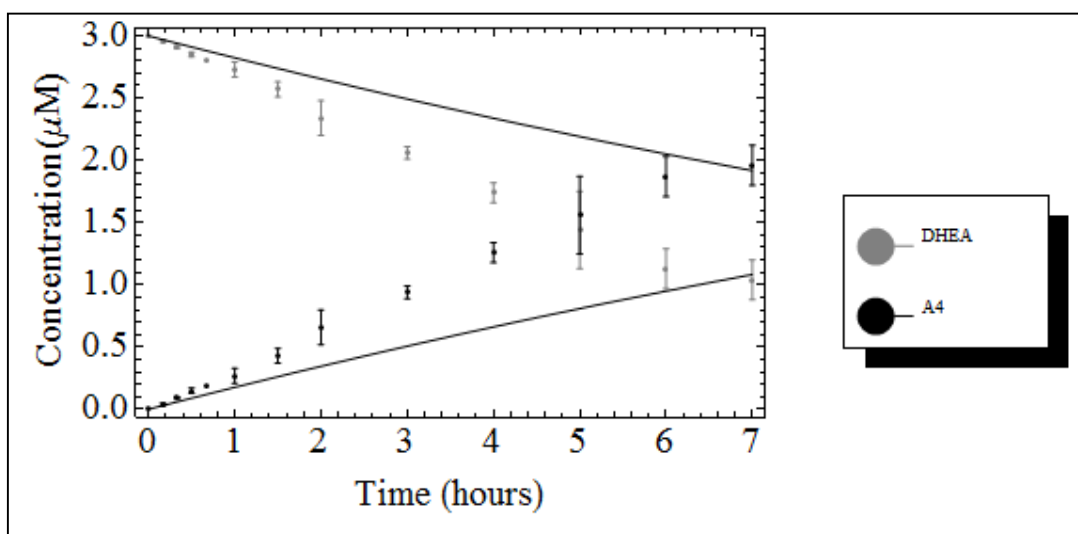


Figure 5.9: Conversion of 3µM dehydroepiandrosterone to androstenedione in the presence of 3µM pregnenolone and 3µM 17-hydroxypregnenolone over 7 hours in 1.0×10^5 cells/ml 3β-HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.475µM/h.

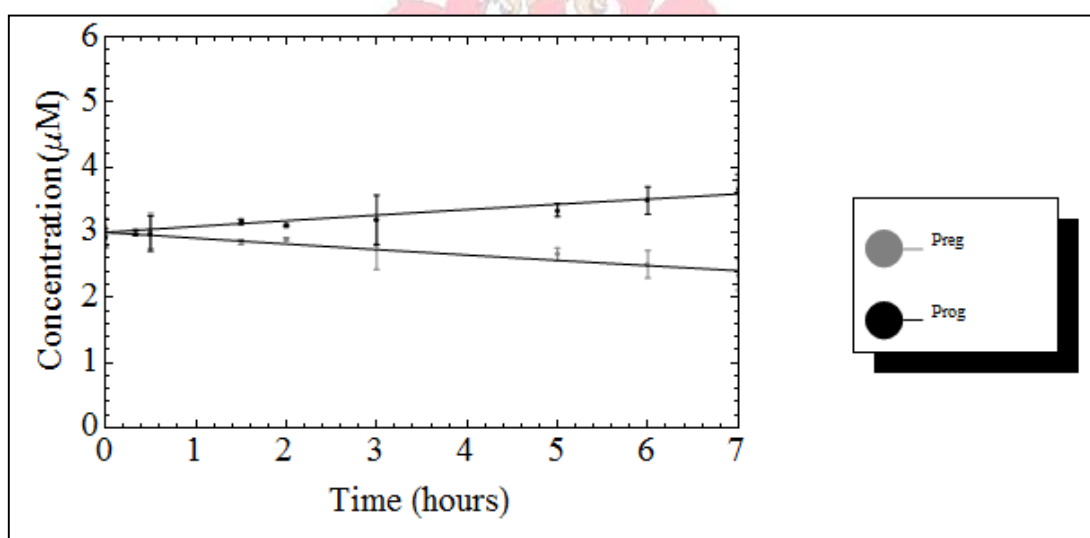


Figure 5.10: Conversion of 3µM pregnenolone and 3µM progesterone in the presence of 3µM 17-hydroxypregnenolone, 3µM 17-hydroxyprogesterone, 3µM dehydroepiandrosterone and 3µM androstenedione over 7 hours in 1.0×10^5 cells/ml 3β-HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.475µM/h.

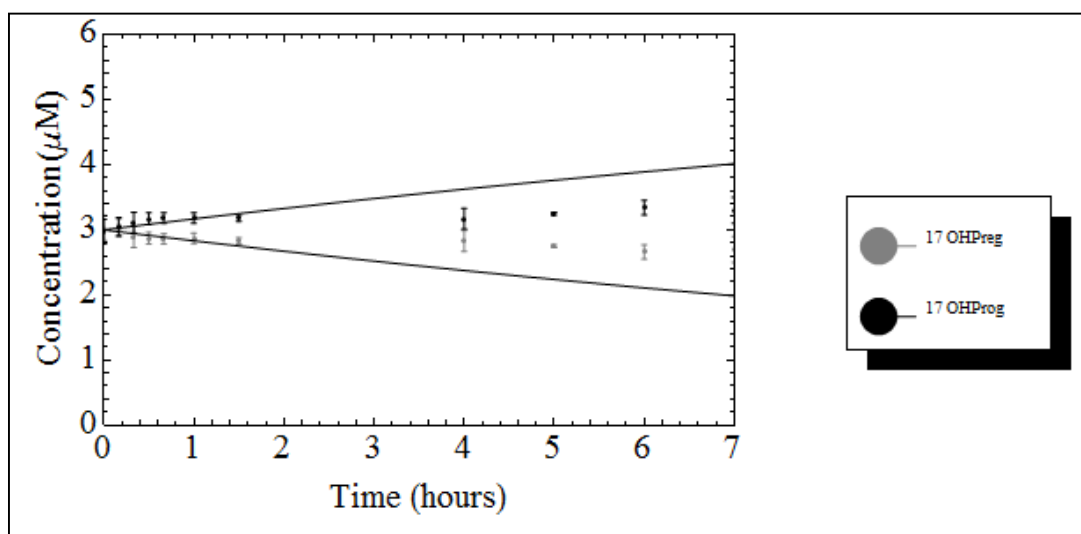


Figure 5.11: Conversion of 3µM 17-hydroxy pregnenolone and 3µM 17-hydroxyprogesterone in the presence of 3µM pregnenolone, 3µM progesterone, 3µM dehydroepiandrosterone and 3µM androstenedione over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is $0.475 \mu\text{M/h}$.

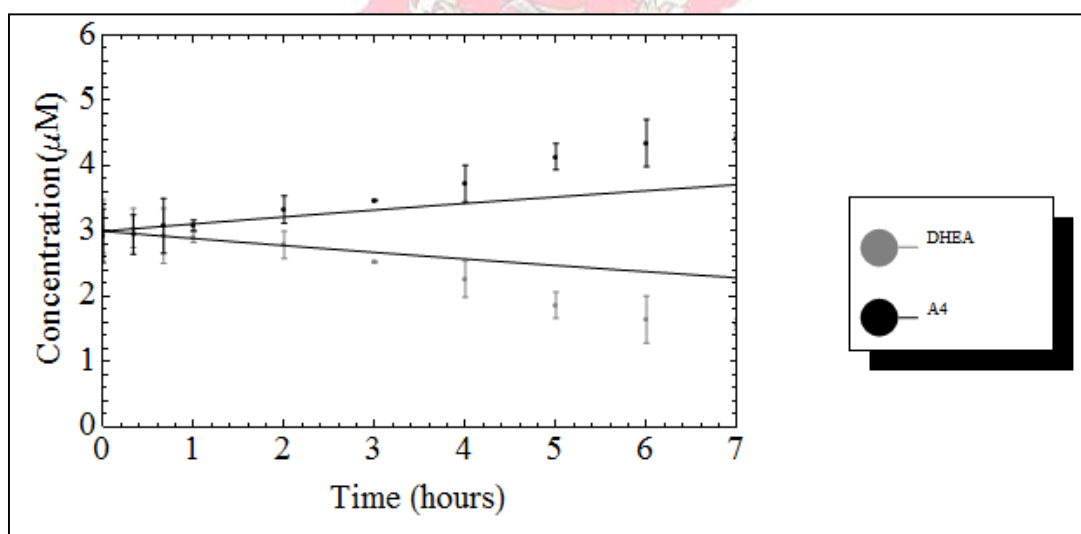


Figure 5.12: Conversion of 3µM dehydroepiandrosterone and 3µM androstenedione in the presence of 3µM pregnenolone, 3µM progesterone, 3µM 17-hydroxy pregnenolone and 3µM 17-hydroxyprogesterone over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is $0.475 \mu\text{M/h}$.

5.2.3.2. Method 2 parameters

The process described above was repeated using the parameters obtained from method 2 in Table 5.2. This can be seen in figures 5.13, 5.14, 5.15, 5.16, 5.17 and 5.18. Graphs showing

conversion of PREG to PROG (Figure 5.13, Figure 5.16), 17-OHPREG to 17-OHPROG (Figure 5.14, Figure 5.17) and DHEA to A4 (Figure 5.15, Figure 5.18) concentrations over time, were plotted as dots with their respective standard error (SE) bars. The concentrations of each steroid intermediate were calculated from 500 μ l aliquots, obtained from 1.0×10^5 3β -HSD transfected cells in a 1ml volume (see section 5.1.2). The continuous lines on the same graphs represent the steroid intermediate concentrations calculated by the kinetic model over time (see section 5.1.5).

When looking at the results, only figures 5.14 and 5.15 show slight over-prediction and under-prediction respectively. In contrast, the overall model predictions of all the reactions are quite well, rendering the slight over- and under-predictions of figures 5.14 and 5.15, insignificant.

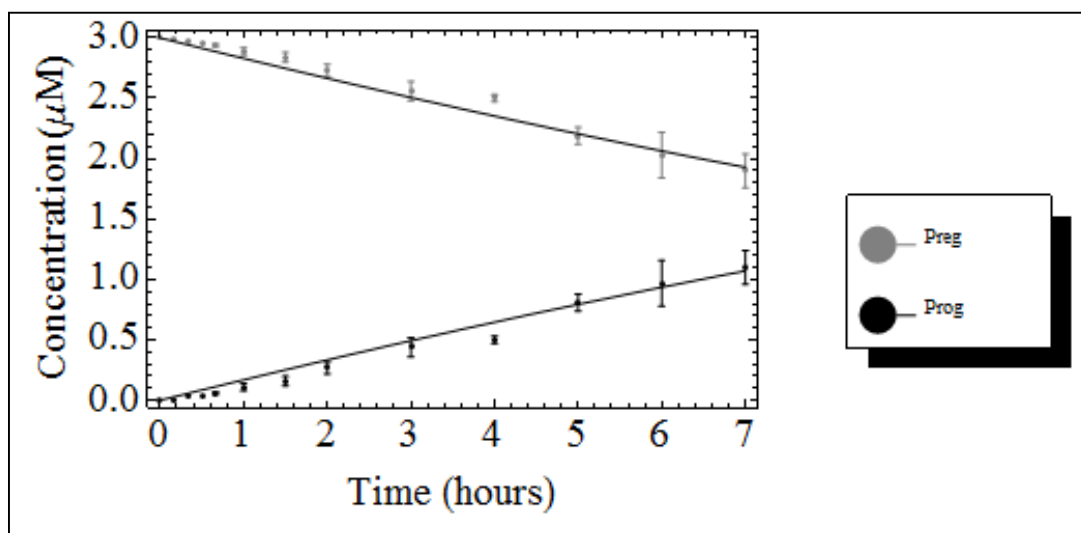


Figure 5.13: Conversion of 3 μ M pregnenolone to progesterone in the presence of 3 μ M 17-hydroxypregnenolone and 3 μ M dehydroepiandrosterone over 7 hours in 1.0×10^5 cells/ml 3β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μ l aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.475 μ M/h.

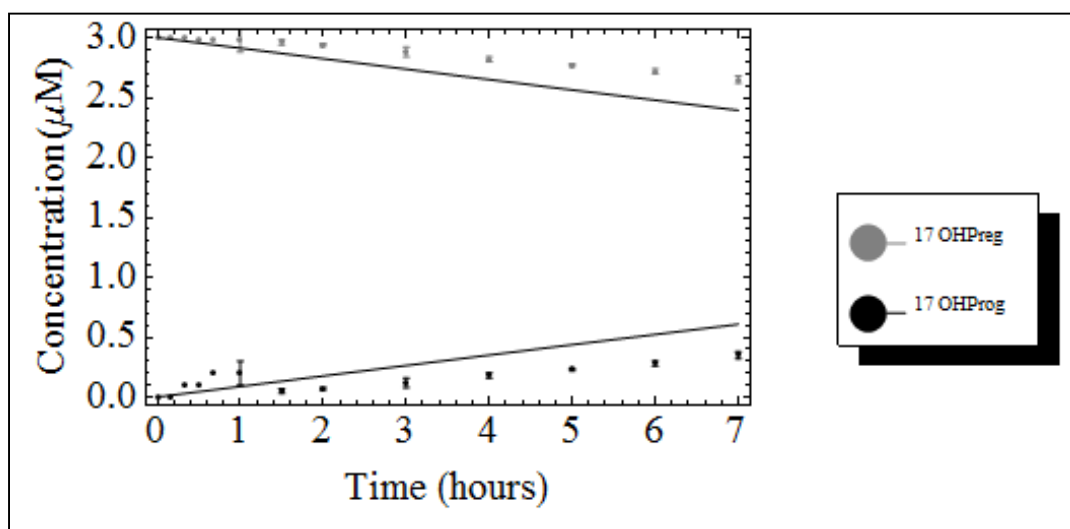


Figure 5.14: Conversion of 3µM 17-hydroxypregnenolone to 17-hydroxy progesterone in the presence of 3µM pregnenolone and 3µM dehydroepiandrosterone over 7 hours in 1.0×10^5 cells/ml β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is $0.475 \mu\text{M}/\text{h}$.

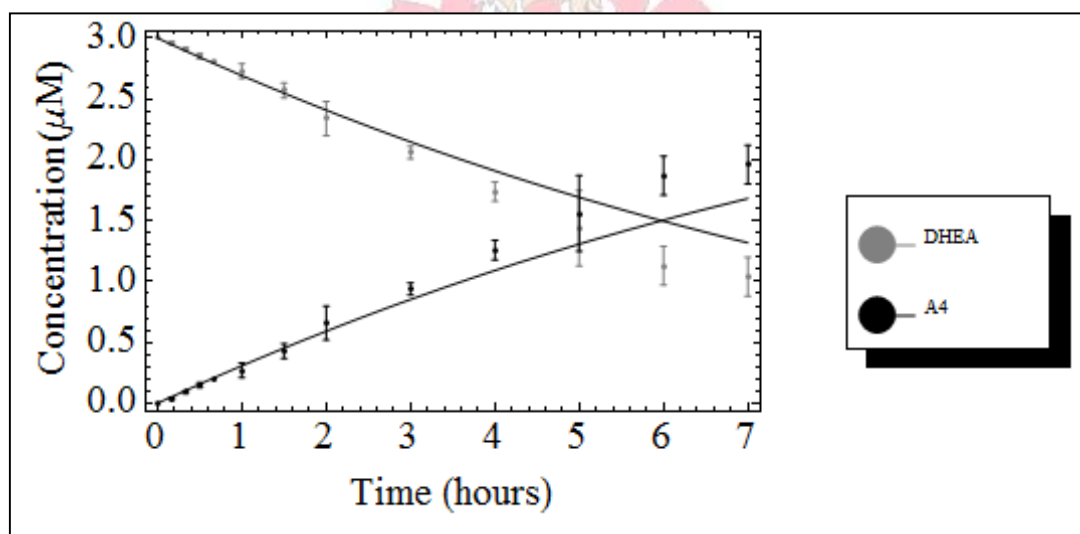


Figure 5.15: Conversion of 3µM dehydroepiandrosterone to androstenedione in the presence of 3µM pregnenolone and 3µM 17-hydroxypregnenolone over 7 hours in 1.0×10^5 cells/ml β -HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is $0.475 \mu\text{M}/\text{h}$.

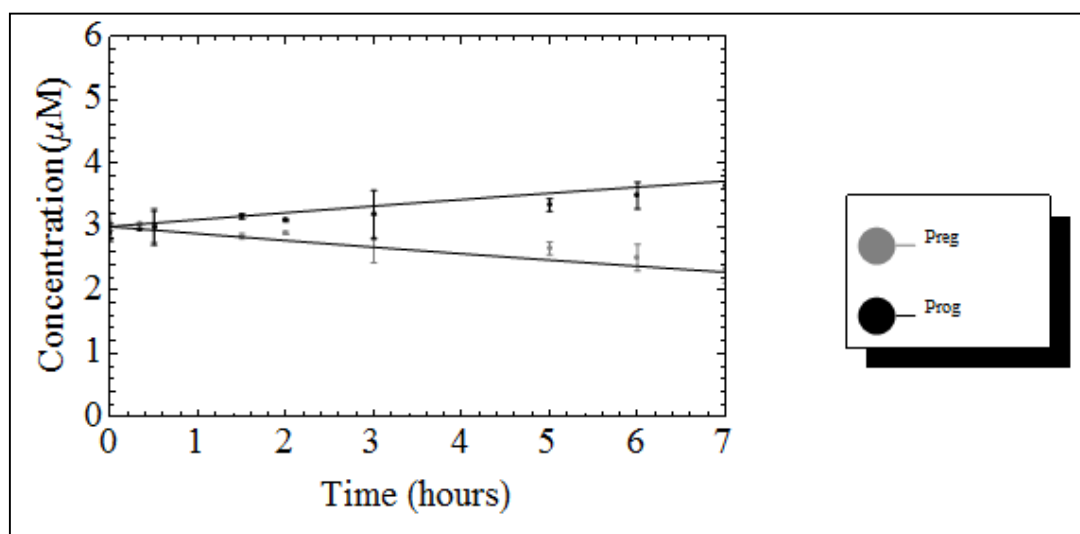


Figure 5.16: Conversion of 3µM pregnenolone and 3µM progesterone in the presence of 3µM 17-hydroxypregnenolone, 3µM 17-hydroxyprogesterone, 3µM dehydroepiandrosterone and 3µM androstenedione over 7 hours in 1.0×10^5 cells/ml 3β-HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is 0.475µM/h.

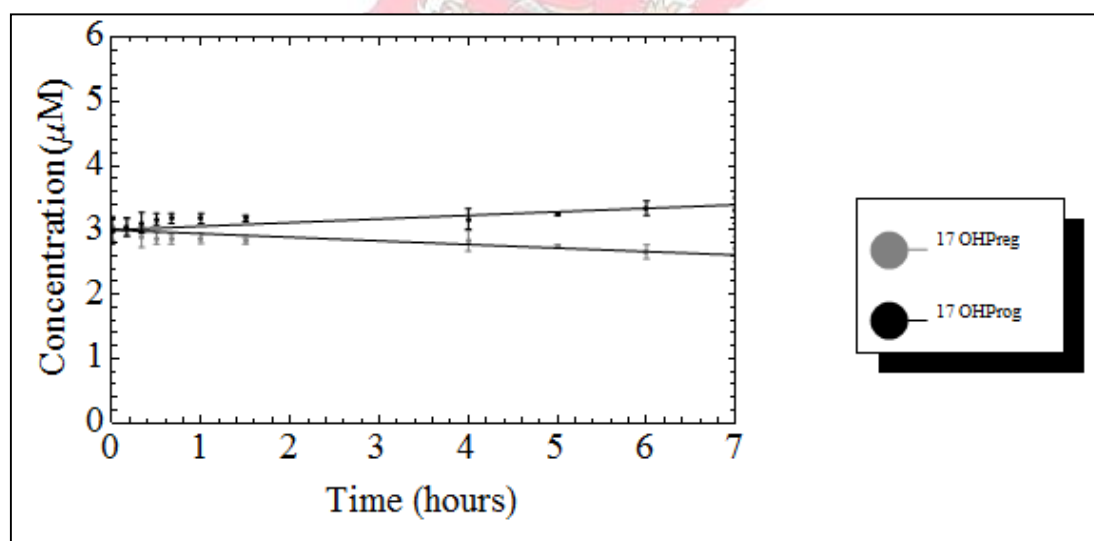


Figure 5.17: Conversion of 3µM 17-hydroxy pregnenolone and 3µM 17-hydroxyprogesterone in the presence of 3µM pregnenolone, 3µM progesterone, 3µM dehydroepiandrosterone and 3µM androstenedione over 7 hours in 1.0×10^5 cells/ml 3β-HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is 0.475µM/h.

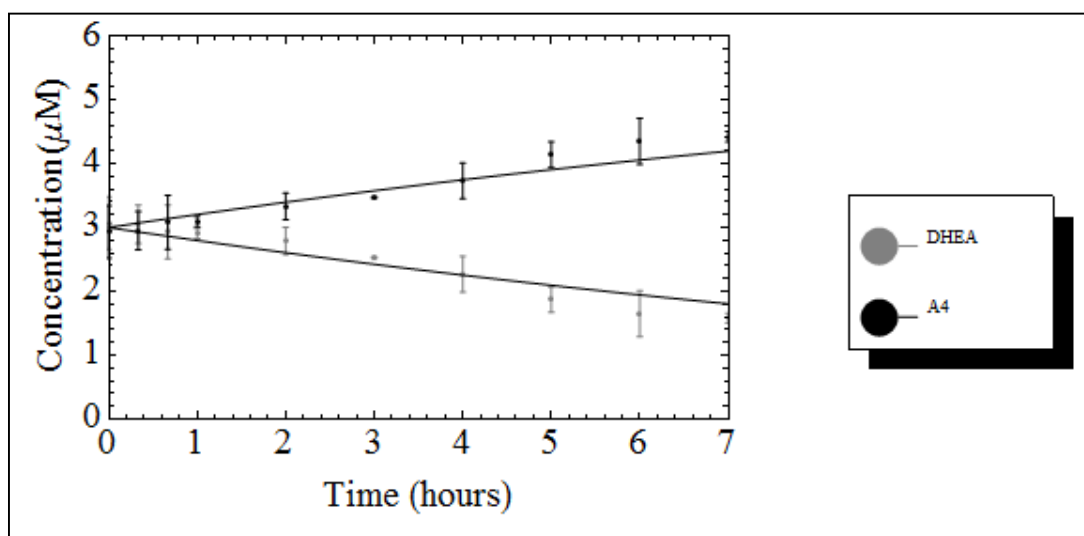


Figure 5.18: Conversion of 3µM dehydroepiandrosterone and 3µM androstenedione in the presence of 3µM pregnenolone, 3µM progesterone, 3µM 17-hydroxypregnenolone and 3µM 17-hydroxyprogesterone over 7 hours in 1.0×10^5 cells/ml 3β-HSD transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{max} standardization is 0.475µM/h.

5.3. Results for CYP17 reactions

5.3.1. Ovine CYP17 activity in COS-1 cells

The CYP17 enzyme converts Δ^5 -steroid intermediates PREG to 17OHPREG and 17OHPREG to DHEA as well as Δ^4 -steroid intermediates PROG to 17OHPROG and 16OHPROG respectively, and further converts 17OHPROG to A4. As previously mentioned, nonsteroidogenic COS-1 cells were transfected with CYP17 plasmid constructs (previously cloned by and obtained from Storbeck (2007)) by Conradie (2010, unpublished) and assayed with PREG steroid intermediate. Each reaction was assayed over a period of 12 hours. CYP17 transfected nonsteroidogenic COS-1 cells were assayed respectively with 2µM and 3µM substrate. A graph showing conversion of PREG to 17OHPREG, and 17OHPREG to DHEA (Figure 5.19) over time was constructed.

Building on the work of Conradie (2010, unpublished), nonsteroidogenic COS-1 cells were transfected with CYP17 plasmid constructs (previously cloned by and obtained from Storbeck (2007)) and assayed with PROG steroid intermediate. Each reaction was assayed over a period of 12 hours. CYP17 transfected nonsteroidogenic COS-1 cells were assayed respectively with 1.5µM, 2µM and 3µM substrate. A graph showing conversion of PROG to 17OHPROG, PROG to 16OHPROG and 17OHPROG to A4 (Figure 5.20) over time was constructed.

5.3.2. Kinetic analysis of CYP17

A computer model consisting of ODEs was constructed to estimate the parameters V_{\max} and K_m of CYP17 for its respective Δ^5 -steroid intermediates PREG, 17OHPREG and DHEA as well as for its respective Δ^4 -steroid intermediates PROG, 17OHPROG, 16OHPROG and A4. In both cases of Conradie (2010, unpublished) and this thesis, all the parameters (V_{\max} , K_s , K_a , K_b and K_p) were constrained to be greater than zero.

5.3.2.1. Standardization of CYP17 experiments

The V_{\max} of each reaction is subjected to the activity of the batch cells used within an experiment. Alongside each experiment for both Δ^5 -steroid intermediates (PREG, 17OHPREG and DHEA) and Δ^4 -steroid intermediates (PROG, 17OHPROG, 16OHPROG and A4), CYP17 transfected nonsteroidogenic COS-1 cells were assayed with 5 μ M PREG as substrate for a period of 4 hours and its initial activity (Unit) used to standardize the difference between the activity of each experiment. The Unit (0.7103 μ M/h) of the PREG experiment was used as reference. The PROG experiment was standardized by dividing its Unit by the reference Unit.

5.3.2.2. Estimated parameter values for CYP17 reactions

The estimated V_{\max}/Unit and K_m values obtained from Conradie (2010, unpublished) for the Δ^5 -steroid intermediates are shown in Table 5.3 and the estimated V_{\max}/Unit and K_m values for the Δ^4 -steroid intermediates are shown in Table 5.4.



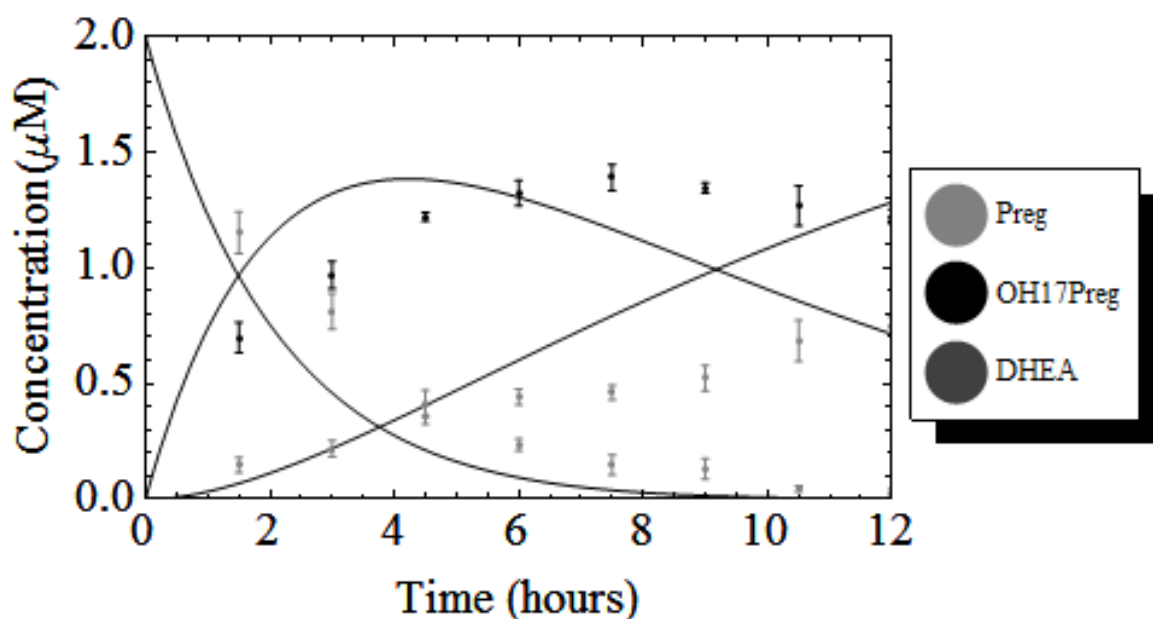


Figure 5.19: Conversion of 2 μM pregnenolone to 17-hydroxypregnenolone and dehydroepiandrosterone over 12 hours in 1.0×10^5 cells/ml CYP17 transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500 μl aliquots of each time interval. The initial activity used for V_{max} standardization is 0.7103 $\mu\text{M}/\text{h}$. Preg: pregnenolone; 17OHPreg: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone.

Table 5.3: The estimated parameter values ($V_{\text{max}}/\text{Unit}$, K_s , K_a and K_p) of CYP17 for its respective Δ^5 -reactions (PREG, 17OHPREG and DHEA). All the parameter values were constrained only to be positive. Preg: pregnenolone; 17OHPreg: 17-hydroxypregnenolone; DHEA: dehydroepiandrosterone; K_s : K_m of CYP17 for PREG; K_a : K_m of CYP17 for 17-OHPREG; K_p : K_m of CYP for DHEA.

| Reaction | PREG | 17OHPREG | DHEA |
|--------------------------------|-------|----------|---------|
| $V_{\text{max}}/\text{Unit}^*$ | 0.733 | 0.162 | N/A |
| K_s (μM) | 0.519 | N/A | N/A |
| K_a (μM) | N/A | 1.000 | N/A |
| K_p (μM) | N/A | N/A | 4430.35 |

* (0.7103 $\mu\text{M}/\text{h}$)

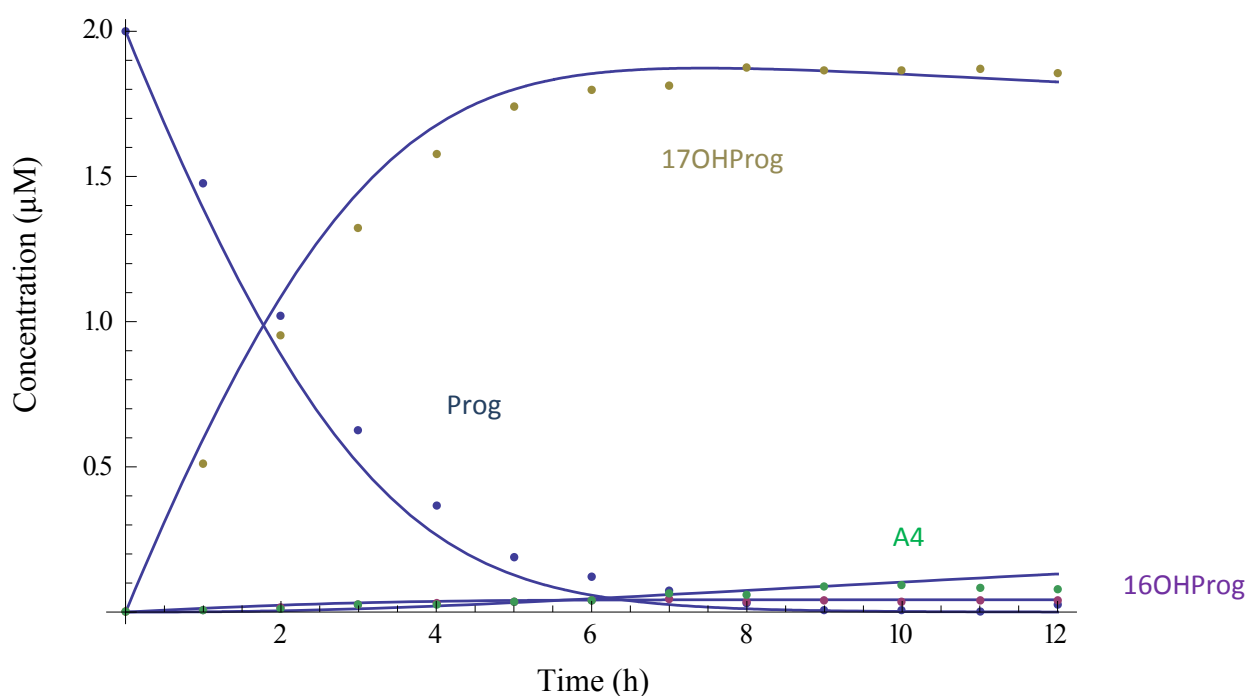


Figure 5.20: Conversion of 2 μ M progesterone to 17-hydroxyprogesterone, 16-hydroxyprogesterone and androstenedione over 12 hours in 1.0×10^5 cells/ml CYP17 transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations calculated from 500 μ l aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.615 μ M/h. Prog: progesterone; 17OHProg: 17-hydroxyprogesterone; 16OHProg: 16-hydroxyprogesterone; A4: androstenedione.

Table 5.4: The estimated parameter values (V_{\max}/Unit , K_s , K_a , K_b and K_p) of CYP17 for its respective Δ^4 -reactions (PROG, 17OHPROG, 16OHPROG and A4). All the parameter values were constrained only to be positive. Prog: progesterone; 17OHProg: 17-hydroxyprogesterone; 16OHProg: 16-hydroxyprogesterone; A4: androstenedione; K_s : K_m of CYP17 for PROG; K_a : K_m of CYP17 for 17-OHPROG; K_b : K_m of CYP17 for 16-OHPROG; K_p : K_m of CYP for A4.

| Reaction | PROG | 17OHPROG | 16OHPROG | A4 |
|--------------------------|-------|----------|----------|------|
| V_{\max}/Unit^* | 1.231 | 0.065 | 0.027 | N/A |
| K_s (μ M) | 1.29 | N/A | N/A | N/A |
| K_a (μ M) | N/A | 4.94 | N/A | N/A |
| K_b (μ M) | N/A | N/A | 2.66 | N/A |
| K_p (μ M) | N/A | N/A | N/A | 1.17 |

* (0.7103 μ M/h)

5.3.3. Validation of CYP17 subsystem

To ensure that the estimated parameters are correct a validation needs to be done. CYP17 transfected nonsteroidogenic COS-1 cells were incubated respectively with $3\mu\text{M}$ of PREG and PROG simultaneously. The estimated parameters and the apparent $V_{\text{max}}/\text{Unit}$ and K_m values obtained from the parameter estimations, were integrated into the model as fixed parameters. The model will give a prediction on how the steroid intermediates change over a period of time according to the fixed parameters. Each reaction with its specific steroid intermediate change over time will then be fit onto the experimental data obtain and a goodness of the fit will be analysed (Figures 5.21 and 5.22).

Looking at the results, figure 5.21 shows a very good prediction of the conversion of intermediates PREG, 17OH-PREG and DHEA over time. In contrast, figure 5.22 shows a little under-prediction of the conversion of the intermediates PROG and 17OH-PROG over time, while the conversion of intermediates 16OH-PROG and A4 show a good prediction over time.

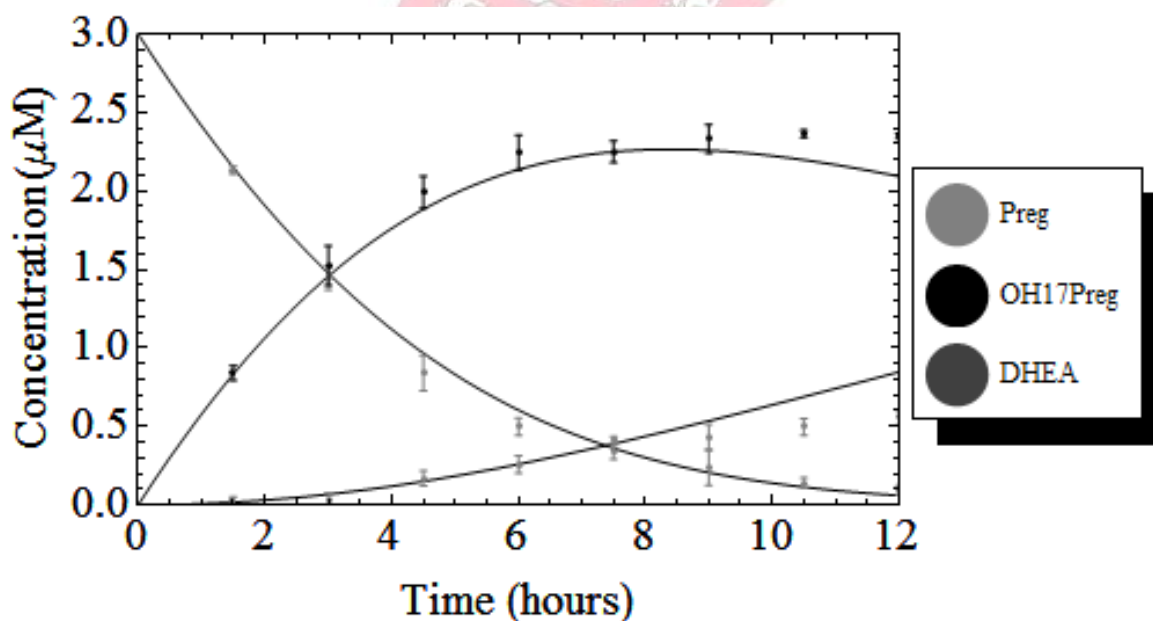


Figure 5.21: Conversion of $3\mu\text{M}$ pregnenolone to 17-hydroxypregnenolone and dehydroepiandrosterone in the presence of $3\mu\text{M}$ progesterone, 17-hydroxyprogesterone, 16-hydroxyprogesterone and adrostenedione, over 12 hours in 1.0×10^5 cells/ml CYP17 transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from $500\mu\text{l}$ aliquots of each time interval. The initial activity used for V_{max} standardization is $0.620\mu\text{M/h}$.

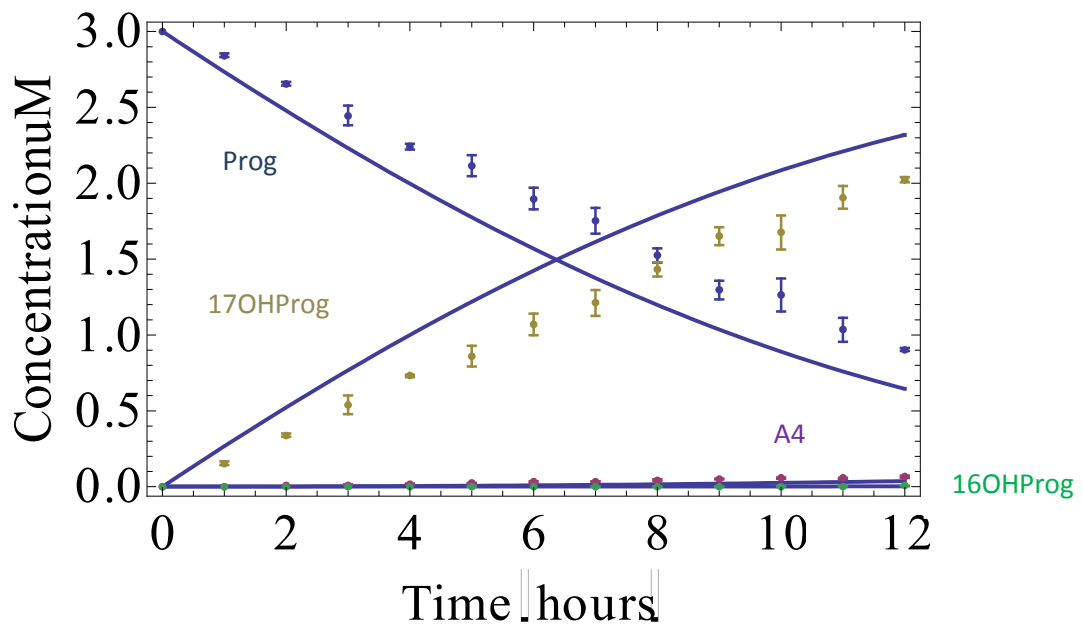


Figure 5.22: Conversion of 3µM progesterone to 17-hydroxyprogesterone and androstenedione in the presence of 3µM pregnenolone, 17-hydroxypregnenolone, and dehydroepiandrosterone, over 12 hours in 1.0×10^5 cells/ml CYP17 transfected COS-1 cells. Each data point represents the average of triplicate intermediate concentrations and error bars (SE) calculated from 500µl aliquots of each time interval. The initial activity used for V_{\max} standardization is 0.620 µM/h.

Chapter 6

GENERAL DISCUSSION

Conley and Bird (1997) suggested that CYP17 and 3 β -HSD levels steroid synthesis in organs by imposing certain constraints. These constraints imply that the levels of 3 β -HSD, and tissue-specific distribution relative to CYP17, play an important role in the balance between androgen and estrogen synthesis. They also stated that understanding the points that have important influences on the flux of substrates in the Δ^5 -and Δ^4 -pathways may help us predict the consequences of increasing or decreasing the levels of these enzymes or their activities. One hypothesised regulatory point in these pathways are the reaction at the junction of the differential metabolism of PREG which are catalyzed by CYP17 and 3 β -HSD in competing reactions (Fevold 1978). Knowledge of these regulatory points can potentially aid in the development of therapeutic approached treatments of steroidogenic or even endocrinological diseases (Conley and Bird 1997). In addition, Breen (2007) showed that a computational model could successfully predict biochemical responses of ovarian steroidogenesis to EAC and that its capabilities could aid the pharmaceutical development and environmental health assessments of EAC, opening the door for Systems Biology to be used as a tool for the investigation of systems such as steroidogenesis.

While investigating the steroidogenic enzyme activity in Angora goats, Engelbrecht and Swart (2000) identified CYP17 and 3 β -HSD as potential contributors to hypocortisolism in these animals. It was found that CYP17 contributes to hypocortisolism by shifting the flux towards the Δ^5 -pathway in adrenal steroidogenesis (Engelbrecht & Swart 2000; Engelbrecht 2000). In addition, increased 17,20 lyase activity towards 17-OHPREG by the CYP17 ACS-isoform of CYP17 was shown to be the cause of the increased androgen precursor production previously identified in the Angora goat adrenal activity (Engelbrecht & Swart 2000; Engelbrecht 2000; Storbeck 2007). By comparing Angora 3 β -HSD to ovine 3 β -HSD, which is considered a hardy species, Goosen (2010) subsequently showed that CYP17 was not the only contributor to hypocortisolism in the Angora goat, but that 3 β -HSD also contributes to this syndrome. However, the kinetic influence that CYP17 and 3 β -HSD has on each other to cause the shift in flux in the subsystem, comprising of these two enzymes has not been addressed. The kinetic parameters of these two ovine enzymes for their respective substrates was previously

determined by means of individual experiments [(Goosen 2010) for 3 β -HSD and (Hough 2012) for CYP17]. In an attempt to answer the latter question, the kinetic parameters of CYP17 and 3 β -HSD for its respective substrates were determined by computational modelling comprising of ODE's. This was done to validate the models constructed for each enzyme and its respective substrates.

To estimate and validate the kinetic parameters for 3 β -HSD, and its respective substrates, experimental data was obtained for each substrate and assayed over a period of 7 hours using transfected nonsteroidogenic COS-1 cells. The computational model constructed estimated the V_{\max}/Unit and K_p (Method 1) and V_{\max}/Unit , K_s and K_p (Method 2) values for each reaction and substrate respectively. Through comparing the two methods it can be seen that fixing the K_s values obtained from Goosen (2010) in method 1, different parameter values are obtained compared to method 2. As already discussed in section 5.2.2.2, comparing the two methods require the study of the estimated parameters for each reaction, as a whole. Studying the estimated parameters of 3 β -HSD for both methods show that the PREG to PROG reaction will give approximately the same amount of intermediate conversion at the same time interval. In both methods 3 β -HSD shows a low affinity towards PROG in the reaction of PREG to PROG conversion. This stands in contrast with both the 17OHPREG to 17OGPROG and DHEA to A4 conversions where the affinity for 3 β -HSD towards the substrate and product are similar in the respective reactions. This might indicate that product inhibition does not have a great effect on the reaction for PREG to PROG conversion, but does play a role in both the reactions of 17OHPREG to 17OGPROG and DHEA to A4 conversions, coinciding with the previous notion that more PROG may be produced from the Δ^5 -pathway compared to 17OHPROG due to less product inhibition. This is confirmed with the results shown in Hough (2012), where the predominantly steroids detected in ovine primary cultures incubated with 100 μM PREG, were PROG and 17OHPROG. Moreover, the addition of 1 μM ACTH resulted in an increase in PROG and 17OHPROG as well as cortisol. This may show that using method 2 parameters, the optimal contribution for 17OHPROG production (a cortisol precursor) rests on the capacity of CYP17 (17 α -hydroxylase) to convert PROG to 17OHPROG. However, the parameter values obtained for CYP17 (See Tables 5.3 and 5.4) cannot be excluded in the analysis of the steroid flux in this subsystem of steroidogenesis. The results show that CYP17 has a better affinity (0.52 μM) and faster catalytic activity than 3 β -HSD for PREG. This indicates that the flux of

steroids might choose to follow the Δ^5 -pathway, a hypothesis still needs to be tested and validated.

Furthermore, the V_{\max}/Unit for 17OHPREG obtained in method 1 (1.44) is approximately 1.6 times slower than method 2 (2.35), while the affinity of 3β -HSD for 17OHPREG in method 1 ($0.9\mu\text{M}$) is 4 times higher than in method 2 ($3.64\mu\text{M}$). This indicates that although the V_{\max}/Unit for method 2 is faster, the overall conversion of 17OHPREG to 17OHPROG will be faster using the estimated parameters in method 1. While the V_{\max}/Unit for DHEA does not significantly differ between the two methods, the affinity for of DHEA for 3β -HSD in method 1 ($1.8\mu\text{M}$) is three times lower than for method 2 ($0.6\mu\text{M}$). This data indicates that the conversion of DHEA to A4 will be much faster using estimated parameters from method 2, than method 1, supporting results obtained by Goosen (2010) in the Angora goat. This anomaly shows that, in sheep, the flux may shift towards the Δ^5 -pathway to produce DHEA and eventually A4 as very little A4 is produced from 17OHPROG as shown in Swart (2003) as well as in Figure 5.20.

In validating the computational model with estimated parameters from the different methods, the same initial concentrations ($3\mu\text{M}$) of all the different substrates and products (PREG, 17OHPREG, DHEA, PROG, 17OHPROG and A4) were added to transfected nonsteroidogenic COS-1 cells simultaneously and the data was obtained. The prediction of the computational model for each reaction, using the estimated parameters and same initial concentrations as the above mentioned experiments, was constructed and compared to the latter obtained data. In both methods, the model show a good prediction for the conversion of PREG to PROG, but show considerable differences in predicting the 17OHPREG to 17OHPROG and DHEA to A4 conversions, probably because the PREG to PROG reaction was used to standardize the differences between all the experiments. Using the parameters estimated from method 2, the model shows a good prediction of the 17OHPREG to 17OHPROG and DHEA to A4 reactions, while using estimated parameters from method 1 shows an over-prediction and under-prediction for the reactions respectively. These results show that the parameters estimated using method 2 are more accurate than the estimated parameters in method 1. This may be due to the estimated parameters in method 2 not having any constraints while fitted onto the experimental data, compared to method 1 having constraints on the K_s parameter of each reaction. In addition, different methods were used in obtaining the K_s parameter values of each reaction. While progress curve analysis was used in this study, Goosen (2010) used the Lineweaver-

Burk reciprocal plot through the initial reaction rate method. Furthermore, using method 2, the parameters suggest that 17OHPROG (a cortisol precursor) may not be predominantly produced through the 17OHPREG conversion via 3β -HSD catalysis as suggested by Goosen (2010), but rather through the conversion of PROG via CYP17, which in turn is produced through the PREG conversion via 3β -HSD. The latter observation, however, still needs to be investigated and confirmed by cotransfection of CYP17, 3β -HSD and CYP21 with addition of all six Δ^5 - and Δ^4 -steroid intermediates. The problem however is that the current nonsteroidogenic COS-1 expression system does not support such an experimental design, since results become less accurate when transfecting cells with more than three plasmid constructs (Hough 2012). The fact that the results show a faster conversion of DHEA, compared to 17OGPREG, by 3β -HSD, coincides with the notion that the flux is shifted towards the Δ^5 -pathway when production of A4 is needed (Engelbrecht and Swart 2000), supporting the data showing that CYP17 has a higher affinity and catalytic activity for PREG compared to 3β -HSD. Due to the assumption that 17OHPROG may predominantly be produced through the Δ^4 -pathway and that A4 production through DHEA may shift the flux towards the Δ^5 -pathway, the hypothesised regulatory point in the sub-system comprising of CYP17 and 3β -HSD is the junction at the differential metabolism of PREG (Fevold 1978).

Summary

The influence that CYP17 and 3β -HSD has on the steroid flux in the ovine adrenal steroidogenic sub-system could be studied and analyzed in light of the data obtained. A generic rate equation describing the competitive influence of the intermediates on CYP17 and 3β -HSD respectively, was constructed. Using this generic rate equation, computational models describing the CYP17 and 3β -HSD reactions respectively, was constructed and used to estimate and validate the kinetic parameters for each reaction and substrate. Using these models, predictions of the intermediate concentrations over time can be made. Further studies still need to confirm the flux of steroids in this sub-system with the addition of cytochrome b5 which has been shown to augment both CYP17 (Auchus 1998) and 3β -HSD (Goosen et al. 2011). With the addition of the latter, constructing and validating a model that describes the competitive influences of the intermediates on both CYP17 and 3β -HSD simultaneously, may

lead to a better understanding of steroid flux in this ovine adrenal steroidogenic sub-system. In addition, this generic rate equation is not limited to the studies of ovine adrenal steroidogenesis, but may be used in studies of adrenal steroidogenesis in different species. This opens the door for cross-species comparison studies for the adrenal steroidogenic pathways using systems biology tools such as kinetic modelling and metabolic control analysis (MCA).



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Addendum A

Construction and validation of a kinetic model for the reactions catalyzed by 3 β -HSD

Reaction for PREG to PROG:

```

req[s1_,p1_,var1_,var2_,var3_]:= (var1*(s1/var2))/(1+s1/var2+p1/var3)
ratenames={v1};
rateequations={req[Preg[t],Prog[t],Vm1,Ks1,Kp1]};
ratefuncs=Thread[ratenames->rateequations];
variables={Preg[t],Prog[t]};
Solvefunc[t1_,t2_]:=NDSolve[Join[ODEs,initvars],variables,{t,t1,t2}]
data1=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie Preg\\2011 werk\\2PP via 3B-HSD.csv"]][[1;;4]]
data2=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie Preg\\2011 werk\\5PP via 3B-HSD.csv"]][[1;;4]]
data3=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie Preg\\2011 werk\\2uM P5 via 3B-HSD.csv"]][[1;;4]]
data4=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie Preg\\2011 werk\\5uM P5 via 3B-HSD.csv"]][[1;;4]]
datfunc[points_,result_]:=Join[{points},Flatten[variables/.result/.{t->points},1]];
points1=data1[[1]];
points2=data2[[1]];
points3=data3[[1]];
points4=data4[[1]];
Onerun[par1_,par2_,par3_,init1_,init2_,points_]:=
(
  initvars={init1,init2};
  params={Vm1->par1,Ks1->par2,Kp1->par3};
  ODEs={Preg'[t]-v1,Prog'[t]-v1}/.ratefuncs/.params;
  initvars=Thread[(variables/.t->0)==initvars];
  result=Solvefunc[0,12];
  datfunc[points,result]
)
objfunc[par1_/;NumberQ[par1],par2_/;NumberQ[par2],par3_/;NumberQ[par3],inita1_/;Num
berQ[inita1],inita2_/;NumberQ[inita2],initb1_/;NumberQ[initb1],initb2_/;NumberQ[initb2],i
nitc1_/;NumberQ[initc1],initc2_/;NumberQ[initc2],initd1_/;NumberQ[initd1],initd2_/;Numb
erQ[initd2]]:=
(
  newdata1=Onerun[par1,par2,par3,inita1,inita2,points1];
  newdata2=Onerun[par1,par2,par3,initb1,initb2,points2];
  newdata3=Onerun[par1,par2,par3,initc1,initc2,points3];
  newdata4=Onerun[par1,par2,par3,initd1,initd2,points4];

```

```

Plus@@(Flatten[newdata1-data1]^2)+Plus@@(Flatten[newdata2-
data2]^2)+Plus@@(Flatten[newdata3-data3]^2)+Plus@@(Flatten[newdata4-data4]^2)
)
NMinimize[{objfunc[par1,par2,par3,2,2,5,5,2,0,5,0],par1>0.0001,par2>0.0001,par3>0.0001}
,{par1,par2,par3},Method->"Automatic"
]
{2.2003324411059775`,{par1->2.498633943957759`,par2->1.9813116532563417`,par3->1
0.059008518716267`}}
{2.20033,{par1->2.49863,par2->1.98131,par3->10.059}}

```

Reaction for 17OH-PREG to 17OH-PROG:

```

req[s1_,p1_,var1_,var2_,var3_]:= (var1*(s1/var2))/(1+s1/var2+p1/var3)
ratenames={v1};
rateequations={req[OH17Preg[t],OH17Prog[t],Vm1,Ks1,Kp1]};
ratefuncs=Thread[ratenames->rateequations];
variables={OH17Preg[t],OH17Prog[t]};
Solvefunc[t1_,t2_]:=NDSolve[Join[ODEs,inits],variables,{t,t1,t2}]
data1=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\2uM 17OHPreg
29022012.csv"]][[1;;4]]
data2=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\5uM 17OHPreg
29022012.csv"]][[1;;4]]
data3=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\2uM 17OHPP
29022012.csv"]][[1;;4]]
data4=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\5uM 17OHPP
29022012.csv"]][[1;;4]]
data5=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\2uM
17OHPreg17052012.csv"]][[1;;4]]

```

```

data6=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
    2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\5uM 17OHPreg
    17052012.csv"]][[1;;4]]
data7=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
    2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\2uM 17OHPP
    17052012.csv"]][[1;;4]]
data8=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
    2011\\3B-HSD werk\\3B-HSD reaksie 17-OHPreg\\2012 werk\\5uM 17OHPP
    17052012.csv"]][[1;;4]]
datfunc[points_,result_]:=Join[{points},Flatten[variables/.result/.{t->points},1]];
points1=data1[[1]];
points2=data2[[1]];
points3=data3[[1]];
points4=data4[[1]];
points5=data5[[1]];
points6=data6[[1]];
points7=data7[[1]];
points8=data8[[1]];
Onerun[par1_,par2_,par3_,init1_,init2_,points_]:=
(
initvars={init1,init2};
params={Vm1->par1,Ks1->par2,Kp1->par3};
ODEs={OH17Preg'[t]==-v1,OH17Prog'[t]==v1}/.ratefuncs/.params;
inits=Thread[(variables/.t->0)==initvars];
result=Solvefunc[0,7];
datfunc[points,result]
)
objfunc[par1_/;NumberQ[par1],par2_/;NumberQ[par2],par3_/;NumberQ[par3],inita1_/;Num
berQ[inita1],inita2_/;NumberQ[inita2],initb1_/;NumberQ[initb1],initb2_/;NumberQ[init
b2],inite1_/;NumberQ[initc1],initc2_/;NumberQ[initc2],initd1_/;NumberQ[initd1],initd2
_/;NumberQ[initd2]]:=
(

```

```

newdata1=Onerun[(1.066/1.254)*par1,par2,par3,inita1,inita2,points1];
newdata2=Onerun[(1.066/1.254)*par1,par2,par3,initb1,initb2,points2];
newdata3=Onerun[(1.066/1.254)*par1,par2,par3,initc1,initc2,points3];
newdata4=Onerun[(1.066/1.254)*par1,par2,par3,initd1,initd2,points4];
newdata5=Onerun[(0.662/1.254)*par1,par2,par3,inita1,inita2,points5];
newdata6=Onerun[(0.662/1.254)*par1,par2,par3,initb1,initb2,points6];
newdata7=Onerun[(0.662/1.254)*par1,par2,par3,initc1,initc2,points7];
newdata8=Onerun[(0.662/1.254)*par1,par2,par3,initd1,initd2,points8];
Plus@@(Flatten[newdata1-data1]^2)+Plus@@(Flatten[newdata2-
  data2]^2)+Plus@@(Flatten[newdata3-data3]^2)+Plus@@(Flatten[newdata4-
  data4]^2)+Plus@@(Flatten[newdata5-data5]^2)+Plus@@(Flatten[newdata6-
  data6]^2)+Plus@@(Flatten[newdata7-data7]^2)+Plus@@(Flatten[newdata8-data8]^2)
)
NMinimize[{objfunc[par1,par2,par3,2,0,5,0,1.15,2.85,3.38,6.62],par1>0.0001,par2>0.0001,par3>0.0001},{par1,par2,par3},Method->"Automatic"]
]
params={Vm1->2.358377531719676`,Ks1->3.6438050004635345`,Kp1->3.9767007326223736`};

```

Reaction for DHEA to A4:

```

req[s1_,p1_,var1_,var2_,var3_]:= (var1*(s1/var2))/(1+s1/var2+p1/var3)
ratenames={v1};
rateequations={req[DHEA[t],A4[t],Vm1,Ks1,Kp1]};
ratefuncs=Thread[ratenames->rateequations];
variables={DHEA[t],A4[t]};
Solvefunc[t1_,t2_]:=NDSolve[Join[ODEs,init],variables,{t,t1,t2}]
data1=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
  2011\\3B-HSD werk\\3B-HSD reaksie DHEA\\2012 werk\\2uM A5
  15032012.csv"]][[1;;4]]
data2=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
  2011\\3B-HSD werk\\3B-HSD reaksie DHEA\\2012 werk\\5uM A5
  15032012.csv"]][[1;;4]]

```

```

data3=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie DHEA\\2012 werk\\2uM AA
15032012.csv"]][[1;;4]]
data4=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
2011\\3B-HSD werk\\3B-HSD reaksie DHEA\\2012 werk\\5uM AA
15032012.csv"]][[1;;4]]
datfunc[points_,result_]:=Join[{points},Flatten[variables/.result/.{t->points},1]];
points1=data1[[1]];
points2=data2[[1]];
points3=data3[[1]];
points4=data4[[1]];
Onerun[par1_,par2_,par3_,init1_,init2_,points_]:=
(
initvars={init1,init2};
params={Vm1->par1,Ks1->par2,Kp1->par3};
ODEs={DHEA'[t]==-v1,A4'[t]==v1}/.ratefuncs/.params;
inits=Thread[(variables/.t->0)==initvars];
result=Solvefunc[0,7];
datfunc[points,result]
)
objfunc[par1_/;NumberQ[par1],par2_/;NumberQ[par2],par3_/;NumberQ[par3],inita1_/;Num
berQ[inita1],inita2_/;NumberQ[inita2],initb1_/;NumberQ[initb1],initb2_/;NumberQ[init
b2],initc1_/;NumberQ[initc1],initc2_/;NumberQ[initc2],initd1_/;NumberQ[initd1],initd2
_/;NumberQ[initd2]]:=
(
newdata1=Onerun[(0.628/1.254)*par1,par2,par3,inita1,inita2,points1];
newdata2=Onerun[(0.628/1.254)*par1,par2,par3,initb1,initb2,points2];
newdata3=Onerun[(0.628/1.254)*par1,par2,par3,initc1,initc2,points3];
newdata4=Onerun[(0.628/1.254)*par1,par2,par3,initd1,initd2,points4];
Plus@@(Flatten[newdata1-data1]^2)+Plus@@(Flatten[newdata2-
data2]^2)+Plus@@(Flatten[newdata3-data3]^2)+Plus@@(Flatten[newdata4-data4]^2)
)

```



```
NMinimize[{objfunc[par1,par2,par3,2,0,5,0,2.23,1.77,5.23,4.78],par1>0.0001,par2>0.0001,par3>0.0001},{par1,par2,par3},Method->"Automatic"]
params={Vm1->1.4144793380470118`,Ks1->0.6031622950844974`,Kp1->0.8998545490204655`};
```

Validation for all reactions catalyzed by 3 β -HSD simultaneously:

```
List1=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\3B-HSD werk\\3B-HSD
validasie\\2012 werk\\3uM Preg.csv"]
List1trans=Transpose[List1]
List2=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\3B-HSD werk\\3B-HSD
validasie\\2012 werk\\3uM 17OHPreg.csv"]
List2trans=Transpose[List2]
List3=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\3B-HSD werk\\3B-HSD
validasie\\2012 werk\\3uM DHEA.csv"]
List3trans=Transpose[List3]
List4=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\3B-HSD werk\\3B-HSD
validasie\\2012 werk\\3uM PP.csv"]
List4trans=Transpose[List4]
List5=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\3B-HSD werk\\3B-HSD
validasie\\2012 werk\\3uM 17OHPP.csv"]
List5trans=Transpose[List5]
List6=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\3B-HSD werk\\3B-HSD
validasie\\2012 werk\\3uM AA.csv"]
List6trans=Transpose[List6]
Needs["ErrorBarPlots`"]
Needs["PlotLegends`"]
ploya=ErrorListPlot[{Table[{Thread[{List1trans[[1]],List1trans[[2]]}][[i]],ErrorBar[List1trans[[4]][[i]]}],{i,1,Length[List1trans[[4]]}],Table[{Thread[{List1trans[[1]],List1trans[[3]]}][[i]],ErrorBar[List1trans[[4]][[i]]}],{i,1,Length[List1trans[[4]]}],PlotStyle->{GrayLevel[.5],GrayLevel[0]},Frame->True,FrameLabel->{"Time
(hours)","Concentration (\\[Mu]M)"},BaseStyle->{"Times New Roman",20}]
```

```

ployd=ErrorListPlot[ {Table[ {Thread[ {List2trans[[1]],List2trans[[2]]}][[i]],ErrorBar[List2tra
ns[[4]][[i]]}, {i,1,Length[List2trans[[4]]}],Table[ {Thread[ {List2trans[[1]],List2trans[[3
]]}][[i]],ErrorBar[List2trans[[4]][[i]]}, {i,1,Length[List2trans[[4]]}],PlotStyle-
>{GrayLevel[.5],GrayLevel[0]},Frame->True,FrameLabel->{"Time
(hours)","Concentration (\[Mu]M)",BaseStyle->{"Times New Roman",20}]

```

```

plocy=ErrorListPlot[ {Table[ {Thread[ {List3trans[[1]],List3trans[[2]]}][[i]],ErrorBar[List3tra
ns[[4]][[i]]}, {i,1,Length[List3trans[[4]]}],Table[ {Thread[ {List3trans[[1]],List3trans[[3
]]}][[i]],ErrorBar[List3trans[[4]][[i]]}, {i,1,Length[List3trans[[4]]}],PlotStyle-
>{GrayLevel[.5],GrayLevel[0]},Frame->True,FrameLabel->{"Time
(hours)","Concentration (\[Mu]M)",BaseStyle->{"Times New Roman",20}]

```

```

ployd=ErrorListPlot[ {Table[ {Thread[ {List4trans[[1]],List4trans[[2]]}][[i]],ErrorBar[List4tra
ns[[4]][[i]]}, {i,1,Length[List4trans[[4]]}],Table[ {Thread[ {List4trans[[1]],List4trans[[3
]]}][[i]],ErrorBar[List4trans[[4]][[i]]}, {i,1,Length[List4trans[[4]]}],PlotStyle-
>{GrayLevel[.5],GrayLevel[0]},PlotRange->{{0,7},{0,6}},Frame->True,FrameLabel-
>{"Time (hours)","Concentration (\[Mu]M)",BaseStyle->{"Times New Roman",20}]

```

```

plocy=ErrorListPlot[ {Table[ {Thread[ {List5trans[[1]],List5trans[[2]]}][[i]],ErrorBar[List5tra
ns[[4]][[i]]}, {i,1,Length[List5trans[[4]]}],Table[ {Thread[ {List5trans[[1]],List5trans[[3
]]}][[i]],ErrorBar[List5trans[[4]][[i]]}, {i,1,Length[List5trans[[4]]}],PlotStyle-
>{GrayLevel[.5],GrayLevel[0]},PlotRange->{{0,7},{0,6}},Frame->True,FrameLabel-
>{"Time (hours)","Concentration (\[Mu]M)",BaseStyle->{"Times New Roman",20}]

```

```

ployd=ErrorListPlot[ {Table[ {Thread[ {List6trans[[1]],List6trans[[2]]}][[i]],ErrorBar[List6tran
s[[4]][[i]]}, {i,1,Length[List6trans[[4]]}],Table[ {Thread[ {List6trans[[1]],List6trans[[3]]
}][[i]],ErrorBar[List6trans[[4]][[i]]}, {i,1,Length[List6trans[[4]]}],PlotStyle-
>{GrayLevel[.5],GrayLevel[0]},PlotRange->{{0,7},{0,6}},Frame->True,FrameLabel-
>{"Time (hours)","Concentration (\[Mu]M)",BaseStyle->{"Times New Roman",20}]

```

Vmax6=2.5;

Vmax7=2.35;

Vmax8=1.41;

Subscript[K, P5]=1.98;

Subscript[K, P4]=10.06;

Subscript[K, DHEA]=0.6;

Subscript[K, A4]=0.89;

Subscript[K, OH17P5]=3.64;

Subscript[K, OH17P4]=3.97;

bvar=0.475/1.254;

v6=(bvar*Vmax6 P5[t]/Subscript[K, P5])/(1+P5[t]/Subscript[K, P5]+P4[t]/Subscript[K, P4]+DHEA[t]/Subscript[K, DHEA]+A4[t]/Subscript[K, A4]+OH17P5[t]/Subscript[K, OH17P5]+OH17P4[t]/Subscript[K, OH17P4]);

v7=(bvar*Vmax7 OH17P5[t]/Subscript[K, OH17P5])/(1+P5[t]/Subscript[K, P5]+P4[t]/Subscript[K, P4]+DHEA[t]/Subscript[K, DHEA]+A4[t]/Subscript[K, A4]+OH17P5[t]/Subscript[K, OH17P5]+OH17P4[t]/Subscript[K, OH17P4]);

v8=(bvar*Vmax8 DHEA[t]/Subscript[K, DHEA])/(1+P5[t]/Subscript[K, P5]+P4[t]/Subscript[K, P4]+DHEA[t]/Subscript[K, DHEA]+A4[t]/Subscript[K, A4]+OH17P5[t]/Subscript[K, OH17P5]+OH17P4[t]/Subscript[K, OH17P4]);

sol3=NDSolve[{P5'[t]==-v6,P5[0]==3,P4'[t]==v6,P4[0]==0.001,OH17P5'[t]==-v7,OH17P5[0]==3,OH17P4'[t]==v7,OH17P4[0]==0.001,DHEA'[t]==-v8,DHEA[0]==3,A4'[t]==v8,A4[0]==0.001},{P5[t],P4[t],OH17P5[t],OH17P4[t],DHEA[t],A4[t]},{t,0,7}];

sol6=NDSolve[{P5'[t]==-v6,P5[0]==3,P4'[t]==v6,P4[0]==3,OH17P5'[t]==-v7,OH17P5[0]==3,OH17P4'[t]==v7,OH17P4[0]==3,DHEA'[t]==-v8,DHEA[0]==3,A4'[t]==v8,A4[0]==3},{P5[t],P4[t],OH17P5[t],OH17P4[t],DHEA[t],A4[t]},{t,0,7}];

plota=Plot[{P5[t],P4[t]}/.sol3,{t,0,7},PlotRange->{{0,7},{0,3}},PlotStyle->{GrayLevel[.5],GrayLevel[0]},Frame->True,FrameLabel->{"Time (hours)","Concentration (μ M)"},BaseStyle->{"Times New Roman",20}]

ShowLegend[Show[ploya,plota],{{{Graphics[{GrayLevel[.5],Line[{{1,0},{2,0}}],Disk[]]},Preg},{Graphics[{GrayLevel[0],Line[{{1,0},{2,0}}],Disk[]]},Prog}},LegendPosition->{1,-.2},LegendSize->.5}]

ShowLegend[Show[ployb,plotb],{{{Graphics[{GrayLevel[.5],Line[{{1,0},{2,0}}],Disk[]]},17OHProg},{Graphics[{GrayLevel[0],Line[{{1,0},{2,0}}],Disk[]]},17OHProg}},LegendPosition->{1,-.2},LegendSize->.5}]

```
ShowLegend[Show[ployc,plotc],{{{Graphics[{GrayLevel[.5],Line[{{1,0},{2,0}}],Disk[]]},
  DHEA},{Graphics[{GrayLevel[0],Line[{{1,0},{2,0}}],Disk[]],A4}},LegendPosition-
  >{1,-.2},LegendSize->.5]}]
ShowLegend[Show[ployd,plotd],{{{Graphics[{GrayLevel[.5],Line[{{1,0},{2,0}}],Disk[]]},
  Preg},{Graphics[{GrayLevel[0],Line[{{1,0},{2,0}}],Disk[]],Prog}},LegendPosition-
  >{1,-.2},LegendSize->.5]}]
ShowLegend[Show[ploye,plote],{{{Graphics[{GrayLevel[.5],Line[{{1,0},{2,0}}],Disk[]]},1
  7OHPreg},{Graphics[{GrayLevel[0],Line[{{1,0},{2,0}}],Disk[]],17OHProg}},Legend
  Position->{1,-.2},LegendSize->.5]}]
ShowLegend[Show[ployf,plotf],{{{Graphics[{GrayLevel[.5],Line[{{1,0},{2,0}}],Disk[]]},D
  HEA},{Graphics[{GrayLevel[0],Line[{{1,0},{2,0}}],Disk[]],A4}},LegendPosition-
  >{1,-.2},LegendSize->.5]}]
```

Construction and validation of a kinetic model for the reactions catalyzed by CYP17

Reaction for PROG to 17OH-PROG and 16OH-PROG in turn 17OH-PROG to A4:

```
req[s1_,a1_,b1_,p1_,var1_,var2_,var3_,var4_,var5_]:=
  (var1*(s1/var2))/(1+s1/var2+a1/var3+b1/var4+p1/var5)
ratenames={v1,v2,v3};
rateequations={req[Prog[t],OH17Prog[t],OH16Prog[t],A4[t],Vm1,Ks1,Ka1,Kb1,Kp1],req[OH17Prog[t],Prog[t],OH16Prog[t],A4[t],Vm2,Ka1,Ks1,Kb1,Kp1],req[Prog[t],OH17Prog[t],OH16Prog[t],A4[t],Vm3,Ks1,Ka1,Kb1,Kp1]};
ratefuncs=Thread[ratenames->rateequations];
variables={Prog[t],OH16Prog[t],OH17Prog[t],A4[t]};
Solvefunc[t1_,t2_]:=NDSolve[Join[ODEs,inits],variables,{t,t1,t2}]
data1=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem 2011\\C17 werk\\2011 werk\\Prog werk\\1.5uM Prog with C17.csv"]][[1;;7]]
data2=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem 2011\\C17 werk\\2011 werk\\Prog werk\\2uM Prog with C17.csv"]][[1;;7]]
```

```

data3=Transpose[Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem
    2011\\C17 werk\\2011 werk\\Prog werk\\3uM Prog with C17.csv"]][[1;;7]]
datfunc[points_,result_]:=Join[{points},Flatten[{Prog[t],OH16Prog[t],OH17Prog[t],A4[t]}/.r
    esult/.{t->points},1]];
points1=data1[[1]];
points2=data2[[1]];
points3=data3[[1]];
Onerun[par1_,par2_,par3_,par4_,par5_,par6_,par7_,init1_,init2_,init3_,init4_,points_]:=
(
initvars={init1,init2,init3,init4};
params={Vm1->par1,Ks1->par2,Ka1->par3,Kb1->par4,Kp1->par5,Vm2->par6,Vm3->par7};
ODEs={Prog'[t]==-v1-v3,OH16Prog'[t]==v3,OH17Prog'[t]==v1-
    v2,A4'[t]==v2}/.ratefuncs/.params;
inits=Thread[(variables/.t->0)==initvars];
result=Solvefunc[0,12];
datfunc[points,result]
)
newdata1=Onerun[1,2,3,4,5,6,7,1.5,0,0,0,points1]
newdata2=Onerun[1,2,3,4,5,6,7,2,0,0,0,points2]
newdata3=Onerun[1,2,3,4,5,6,7,3,0,0,0,points3]
objfunc[par1_/;NumberQ[par1],par2_/;NumberQ[par2],par3_/;NumberQ[par3],par4_/;Numb
    erQ[par4],par5_/;NumberQ[par5],par6_/;NumberQ[par6],par7_/;NumberQ[par7],inita1_/
    ;NumberQ[inita1],inita2_/;NumberQ[inita2],inita3_/;NumberQ[inita3],inita4_/;Number
    Q[inita4],initb1_/;NumberQ[initb1],initb2_/;NumberQ[initb2],initb3_/;NumberQ[initb3],
    initb4_/;NumberQ[initb4],initc1_/;NumberQ[initc1],initc2_/;NumberQ[initc2],initc3_/;N
    umberQ[initc3],initc4_/;NumberQ[initc4]]:=
(
newdata1=Onerun[(0.615/0.7103)*par1,par2,par3,par4,par5,(0.615/0.7103)*par6,(0.615/0.71
    03)*par7,inita1,inita2,inita3,inita4,points1];
newdata2=Onerun[(0.615/0.7103)*par1,par2,par3,par4,par5,(0.615/0.7103)*par6,(0.615/0.71
    03)*par7,initb1,initb2,initb3,initb4,points2];

```

```

newdata3=Onerun[(0.321/0.7103)*par1,par2,par3,par4,par5,(0.321/0.7103)*par6,(0.321/0.7103)*par7,initc1,initc2,initc3,initc4,points3];
Plus@@(Flatten[newdata1-data1]^2)+Plus@@(Flatten[newdata2-data2]^2)+Plus@@(Flatten[newdata3-data3]^2)
)
NMinimize[{objfunc[par1,par2,par3,par4,par5,par6,par7,1.5,0,0,0,2,0,0,0,3,0,0,0],par1>0.000001,par2>0.000001,par3>0.000001,par4>0.000001,par5>0.000001,par6>0.000001,par7>0.000001},{par1,par2,par3,par4,par5,par6,par7},Method->"Automatic"]
]
params={Vm1->1.1441436153194071`,Ks1->1.2146454291126914`,Ka1->6.090319576740303`,Kb1->5.5789827167780945`,Kp1->0.20222941801335942`,Vm2->0.07086031325903548`,Vm3->0.02591511568881708`};

```

Validation for all reactions catalyzed by CYP17 simultaneously:

```

List1=Import["C:\\Users\\Public\\Documents\\MSc Biochem 2011\\C17 werk\\2011 werk\\C17 validasie\\3PP PregC17.csv"]
List1trans=Transpose[List1]
List2=Import["C:\\Users\\user\\Documents\\Stellenbosch\\MSc Biochem 2011\\C17 werk\\2011 werk\\C17 validasie\\3uM Preg en Prog.csv"](*Import die file as CVS*)
List2trans=Transpose[List2]
Needs["ErrorBarPlots`"]
Needs["PlotLegends`"]
ploya=ErrorListPlot[{Table[{Thread[{List1trans[[1]],List1trans[[2]]}][[i]],ErrorBar[List1trans[[5]][[i]]}],{i,1,Length[List1trans[[5]]}],Table[{Thread[{List1trans[[1]],List1trans[[3]]}][[i]],ErrorBar[List1trans[[6]][[i]]}],{i,1,Length[List1trans[[6]]}],Table[{Thread[{List1trans[[1]],List1trans[[4]]}][[i]],ErrorBar[List1trans[[7]][[i]]}],{i,1,Length[List1trans[[7]]}],PlotStyle->PointSize[0.01],Frame->True,FrameLabel->{"Time (hours)","Intermedaite (\\[Mu]mol)"},BaseStyle->{"Helvetica",20}]
]
ployx=
ShowLegend[ErrorListPlot[{Table[{Thread[{List2trans[[1]],List2trans[[2]]}][[i]],ErrorBar[List2trans[[6]][[i]]}],{i,1,Length[List2trans[[6]]}],Table[{Thread[{List2trans[[1]],List2tr

```



```
ans[[3]]][[i]],ErrorBar[List2trans[[7]][[i]]],{i,1,Length[List2trans[[7]]]},Table[{Thread[
d[{List2trans[[1]],List2trans[[4]]][[i]],ErrorBar[List2trans[[8]][[i]]],{i,1,Length[List2t
rans[[8]]]},Table[{Thread[{List2trans[[1]],List2trans[[5]]][[i]],ErrorBar[List2trans[[9]
][[i]]],{i,1,Length[List2trans[[9]]]},PlotRange->{{0,12},{0,3}},PlotStyle-
>{GrayLevel[.5],GrayLevel[0]},Frame->True,FrameLabel->{"Time
(hours)","Concentration (\[Mu]M)"},BaseStyle-
>{"Helvetica",20}],{{Graphics[{GrayLevel[.75],Disk[]},Style["Prog",FontSize-
>12]},Graphics[{GrayLevel[0],Disk[]},Style["OH17Prog",FontSize-
>12]},Graphics[{GrayLevel[.5],Disk[]},Style["OH16Prog",FontSize-
>12]},Graphics[{GrayLevel[.25],Disk[]},Style["A4",FontSize-
>12]}],LegendPosition->{0.8,-.2},LegendSize->.5}]
```

```
Vmax1=0.7329311619157484`;
```

```
Vmax2=0.16239303138893654`;
```

```
Vmax3=1.231`;
```

```
Vmax4=0.065`;
```

```
Vmax5=0.027`;
```

```
Subscript[K, P4]=1.29`;
```

```
Subscript[K, OH17P4]=4.94`;
```

```
Subscript[K, OH16P4]=2.66`;
```

```
Subscript[K, A4]=1.17`;
```

```
Subscript[K, P5]=0.5188381175053178`;
```

```
Subscript[K, OH17P5]=1.0000000000051403`;
```

```
Subscript[K, DHEA]=4430.349465240219`;
```

```
bvar=0.620/0.7103;
```

```
v1=((Vmax1*bvar) P5[t]/Subscript[K, P5])/(1+P4[t]/Subscript[K,
P4]+OH17P4[t]/Subscript[K, OH17P4]+A4[t]/Subscript[K,
A4]+OH16P4[t]/Subscript[K, OH16P4]+P5[t]/Subscript[K,
P5]+OH17P5[t]/Subscript[K, OH17P5]+DHEA[t]/Subscript[K, DHEA]);
v2=((Vmax2*bvar) OH17P5[t]/Subscript[K, OH17P5])/(1+P4[t]/Subscript[K,
P4]+OH17P4[t]/Subscript[K, OH17P4]+A4[t]/Subscript[K,
```

```

A4]+OH16P4[t]/Subscript[K, OH16P4]+P5[t]/Subscript[K,
P5]+OH17P5[t]/Subscript[K, OH17P5]+DHEA[t]/Subscript[K, DHEA]);
v3=((Vmax3*bvar) P4[t]/Subscript[K, P4])/(1+P4[t]/Subscript[K,
P4]+OH17P4[t]/Subscript[K, OH17P4]+A4[t]/Subscript[K,
A4]+OH16P4[t]/Subscript[K, OH16P4]+P5[t]/Subscript[K,
P5]+OH17P5[t]/Subscript[K, OH17P5]+DHEA[t]/Subscript[K, DHEA]);
v4=((Vmax4*bvar) OH17P4[t]/Subscript[K, OH17P4])/(1+P4[t]/Subscript[K,
P4]+OH17P4[t]/Subscript[K, OH17P4]+A4[t]/Subscript[K,
A4]+OH16P4[t]/Subscript[K, OH16P4]+P5[t]/Subscript[K,
P5]+OH17P5[t]/Subscript[K, OH17P5]+DHEA[t]/Subscript[K, DHEA]);
v5=((Vmax5*bvar) P4[t]/Subscript[K, P4])/(1+P4[t]/Subscript[K,
P4]+OH17P4[t]/Subscript[K, OH17P4]+A4[t]/Subscript[K,
A4]+OH16P4[t]/Subscript[K, OH16P4]+P5[t]/Subscript[K,
P5]+OH17P5[t]/Subscript[K, OH17P5]+DHEA[t]/Subscript[K, DHEA]);
sol3=NDSolve[{P5'[t]==-v1,P5[0]==3,OH17P5'[t]==v1-
v2,OH17P5[0]==0.001,DHEA'[t]==v2,DHEA[0]==0.001,P4'[t]==-v3-
v5,P4[0]==3,OH17P4'[t]==v3-
v4,OH17P4[0]==0.001,OH16P4'[t]==v5,OH16P4[0]==0.001,A4'[t]==v4,A4[0]==0.001}
,{P5[t],OH17P5[t],DHEA[t],P4[t],OH17P4[t],OH16P4,A4[t]},{t,0,12}];
plota=Plot[{P5[t],OH17P5[t],DHEA[t]}/.sol3},{t,0,12},PlotStyle->
Thickness[0.005],Frame->True,FrameLabel->{"Time (hours)","Intermediate
(\[Mu]mol)"},BaseStyle->{"Helvetica",20}]
Show[plota,ploya]
plotx=ShowLegend[Plot[{P4[t],OH16P4[t],OH17P4[t],A4[t]}/.sol3},{t,0,12},PlotRange-
>{{0,12},{0,3}},PlotStyle->{GrayLevel[.5],GrayLevel[0]},Frame->True,FrameLabel-
>{"Time (hours)","Concentration (\[Mu]M)"},BaseStyle-
>{"Helvetica",20}],{{Graphics[{GrayLevel[.75],Disk[]}],Style["Prog",FontSize-
>12]},Graphics[{GrayLevel[0],Disk[]}],Style["OH17Prog",FontSize-
>12]},Graphics[{GrayLevel[.5],Disk[]}],Style["OH16Prog",FontSize-
>12]},Graphics[{GrayLevel[.25],Disk[]}],Style["A4",FontSize-
>12]}},LegendPosition->{0.8,-.2},LegendSize->.5}]
Show[plotx,ployx]

```