FINAL DETAILED REVIEW PAPER

ON

STEROIDOGENESIS SCREENING ASSAYS
AND ENDOCRINE DISRUPTORS

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Gary E. Timm
Work Assignment Manager
U.S. Environmental Protection Agency
Endocrine Disruptor Screening Program
Washington, D.C. 20004

by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

AUTHORS

Patricia A. Fail, Ph.D.
Carol S. Sloan, M.S.
Research Triangle Institute
Research Triangle Park, North Carolina

Jerry D. Johnson, Ph.D.
Vincent J. Brown, Ph.D.
Battelle Memorial Institute
Columbus, Ohio

PREFACE

The original draft of this DRP was prepared in May 2002. This final draft incorporates comments based on EPA and OECD reviews. No update of the references was performed for this final draft.

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List of Abbreviations

The following abbreviations are used in this DRP:

ACTH	adrenocorticotropic hormone
ANOVA	Analysis of Variance
AR	androgen receptor
ATP	adenosine triphosphate
BSA	bovine serum albumin
C8	ammonium perfluorooctanoate
cAMP	cyclic adenosine monophosphate
CEMS	chloroethylmethanesulfonate
CNS	central nervous system
CRH	corticotropin releasing hormone
DBA	dibromoacetic acid
DEHP	diethylhexylphthalate
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DMSO	dimethylsulfoxide
DRP	Detailed Review Paper
E2	17β-estradiol
ECT	Endocrine Challenge Test
ED_{50}	effective dose 50
EDMVS	Endocrine Disruptor Methods Validation Subcommittee
EDS	ethane dimethanesulfonate
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EPA	U. S. Environmental Protection Agency
ER	estrogen receptor
FFDCA	Federal Food, Drug and Cosmetics Act
FGF	fibroblast growth factor
FQPA	Food Quality Protection Act
FRF	follicle releasing factor
FSH	follicle-stimulating hormone
GD	gestation day
GLM	General Linear Model
GnRH	gonadotropin-releasing hormone

hCG	human chorionic gonadotropin
HDL	high-density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
3β-HSD	3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase
IC	inhibitory concentration
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IGF	Insulin Growth Factor
IVF	*in vitro* fertilization
17 KSR	17-ketosteroid reductase
LDL	low-density lipoprotein
LH	luteinizing hormone
LRF	luteinizing hormone releasing factor
mRNA	messenger ribonucleic acid
NIEHS	National Institute of Environmental Health Science
NRC	National Research Council
OECD	Organization for Economic Cooperation and Development
P4	progesterone
PBS	phosphate buffered saline
PIF	prolactin-inhibiting factor
PMSG	pregnant mare serum gonadotropin
Prl	prolactin
REGRESS	procedure from SUDAAN
RIA	radioimmunoassay
RMD	Reference Manager Database
RNA	ribonucleic acid
RTI	Research Triangle Institute
SAB	Science Advisory Board
SAP	Scientific Advisory Panel
SCC	side chain cleavage
SDWA	Safe Drinking Water Act
StAR	steroidogenic acute regulatory protein
STP	steroidogenic stimulatory protein

T	testosterone
T1S	Tier 1 Screening
T2T	Tier 2 Testing
T3	triiodothyronine
T4	thyroxin
TCDD	tetrachlorodibenzodioxin
TSH	thyroid stimulating hormone
VMG	Validation Management Group
VMG-NA	Validation Management Grup–Non-animal Methods

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1.0 EXECUTIVE SUMMARY

The purpose of this Detailed Review Paper (DRP) on gonadal steroidogenesis is to (1) survey and review the biological mammalian methods that identify substances with direct effects on the steroidogenic pathway*;* (2) critically evaluate the methods and the individual assays of a given method for their potential use as screening test(s) to identify potential endocrine disruptors, endocrine modulators, or endocrine toxicants; and (3) recommend the next step in developing and evaluating an appropriate screening study protocol using these assays for further standardization and validation. In addition, this document has been written in a manner that can be understood by individuals trained in the biological sciences at the collegiate level but who may have little or no specialty training in reproductive physiology or toxicology, nor any reproductive laboratory experience.

This DRP (1) summarizes the state of the science of the *in vivo, ex vivo,* and *in vitro* methodologies available for measuring gonadal steroidogenesis; (2) for each methodology, presents a review of the individual assays and representative data generated by investigators that used the assay to evaluate a substance for steroidogenic-altering activity; (3) provides an evaluation of the various methodologies and the assays as tools for screening substances with suspected steroidogenic activity; (4) recommends a particular screening method and assay as a screening tool; and (5) describes the strengths, weaknesses, and implications for further research associated with the recommended screening assay.

In addition to the Executive Summary (Section 1), this DRP is organized into five other sections:

2. Introduction to the Endocrine Disruptor Screening Program
3. General background on reproductive endocrinology and steroidogenesis
4. Bioanalytical methodologies for measuring gonadal steroidogenesis
5. Candidate protocol
6. Developmental status of the assay.

References and appendices provide additional, more detailed information.

During the development of this DRP, a thorough review of the published and unpublished literature was conducted. Over 230 journal articles germane to the effort were reviewed. A Reference Manager Database (RMD) was created from the retrieved literature. The title and abstracts were included in the RMD, along with key information obtained from individual articles, such as test substance and species. In addition, personal interviews with five leading experts in the field of steroidogenesis were conducted to gather additional information on known test methods, procedures, and measurement endpoints that could be used for identifying impacts from substances that can directly alter the function of the steroidogenic pathway.

The steroidogenic pathway of interest in this DRP was limited to the biochemical pathway located in the gonads of male and female animals. In the male, steroidogenesis occurs in the Leydig cells of the testis and, in the female, this pathway is found in the follicle of the ovary. At the cellular level, a series of biochemical reactions are initiated upon stimulation of

receptors located in the membranes of these cells. Activation of the LH/FSH receptors initiates a series of enzymatic reactions that culminate in the biosynthesis of end-product hormones, i.e., testosterone (male) and estradiol/estrone (female). This DRP is intended to address the pathway after the receptor, beginning with the second messenger and ending with the end-product hormone. In particular, this DRP seeks to survey and review methodologies that can be used to identify substances that alter steroidogenesis by a direct interaction with one or more of the substrates, enzymes, or other cellular components that constitute this pathway. Only assays that evaluate these components of the pathway were reviewed and assessed as a possible steroidogenic screening tool.

The most promising assay for use as a screening tool was based on advantages and disadvantages of the various methodologies and criteria established for the optimal screen. These attributes were reported by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) in their final report, as well as other literature references (Gray et al., 1997; EDSTAC, 1998). Based on this information, the methodologies and their respective assays were compared to the optimal screen criteria in order to select the most promising assay for use as a screening tool. For a given method, the assays evaluated were the Endocrine Challenge Test (ECT), which is an *in vivo* method; the whole testis or ovary used in simple incubation, perifusion, or perfusion assays, as well as the sectioned testis, minced ovary, isolated and cultured crude or purified Leydig cells, isolated and cultured granulosa cells, and cell line assays, which are all *in vitro* assays; or a combination of the *in vivo* and *in vitro* methods, which constitute the *ex vivo* assays.

The ***in vitro*** **sectioned testis assay** was selected as the most promising screening tool for identifying substances with steroidogenic-altering activity. Based on the advantages and disadvantages of the *in vivo* and *in vitro* methods, the *in vitro* methodology was in better agreement with the characteristics of an optimal screen. In addition, it was determined that this single assay would suffice as a screen for females as well, because the steroidogenic pathways of the two genders are very similar, the testes provide more organ for testing, and organ isolation and preparation are technically easier to accomplish using the male organs. Finally, based on the criteria used, the sectioned testis assay is recommended, because it can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory training; the preparation is stable; the organ remains viable (over several hours); the assay is relatively sensitive and specific; it maintains the cytoarchitecture of the organ; the assay uses a reduced number of animals (quartered sections); the assay will be relatively easy to standardize (by optimization or consensus); and the assay has well-defined and multiple endpoints.

2.0 INTRODUCTION

2.1 Developing and Implementing the Endocrine Disruptor Screening Program (EDSP)

In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) mandated the United States Environmental Protection Agency (U.S. EPA) to screen pesticides and authorized the U.S. EPA to screen chemicals found in drinking water to determine whether they possess estrogenic or other endocrine activity (Federal Register, 1998a, 1998b). The U.S. EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect..." (FQPA, 1996). The U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally-occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC, 1998), the U.S. EPA established the Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, e.g. a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial substances, and environmental contaminants. The Organization for Economic Cooperation and Development (OECD) initiated a high-priority activity in 1997 to develop new test guidelines and revise existing test guidelines for the screening and testing of potential endocrine disruptors. This activity is organized under the Task Force on Endocrine Disrupters Testing and Assessment as part of the OECD test guidelines program and managed by three Validation Management Groups (VMGs) covering mammalian, ecotoxicity, and non-animal methods.

This Detailed Review Paper was prepared for the U.S. EPA in 2002 to review the scientific basis of the steroidogenesis assay and examine assays reported in the literature used to measure the effect of chemical substances on steroidogenesis. It was presented and discussed at the first meeting of the OECD Validation Management Group for Non-Animal methods (VMG-NA) and is being adopted for use by the OECD. As the document has served its original purpose of identifying promising methods for further development and prevalidation, it has not been updated to reflect the literature published after April 2002, but does reflect comments submitted by members of the VMG-NA.

2.2 Purpose of the Review on Steroidogenesis

The purpose of this Detailed Review Paper (DRP) is to survey and investigate the status of steroidogenic assay methodologies. The steroidogenic pathway includes several potential target sites for pesticides, industrial substances, environmental contaminants, and other such substances. For this reason, an *in vitro* steroidogenic assay is proposed to be used as a Tier 1 assay. It is also the purpose of this DRP to identify the most promising assay that could be used as a screen from among the various steroidogenic assays, as well as the steps that are necessary to develop and evaluate the assay's protocol for further standardization and validation.

2.3 Objective of the Steroidogenic Screen Assay

The objective of the steroidogenic screen assay is to detect any substance that would disrupt estrogen and/or androgen gonadal steroid hormone production. In this way, the assay will complement the other Tier 1 assays so as to provide the necessary breadth and depth to detect substances that could be classified as endocrine disruptors. The steroidogenic assay is intended to identify xenobiotics that have as their target site(s) the endogenous components that comprise the intracellular biochemical pathway beginning with the sequence of reactions occurring after the receptor, up through and including the production of the terminal steroid hormones, i.e. testosterone (males) and estradiol/estrone (females). The steroidogenic assay was not intended to be used to evaluate androgen (AR) or estrogen (ER) receptor binding because substances that effect these sites will be evaluated using separate assays, i.e. AR/ER binding assays. Furthermore, the steroidogenic assay is not intended to identify substances that effect steroidogenesis due to effects on the hypothalamus, pituitary gland, and storage or release of gonadal steroid hormones. The most promising assay for use as a screen, which will meet the objectives as described above, will be a relatively fast, inexpensive, technically simple, animal-limited assay that identifies substances that alter gonadal steroid hormone production due to direct effects on the enzymes or other endogenous components of the steroidogenic pathway found in the testis and ovary.

2.4 Methodology Used in the Analysis

Appendix A describes the methods employed for the literature search (i.e., key words, databases used, results, etc.). Briefly, after key papers were identified, retrieved, and read for content, pertinent information was extracted and synthesized to generate this DRP. In addition to the literature review, interviews with experts were conducted to obtain current views and opinions regarding assays, methods, procedures, and measurement endpoints that hold promise for identifying and developing the most promising screening assay to identify substances that affect (i.e., inhibit or enhance) steroidogenesis. The results of the interviews are found in Appendix B. Finally, accompanying this report is a CD ROM that has the Reference Manager Database of all documents reviewed. This database includes the reference citation and abstract, in addition to summary information from each article.

2.5 Definitions

The definitions of terms that were utilized throughout the DRP are presented below:

ex vivo - treated *in vivo* but observed *in vitro*

hypophysectomized - removal of the pituitary gland

in vitro - outside the body, in an artificial environment

in vivo - within the body

perfusion - media is pumped into the organ via cannulated blood vessels

perifusion - media is pumped through a chamber containing the organ (the media surrounds the organ)

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3.0 GENERAL BACKGROUND ON REPRODUCTIVE ENDOCRINOLOGY

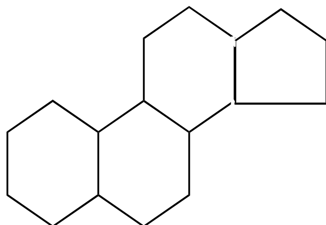
The following material provides background information about the mammalian endocrine system, especially as it pertains to relevant aspects of the reproductive system. The hormones of the reproductive system are synthesized in a steroid-producing sequence of reactions termed steroidogenesis, which is the primary focus of this DRP. The information presented in this section is adapted from textbook chapters on endocrinology (Harrison, 1994; Klaassen, 1996).

3.1 General Endocrinology

The endocrine system, also referred to as the hormonal system, is one of three very important control systems in mammals (the others being the immune and nervous systems). An endocrine system is common to most animals. This system is found in mammals, nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species). In vertebrates, the function of the endocrine system is to regulate a wide range of biological processes including blood sugar levels (through the hormone insulin from the pancreas), growth and function of reproductive systems (through the hormones testosterone and estradiol and related compounds from the testes and ovaries), regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland), development of the brain and the rest of the nervous system (estrogen and thyroid hormones), and development of an organism from conception through adulthood and old age. Therefore, normal functioning of the endocrine system contributes to homeostasis and to the body's ability to control and regulate reproduction, development, and behavior.

3.1.1 Hormone Synthesis and Storage

In humans, the endocrine system comprises more than 50 different hormones, and the complexity in other species appears to be comparable. Tissues or glands contain specialized types of cells that synthesize, store, and release hormones directly into the bloodstream. Endocrine-producing cells produce one of three types of hormones: amines, polypeptides, and steroid hormones. The focus of this review will be on the steroid hormones of the reproductive system. The steroid hormones have the following structure as their nucleus:



Steroid hormones are synthesized following a series of chemical reactions using cholesterol as the precursor. A constant supply of cholesterol is necessary for steroid hormone production. Cholesterol is supplied to the cell from serum via protein carriers (high-density or low-density lipoprotein). A minor source of cholesterol involves *de novo* synthesis from acetate. (The

complete biosynthetic pathways for the reproductive system steroid hormones are reviewed in

the section below on Steroidogenesis.)

Storage of hormones depends on the type of hormone. Amine and polypeptide hormones are packaged into granules for intracellular storage and transport. These granules are unique to catecholamine-secreting and polypeptide hormones. In contrast, steroid hormones are not packaged for storage. Steroid hormones are too polar relative to the lipid-like storage vessels of the body, thereby precluding build-up of extra reserves for later use. Storage in such vessels would allow steroid hormones to leak, thereby negating the tight control needed for such active substances. Instead, the availability of steroid hormones is dependent on continual biosynthesis in order for secretion to occur when needed for a physiological response or developmental change to occur.

3.1.2 Hormone Release and Transport

Release of hormones varies by the type of hormone and the way in which the hormone is stored. Hormones stored in storage granuoles, i.e., amines and polypeptides, are released by exocytosis. As for the steroid hormones, their release is dependent on a stimulus for production and, once synthesized, their release can occur as a result of passive diffusion. The steroid hormone flows down a concentration gradient created by the site of production relative to the dynamic flow of the blood circulation. Thus, for steroid hormones, the limiting factor in their release is the rate of production.

The rate of release of hormones varies, at times, in a rhythmic fashion. For example, the hypothalamus releases gonadotropin releasing hormone (GnRH) in a pulsatile pattern. GnRH, in a receptor-mediated process, stimulates cells of the anterior lobe of the pituitary to secrete the gonadotrophins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are also released in a similar pulsatile pattern. Pulsatile patterns of GnRH releases continue throughout adulthood. The specific patterns of GnRH release define the effect on release of FSH and/or LH in synchronous or asynchronous patterns.

Transport of hormones occurs via the circulatory system. While the amine and peptide hormones are soluble enough to be carried in the plasma, the steroid hormones are not. A transport protein is required for the steroid hormones. The steroid hormones are transported bound to a plasma protein, such as albumin. In addition, there are specific binding transport proteins. For example, testosterone-binding globulin transports testosterone in the circulatory system.

3.1.3 Hormone Action at the Cellular Level

Hormone effects are dependent upon the hormone binding with a specific receptor. Receptors for hormones are found on the cell surface or inside the cell. The receptor site for the steroid hormones is intracellular. Briefly, steroid hormones, once released from a transport binding protein, diffuse into the cell and bind to an intracellular receptor, which can be located in the cytoplasm or nucleus. This hormone-receptor complex initiates a series of reactions in the cytoplasm or regulates transcription. If the complex affects gene transcription, then a mRNA molecule is formed, which is transported to the cytoplasm, where proteins are synthesized for mediating the effect of the steroid hormone.

3.1.4 Hormone Metabolism and Excretion

The pathway for inactivation of a hormone depends on the type of hormone. Peptide hormones are metabolized by proteases. Steroid hormones are metabolized by enzymes that reduce, oxidize, hydroxylate, etc., the molecule for subsequent addition of a glucuronide or sulfate conjugate that can be more readily excreted. Excretion occurs in the urine, bile or feces. Steroid hormones that are excreted in the bile may be hydrolyzed in the gastrointestinal tract and then reabsorbed back into the body through the portal system circulation.

3.1.5 Control of Hormonal Secretion

Feedback systems control the secretion and synthesis of hormones from endocrine glands (Figure 3-1). In general, when affected by a stimulus, a given endocrine gland will increase secretion of its hormone. However, once the desired physiological effect occurs, information is fed back to the endocrine gland responsible for producing the hormone, and further secretions are reduced or stopped. In contrast, under-secretion of a given hormone will stimulate cells in the endocrine gland to increase production and secretion. This negative feedback mechanism is specific for each producing gland and its hormone.

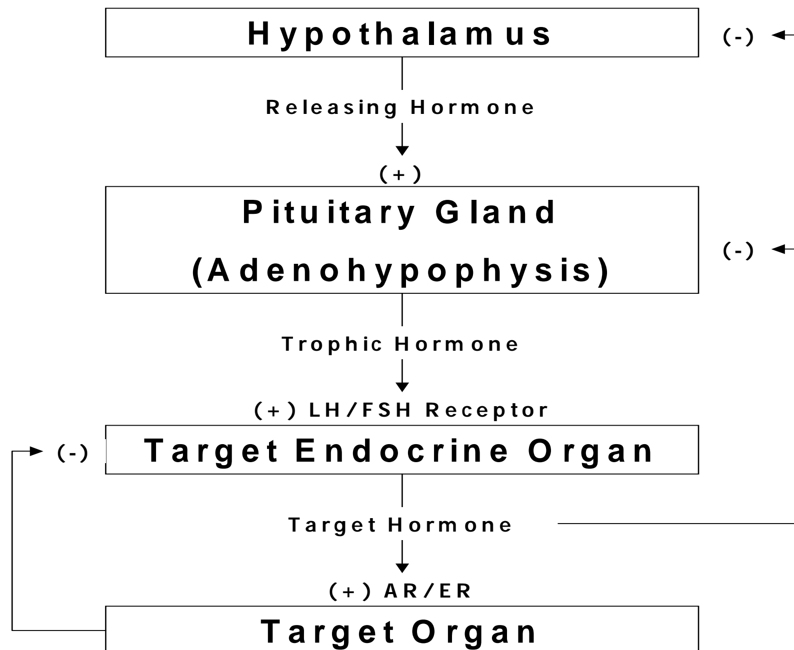


Figure 3-1. Endocrine System Regulation – A Feedback Mechanism

The hormonal feedback system comprises four primary organs: the hypothalamus, the pituitary gland, the hormone secreting target gland, and the target organ. These four sites are in communication with each other via the bloodstream, and they interact to regulate hormone synthesis and secretion. In males, this feedback system is referred to as the Hypothalamic-Pituitary-Testicular Axis; likewise, in females, it is referred to as the Hypothalamic-Pituitary-Ovarian Axis.

The following sections summarize the function and interrelated operation of the hypothalamus, the pituitary, and the hormone secreting target glands. In addition, descriptions using examples from the reproductive system are included to provide additional information about steroidogenesis.

3.1.5.1 Hypothalamus. The hypothalamus, located in the diencephalic region of the brain, receives signals from other components of the central nervous system. These signals are used to control the secretion of substances from the pituitary gland, which is attached by a stalk to the hypothalamus. Hypothalamic-directed control of pituitary gland hormones occurs via a neuro-secretory neuron network (neurohypophysis) or a hypothalamo-hypophyseal portal system blood vessel network (adenohypophysis). The hypothalamus exerts control over the reproductive system by secreting releasing or inhibitory factors into the hypothalamic-pituitary portal system, which then act on the anterior pituitary gland. The hypothalamus releasing factors pertinent to controlling reproduction are the follicle-stimulating releasing factor (FRF), luteinizing hormone releasing factor (LRF), and prolactin-inhibiting factor (PIF). The releasing factors FRF and LRF are also referred to as the gonadotropin-hormone releasing factors (GnRH).

It is generally believed that the central nervous system (CNS) is the trigger point for initiation of sexual maturation in the male and female rat (Goldman et al., 2000; Stoker et al., 2000). GnRH levels can be viewed as an indicator of initiation of sexual maturation. GnRH is present in the fetal brain and slowly increases until the second postnatal week in females, and the third postnatal week in males. At that point, GnRH increases steeply and remains elevated until puberty. At puberty, the GnRH neurons undergo a morphological change, developing spiny-like processes that may be related to an increase in synapses on the cells. It has been shown that, at puberty, the GnRH neurons become more responsive to neurotransmitter (norepinephrine and dopamine) stimulation.

3.1.5.2 Pituitary Gland. The pituitary gland is found at the base of the brain and is composed of two main lobes: the adenohypophysis and neurohypophysis. The adenohypophysis is composed of different cell types that are richly innervated by a capillary system. The different types of cells can be differentiated by specific histological stains, and each is generally associated with the production of a specific hormone. The cells, when stimulated by the hypothalamic releasing hormones, secrete hormones into the blood vessels. The adenohypophysis secretes numerous hormones such as the gonadotropins FSH and LH, and the lactating promoting hormone prolactin (Prl). LH and FSH travel via the blood supply to the target endocrine organs, e.g., the testes (male) and ovaries (female). These hormones are essential for sexual maturation and reproductive activity. The neurohypophysis has no role in the reproductive system or its development.

3.1.5.3 Hormone-Secreting Target Gland. The reproductive system’s target endocrine

organs are the testes and ovaries. These organs release hormones that regulate the target organs, e.g., the mammary glands, uterus, or other structures involved in reproductive activity.

In the testis, regulation of hormone releases involve coordinated communication among different cells and tissues (Figure 3-2). LH binds to receptors on the interstitial cells of Leydig to stimulate the synthesis of testosterone. FSH binds to receptors on Sertoli cells that release and metabolize factors required for spermatogenesis. FSH also increases the number of LH receptors in the testis, which in turn increases testosterone production and testis growth. Sertoli cells have receptors for both FSH and testosterone for additional coordination between the Sertoli and Leydig cell populations within the testis (Russell et al., 1990). The Sertoli cells also produce a glycopeptide, inhibin, that provides negative feedback on the release of FSH from the anterior pituitary gland.

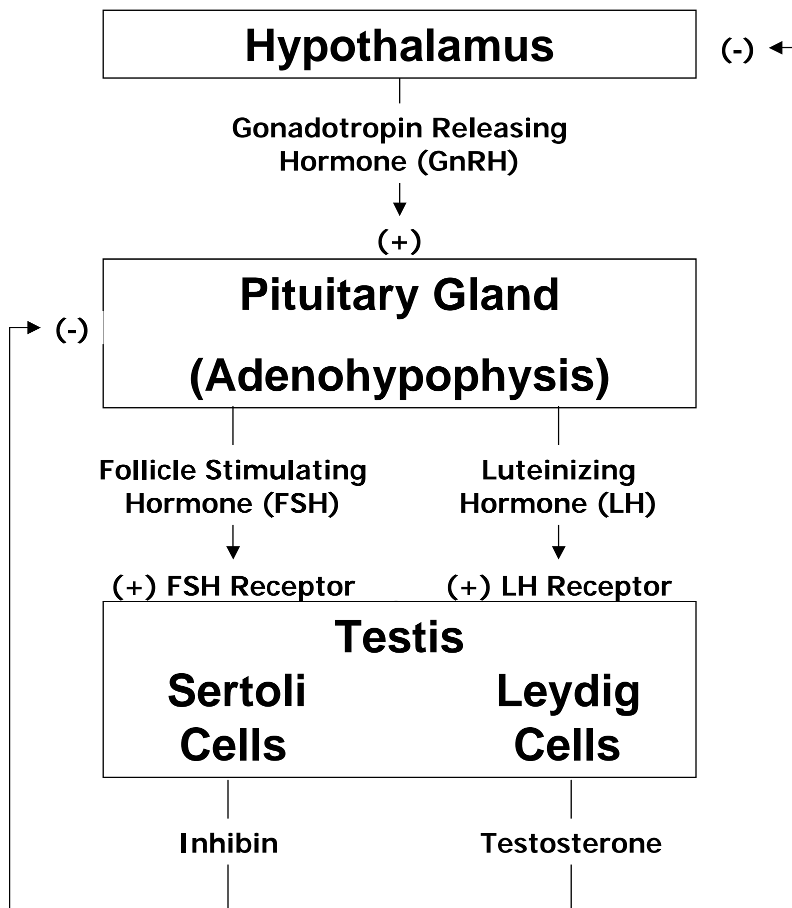


Figure 3-2. Feedback System of the Hypothalamic-pituitary-testicular Axis

In humans, a 28-day cycle is used to describe the changes in the ovary. This cycle has three phases: follicular phase, luteal phase, and menstrual phase (hormonal withdrawal). At the end of one cycle and the beginning of the next, the concentrations of estrogen and progesterone begin to decrease, which stimulates the anterior pituitary gland to secrete FSH. As FSH increases, numerous follicles in the ovary begins to develop. The developing follicle secretes increasing amounts of estradiol from the granulosa cells. A positive feedback mechanism causes the estradiol level to begin to increase.

It is during the follicular phase that LH stimulates thecal cells to increase steroid hormone biosynthesis. The product is androstenedione, which crosses into the granulosa, where it is enzymatically converted by aromatase to estrone (see the section on steroidogenesis). FSH stimulates this conversion. Also during the follicular phase, progesterone, secreted from the follicle, begins to rise. Just before ovulation, estradiol secretion peaks, inducing an LH surge to occur, in turn inducing the follicle to rupture, thereby resulting in ovulation. As the luteal phase begins, FSH and LH decrease and progesterone levels increase. Also, estrogens begin to rise again, which further decreases FSH and LH levels. As the luteal phase nears the end, the estrogen and progesterone levels fall, inducing the menstural period. Low levels of these hormones result in stimulating the release of FSH, and the cycle starts again.

3.1.6 Sexual Development

Androgens and estrogens are essential in the development of the reproductive system. In addition, these hormones are needed for feedback regulation of the hypothalamic-pituitary axis, sex accessory organ development and maintenance, spermatogenesis in males, and oogenesis in females (Goldman et al., 2000; Stoker et al., 2000). The differentiation of tissue into a male or female gonad is chromosome dependent. The presence of the Y chromosome results in testicular development, and, if the Y chromosome is absent, then ovaries develop. Gonadal development dictates the types of hormones produced, which determine whether the embryo exhibits a male or female phenotype. The wolffian and müllerian ducts, found in the early embryo, give rise to either male or female sex characteristics. The presence of testes results in the production of two hormones: müllerian-inhibiting substance (antimüllerian hormone-AmH) and testosterone. AmH causes the müllerian ducts to disappear and suppresses uterine and fallopian tube development. Testosterone causes the wolffian ducts to develop into the epididymides, vasa deferentia, and seminal vesicles. For the female, the müllerian ducts develop into the fallopian tubes, uterus, and upper vagina, but the wolffian ducts disappear. Thus, differentiation of these two tissues into male or female is dependent on the production of androgens from the testes. If present, then male; if absent, then female. The ovary is not necessary for female development.

Testosterone and dihydrotestosterone (DHT) are the two most active androgens. In the male, testicular descent and development; maturation of the epididimydes, vas deferens, seminal vesicles, levator ani/bulbocavernosus; and other aspects of the male reproductive tract are dependent upon testosterone. DHT is responsible for development of the male urethra and prostate and the formation of the penis and scrotum, and male secondary sexual characteristics such as scrotum and penis development.

Target organs in males and females also includes the primary sexual organs (penis and

clitoris), the secondary sex targets (e.g., muscle, bone, skin, hair follicles, and sweat glands), and sex accessory glands (e.g., seminal vesicles and prostate gland in males, and the uterus and breasts in females). These male and female target organs respond to testosterone in males and estradiol or progesterone in females. These target organs require steroids for functional integrity and growth (in juveniles). Changes in hormones in a cyclic manner (over days) are responsible for maintenance of the menstrual cycle in human females and the estrus cycle in domestic animals.

3.2 Steroidogenesis

The endocrine system synthesizes three types of hormones—polypeptides, amines, and steroids. Synthesis of the latter hormone type is referred to as steroidogenesis. In short, steroidogenesis is the biosynthetic pathway that produces steroid hormones. Although steroidogenesis is a general term that refers to the biosynthesis of any chemical substance with a steroid nucleus, in the context of this review paper it will be used to describe the production of gonadal steroid hormones. Steroid hormones participate in the control and regulation of the reproductive system.

The following subsections focus on the sites and pathway of the steroidogenic biosynthetic processes for the reproductive system. It is important to point out that although a lot of detail about the steroidogenic pathway is presented, an assay used as a screen will not provide specific information about the site or mechanism of action of a test substance. However, a thorough description of the steroidogenic pathway is believed necessary in order for one to appreciate the possible number of sites in which an endocrine disruptor compound can act.

3.2.1 Site of Steroidogenesis

The reproductive system steroid hormones are produced primarily in the gonads, although some steroidogenic chemical reactions are also found at peripheral tissue sites. For the male, the steroidogenic pathway is found in the testes and, to a much lesser extent, the adrenal glands. Within the testis, steroidogenesis occurs in the Leydig cell. The Leydig cells are interstitial cells that lie interdispersed among the seminiferous tubules. Inside the Leydig cell, the steroidogenic pathway begins in the cytoplasm and includes chemical reactions that occur in the mitochondria and smooth endoplasmic reticulum, where the final end-product hormone, i.e., testosterone, is produced (Chen et al., 1996). Other active androgenic hormones are produced in the testis and at peripheral tissue sites. Several peripheral tissues are involved in testosterone's role as a prohormone (Figure 3-3). For example, testosterone is converted to estradiol in the liver and brain (hypothalamus) and converted to dihydrotestosterone in the liver, brain, prostate, and external genitalia.

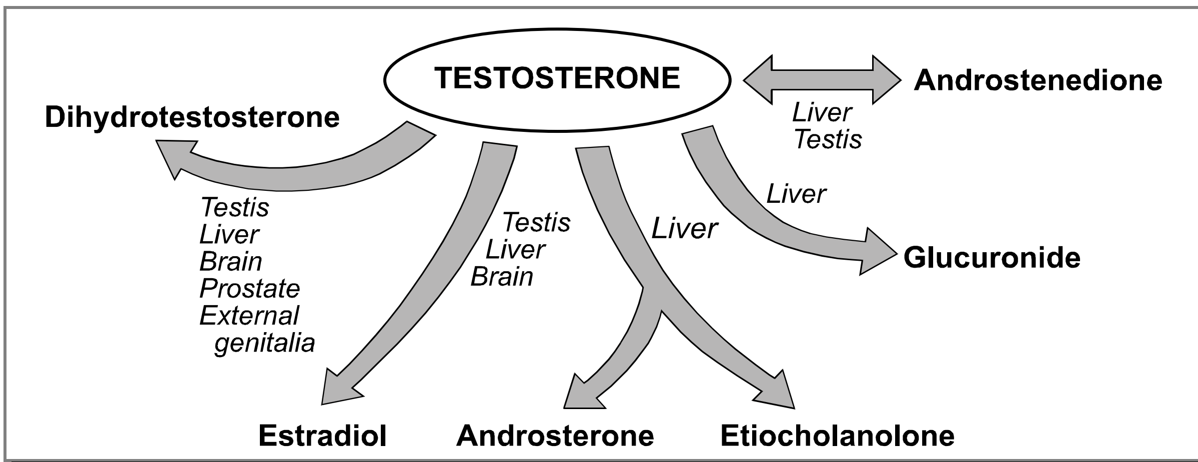


Figure 3-3. Testosterone Conversion in Peripheral Tissues

Source: Federman (1981)

In the female, biosynthesis of the reproductive system steroid hormones occurs in the ovary (Carr and Wilson, 1994). Several cell types in the ovary participate in the synthesis of these steroid hormones. Two of the cell types are follicular cells, the granulosa and theca interna. A third cell type that has been implicated in steroid hormone synthesis is the interstitial cell. These cells are located between the follicles. A fourth cell type is the luteal cells of the corpus luteum, which is formed from the post-ovulatory follicle. Different cell types within the ovary can have varying amounts of given enzymes resulting in some types of cells producing more of one steroid hormone than another. For example, the corpus luteum, which contains primarily theca interna cells and fewer granulosa cells, is the primary source for progesterone and 17β-hydroxy progesterone.

3.2.2 Steroidogenic Biosynthetic Pathway

For the purposes of this DRP, the steroidogenic pathway will have a defined starting point and will include a specified set of chemical reactions that result in the production of gonadal intermediary and end-product hormones. More specifically, the steroidogenic pathway will be those processes in the testis or ovary that occur after stimulation of the gonadotropic receptor. The pathway (1) begins with intracellular signal transduction, (2) continues with cholesterol production in the cytoplasm and transport to the mitochodrial inner membrane, and (3) ends with a set of multi-step enzymatic conversions from cholesterol to the end-product hormones. Each of these stages is described below in further detail and each will also appear in later discussions about the sites of action of substances that disrupt steroidogenesis.

3.2.2.1 Signal Transduction. Signal transduction describes the intracellular biochemical reactions that occur after stimulation of the LH membrane bound receptor and up to initiation of cholesterol transport to the mitochondria. The intracellular pathways that constitute the signal transduction phase are illustrated in Figure 3-4.

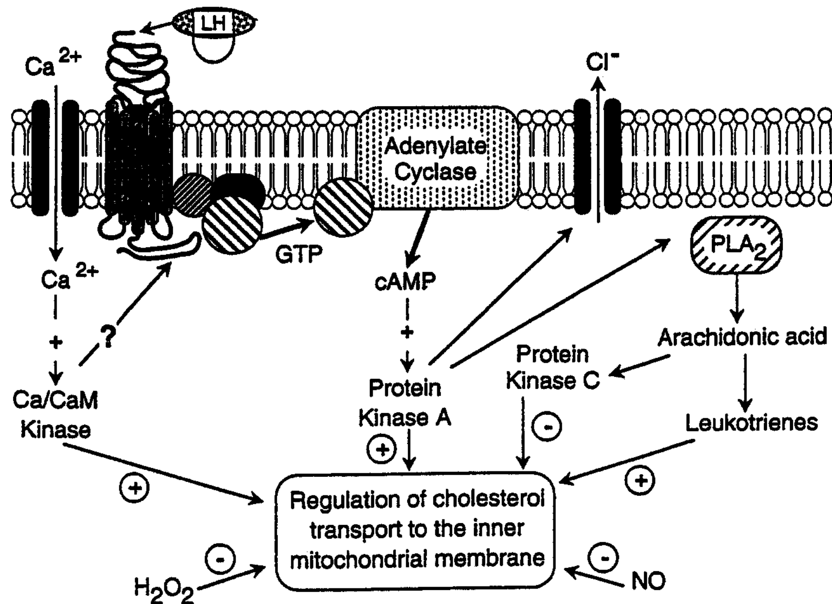


Figure 3-4. Signal Transduction in the Leydig Cell

Source: Cooke (1996)

The intracellular reactions that occur in the cytoplasm of the Leydig cell when LH binds to the membrane bound LH receptor are useful for describing the signal transduction stage of the steroidogenic pathway. The LH receptor is coupled with a G-protein and, when stimulated, interacts with adenylate cyclase to form cyclic adenosine 3',5'-cyclic monophosphate (cAMP). Increased cAMP, the second messenger, stimulates protein kinase A, which initiates cholesterol biosynthesis and cholesterol transport protein synthesis (Cooke, 1996; Stocco, 1999).

Calcium (Ca^{2+}) is involved in the signal transduction of the steroidogenic pathway (Janszen et al., 1976). In order for the maximal stimulation of steroidogenesis to occur, intracellular calcium levels must increase following LH binding. Intracellular calcium increases through the release of calcium from intracellular storage depots and/or passage of extracellular calcium through membrane bound calcium channels. The calcium-mediated reactions also involve calmodulin, a calcium binding protein (Hall et al., 1981). Through this series of events, cholesterol transport into the mitochondria is enhanced.

Chloride (Cl^-) has also been implicated in steroidogenic signal transduction (Choi and Cooke, 1990). Chloride channels were identified in the plasma membrane of the Leydig cell. Both LH and cAMP stimulate chloride conductance. Although a specific role is unclear, chloride is believed to be involved in that part of the steroidogenic pathway that occurs in the mitochondria.

LH stimulation increases the release of arachidonic acid in the Leydig cell (Naor, 1991; Cooke, 1996). Arachidonic acid appears to act as an intracellular mediator and also appears to produce a direct inhibitory effect and an indirect stimulatory effect on steroidogenesis. Steroid hormone production is inhibited when arachidonic acid activates protein kinase C. However, metabolism of arachidonic acid to its metabolites, e.g., leukotrienes, stimulates cholesterol transport into the mitochondria, thereby enhancing steroid hormone production.

Other intracellular substances shown to affect steroidogenesis include free radicals, i.e., superoxide anion and hydroxyl free radical, as well as hydrogen peroxide and nitric oxide. Molecular oxygen is needed for proper function of the cytochrome P_{450} enzymes, which are used in the synthesis of intermediary and end-product hormones. However, molecular oxygen leads to the formation of free radicals, which damage Leydig cells (Kukucka and Misra, 1993). Hydrogen peroxide (H_2O_2) inhibits steroidogenesis by affecting cholesterol transport and inhibiting 3β-hydroxysteroid dehydrogenase, the enzyme that converts pregnenolone to progesterone (Clark et al., 1994). As for nitrous oxide (NO), this molecule diffuses through cell membranes, is formed in the testis, and affects steroidogenesis (Davidoff et al., 1995). Although its site of action is not clear, nitrous oxide has a negative effect on steroid hormone production.

In summary, stimulation of the LH membrane-bound receptors initiates intracellular events, and this post-receptor signal transduction constitutes the beginning stage of steroidogenesis. Steroidogenic signal transduction involves the second messenger, cyclic AMP, and stimulation of protein kinase A, which leads to increased cholesterol transport and utilization for the production of steroid hormones. The pathways and molecules that comprise steroidogenic signal transduction can be altered by several substances, and this alteration can have a stimulatory or inhibitory effect on steroid hormone production. Thus, it is important to understand the role signal transduction plays in steroidogenesis to better identify substances that could potentially alter steroid hormone production through interactions at intracellular sites.

3.2.2.2 Cholesterol Synthesis and Transport. Steroidogenic signal transduction initiates events that result in the next stage of steroidogenesis: intracellular biosynthesis of cholesterol, mobilization of intracellular storage depots of cholesterol, and cholesterol transport from the cytoplasm to the mitochondria. These events were elegantly determined and are reviewed in detail by Stocco (1999). The intracellular events involving cholesterol in the biosynthesis of steroid hormones are illustrated in Figure 3-5.

Cholesterol is the common precursor to the formation of all gonadal steroid hormones. The primary source of cellular cholesterol is the serum. Cholesterol is transported to the cell via serum protein carriers, e.g., high- or low-density lipoprotein (HDL or LDL). Once inside the cell, cholesterol is immediately utilized, or it can be stored, e.g., in lipid droplets. A second, minor source of cholesterol is *de novo* synthesis, which increases following hormone stimulation of the Leydig and follicle cells. This *de novo* cellular synthesis begins with acetate, which goes through a four-step conversion process that produces malonate, squalene, and lantosterol, which is then converted into cholesterol. Upon LH-induced stimulation, mobilization of newly synthesized and stored cholesterol (enzymatic hydrolysis of cholesterol esters) in lipid droplets occurs. Cholesterol is transported out of the cytoplasm and into the mitochondria. In the mitochondria, cholesterol is transported from the outer to the inner membrane. The movement

of cholesterol across this membranous, aqueous, mitochondrial gulf is the rate-limiting step in steroidogenesis.

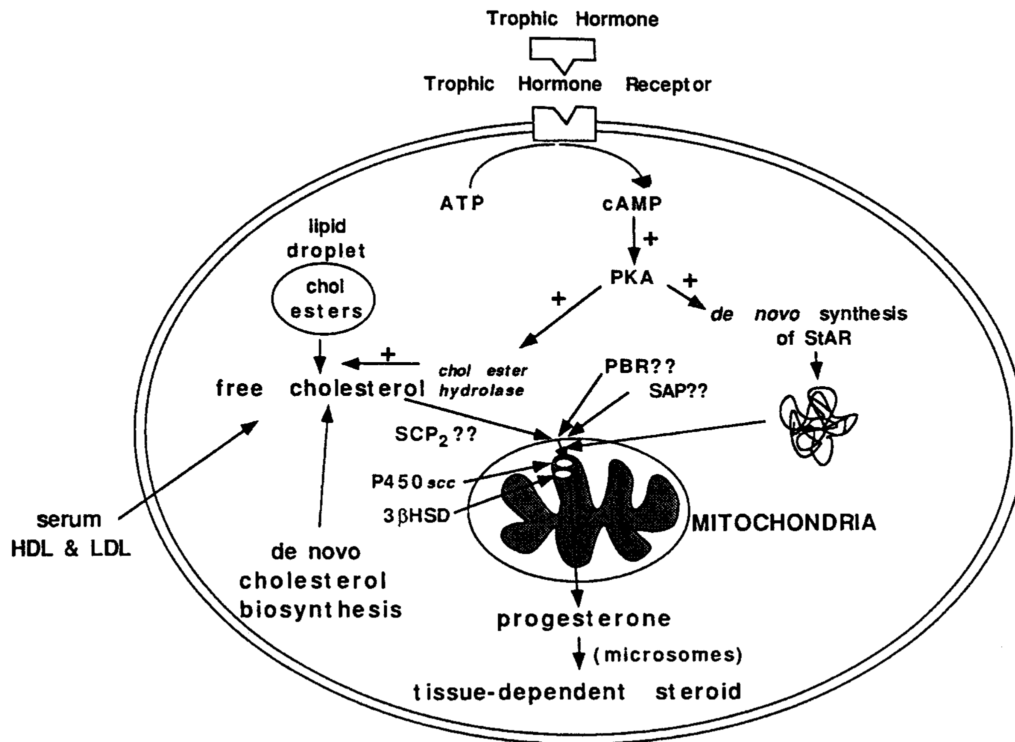


Figure 3-5. Intracellular Biochemical Pathway Following Trophic Hormone Stimulation

Source: Stocco (1999)

The transport of cholesterol from the outer to the inner mitochondrial membrane requires a transport protein. LH stimulation of steroidogenic cells activates *de novo* production of the cholesterol transport protein. This protein is essential for steroidogenesis and, since it mediates the rate-limiting step of steroid hormone production, it is referred to as the steroid acute regulatory (StAR) protein. Investigations that led to the identification of the StAR protein reported that it was rapidly synthesized, cycloheximide-sensitive (dependent on *de novo* protein synthesis), and highly labile (short half-life). The StAR protein is synthesized in the cytoplasm as a precursor molecule. It is transported to the mitochondria, where it is cleaved to its active form. In the mitochondria, StAR protein transports cholesterol to the inner mitochondrial membrane, where the first cytochrome P450 enzymatic conversion takes place. At this site, the side-chain cleavage enzyme ($P450_{SCC}$) is found. This enzyme catalyzes cholesterol into pregnenolone.

Steroidogenesis is controlled through regulation of StAR protein production. The StAR gene is regulated by steroidogenic factor-1 (SF-1). SF-1 regulates the basal and hormone-stimulated expression of the StAR gene. The effect of SF-1 is modulated by cAMP,

thereby linking the signal transduction phase to control of the carrier protein responsible for the rate-limiting step in the biosynthetic process. Other regulators of the StAR gene include estrogen, growth hormone, IGF-1, and calcium, which also cause up-regulation of the StAR gene.

In summary, cholesterol is the common precursor molecule for production of all steroid hormones. Cholesterol is synthesized, mobilized from storage depots, and transported to the mitochondria following LH-stimulated signal transduction. Transport of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step of steroidogenesis. Cholesterol is carried between the membranes by the StAR protein. At the inner membrane, the first of a series of enzymatic reactions occurs, whereby cholesterol is converted to pregnenolone by $P450_{SCC}$.

3.2.2.3 Enzymatic Conversions. Enzymatic conversion of cholesterol to pregnenolone constitutes the initial step in a series of biochemical reactions that culminate in end-product hormone production. Figure 3-6 summarizes the final stage of the steroidogenic biosynthetic pathway, as well as the cell types for males and females and the intracellular location of various enzymatic steps of the steroidogenic pathway. The remainder of this section describes the enzymatic reactions in detail.

The first enzyme reaction is the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme ($P450_{SCC}$). $P450_{SCC}$ activity is also considered to be a rate-limiting step in the production of gonadal steroid hormones. This reaction occurs on the inner membrane of the mitochondria and involves three sequential oxidation reactions, each requiring molecular oxygen and NADPH. The reactions add two hydroxyl groups to cholesterol (at C_{22} and C_{20}) followed by cleavage between the added hydroxyl groups. As a result of these reactions, cholesterol, a 27-carbon sterol, is cleaved of its 6-carbon group termed the "side chain," thereby resulting in production of pregnenolone, a 21-carbon steroid (Kagawa & Waterman, 1995).

The second enzymatic reaction results in the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase/Δ^5 - Δ^4 isomerase (3β-HSD). This reaction is also believed to occur on the inner membrane of the mitochondria. It is hypothesized that an interaction of the StAR protein with the inner mitochondrial membrane could cause the formation of $P450_{SCC}$ and 3β-HSD, thereby allowing cholesterol to be converted to pregnenolone and then to progesterone rather quickly (Stocco, 1999). 3β-HSD catalyzes dehydrogenation and isomeration of pregnenolone to progesterone, which converts a Δ^5-3β-hydroxysteroid to a Δ^4-3-ketosteroid, the active form of steroid hormones. It is also possible that pregnenolone is converted to progesterone in the cytosol by 3β-HSD, as well. Thus, the steroidogenic pathway bifurcates into a Δ^5- hydroxysteroid pathway (starting with pregnenolone) and a Δ^4-ketosteroid pathway (starting with progesterone) and, even though the same enzymes use different substrates along the parallel pathways, both pathways converge. The result is the production of androstenedione. 3β-HSD converts the Δ^5- hydroxysteroid pathway substrates, 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA), into their respective Δ^4-ketosteroids, 17α-hydroxyprogesterone and androstenedione, respectively.

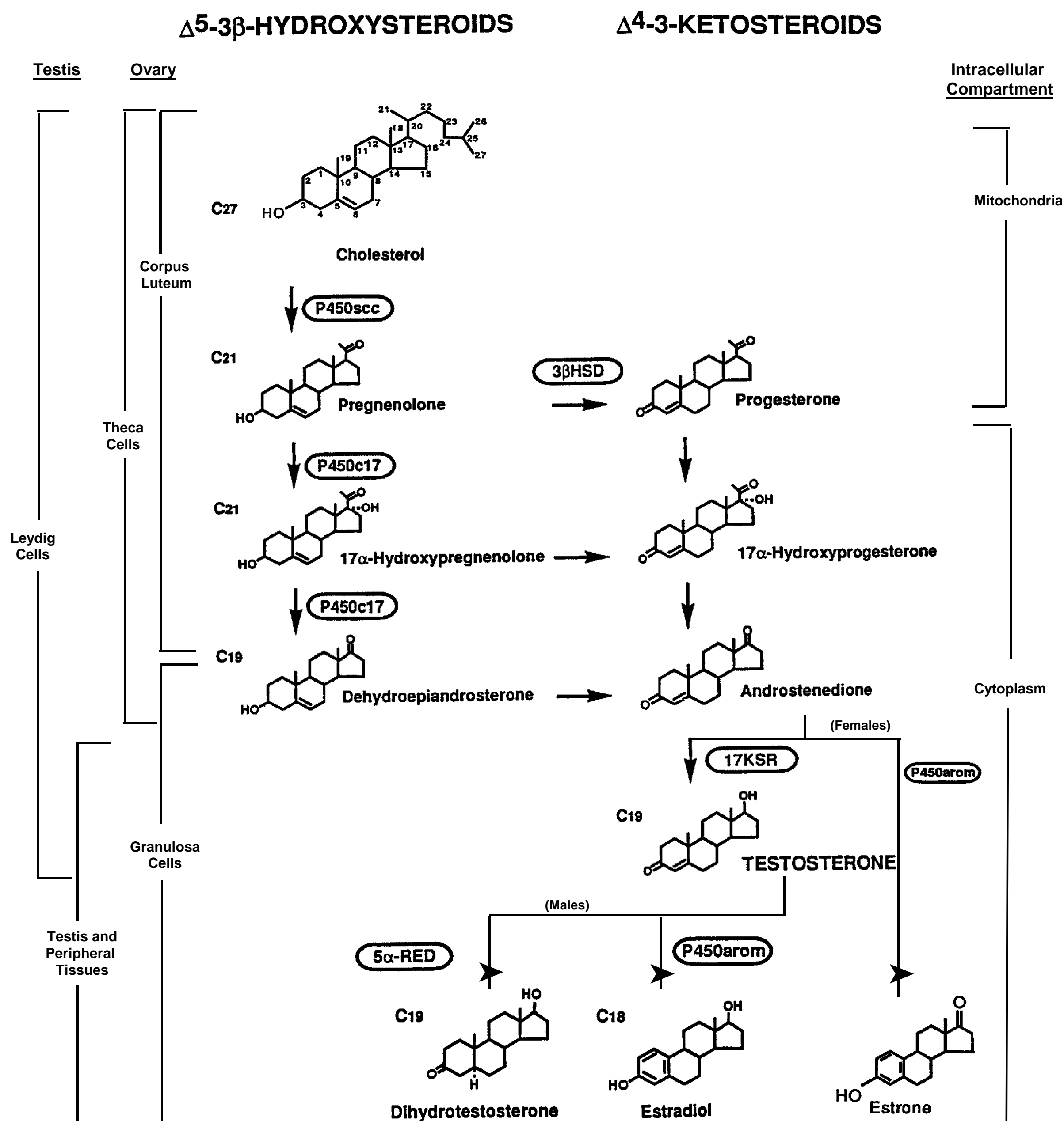


Figure 3-6. Enzymatic Conversions of Cholesterol and Intermediate/End-Product Hormones

The third enzymatic reaction involves cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17). This enzyme catalyzes two chemical reactions, hydroxylation and cleavage (converts the steroid from a 21-carbon to a 19-carbon molecule), and requires molecular oxygen and NADPH. The products after the hydroxylation step are considered intermediates. Thus, for the Δ^5-hydroxysteroid pathway, P450c17 initially catalyzes the conversion of pregnenolone to

17α-hydroxypregnenolone, which is then converted to DHEA. As mentioned above, DHEA is converted to androstenedione by 3β-HSD. Likewise for the Δ^4- ketosteroids, P450c17 converts progesterone to 17α-hydroxyprogesterone, which is then converted to androstenedione. The lyase activity of P450c17 differs for the intermediate substrates among species. For example, in the human, P450c17 converts 17α-hydroxypregnenolone to DHEA (Δ^5- hydroxysteroid pathway) but not 17α-hydroxyprogesterone to androstenedione (Δ^4- ketosteroid pathway). In comparison, in the rat, P450c17 converts the intermediates of both the Δ^5- hydroxysteroid and Δ^4- ketosteroid pathways equally. Such differences in the P450c17 lyase activity may explain species-dependent differences in response to substances that alter steroidogenesis.

The next enzymatic reaction involves the conversion of androstenedione to testosterone by 17-ketosteroid reductase (17KSR), which is also referred to as17β-hydroxysteroid dehydrogenase (17β-HSD). The production of testosterone is considered an end-hormone product. A second possible reaction involving androstenedione occurs in the female, whereby androstenedione is converted to estrone by aromatase. (Aromatase is described in further detail below). The conversion of androstenedione to testosterone is reversible and dependent on product concentrations. 17KSR is able to catalyze either the reduction (forward) or the oxidation (reverse) reactions. The cofactors $NADH/NAD^+$ are used in this interconversion.

In the male, testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase, which is found in the cellular membranes, nuclear envelope, and endoplasmic reticulum. DHT is significantly more potent as an androgen than testosterone and is also considered an end-product hormone. DHT is produced primarily in peripheral tissues, although it is also found in the testis. The activity of 5α-reductase in the Leydig cells and testes varies with age; the highest activity occurs around puberty.

The last enzyme in the steroidogenic pathway is aromatase. Aromatase converts testosterone into estradiol and, in the female, androstenedione into estrone. In short, aromatase converts androgenic substances into estrogenic substances. As mentioned above for testosterone and DHT, estradiol and estrone are considered end-product hormones of the steroidogenic pathway. Aromatase is found in many different peripheral tissues, as well as male and female gonadal tissue. The activity of this enzyme varies with species and age. Aromatase is an enzyme complex (two cytochrome P450 enzymes: a reductase and an aromatase) bound to the endoplasmic reticulum. The complex catalyzes two hydroxylation steps and the aromatization (hence the name aromatase) of Ring A in the steroid nucleus, which results in the loss of the C-19 carbon atom, thereby producing a C-18 molecule characteristic of estrogens. The reaction requires molecular oxygen and NADPH.

Production and activity of these enzymes are under hormonal control. The $P450_{SCC}$ and P450c17 enzymes are regulated by LH. In the male, FSH stimulates release of a Sertoli cell factor that increases the effect of LH on 3β-HSD activity. For females, FSH increases the activity of aromatase, thereby enhancing the conversion of androstenedione to estrone. In addition to regulatory effects of gonadotropins, gonadal hormones regulate steroidogenic enzymes. For example, testosterone inhibits P450c17 activity, which occurs via effects on the second messenger cAMP pathway. Testosterone also suppresses 3β-HSD through inhibitory effects on cAMP-mediated 3β-HSD mRNA.

In summary, cholesterol is the common precursor for production of steroid hormones. A

series of biochemical reactions involving different enzymes results in conversion of cholesterol to end-hormone products: testosterone, DHT, estradiol, and estrone. The steroidogenic pathway is regulated by gonadotropins and end-product hormones. An alteration of the regulatory mechanisms, as well as direct effects on the substrates and enzymes of the steroidogenic pathway, can affect end-hormone product formation, thereby possibly resulting in reproductive system toxicity.

3.3 Steroidogenic Pathway Defects and their Effects on Sexual Development

Alteration of the hormonal steroidogenic pathway can lead to abnormal sexual development. If sex chromosomes are altered at fertilization due to mutations, cross-over abnormalities, chromosomal aberrations, etc., then there will be abnormal gonad development, which, in turn, may lead to defects in hormonal steroidogenesis. A deficiency in an enzyme at any one step of the steroidogenic pathway will change the pattern of production and eventually secretion of steroid hormones. This section summarizes many of the disorders in mammalian male and female sexual development that occur as a result of defects in the steroidogenic pathway. In general, any defect in steroidogenesis at the embryonic stage can lead to pseudohermaphroditism or defective masculinization in mammals. (Kelce and Wilson, 1997; Gray et al., 1999). Although pseudohermaphroditism is caused by many different defects, the following information will focus on gonadal steroidogenic pathway deficiencies and the defects that develop (Wilson and Griffin, 1994).

3.3.1 StAR Gene Suppression

Negative regulation of the StAR gene has been associated with adrenal hypoplasia congenita (AHC) and hypogonadotropic hypogonodism (HHG) (Stocco, 1999). These diseases are attributed to mutations in the DAX-1 gene, which expresses the dosage-sensitive sex reversal transcription factor (DAX-1). Also, duplication of that part of the X chromosone where the DAX-1 gene is found results in a male-to-female sex reversal. Excessive expression of the DAX-1 gene leads to excessive levels of DAX-1, which inhibits expression of the StAR gene (Zazopoulos et al., 1997). Complete blockage of the active StAR protein is the cause of congenital lipoid adrenal hyperplasia (CAH). This disease occurs because the StAR protein is truncated by 28 or 93 amino acids. In addition to blocking gonodal steroid hormones, mineralocorticoid and glucocorticoid production is inhibited, thereby causing death shortly after birth if left undetected.

3.3.2 Cholesterol Side Chain Cleavage ($P450_{scc}$) Enzyme Deficiency

This defect is also called lipoid adrenal hyperplasia. It occurs as a result of the absence of the $P450_{scc}$ enzyme. Apparently, an error on chromosome 15, where this enzyme is encoded, results in no conversion of cholesterol to pregnenolone. Most individuals die at infancy from this defect due largely to adrenal gland insufficiency. A necropsy of these infants shows that males are incompletely masculinized, whereas females have normal genital development. Also, laboratory tests find no detectable steroids in the urine.

3.3.3 3β-Hydroxysteroid Dehydrogenase/Isomerase Deficiency

This defect is attributed to an error on chromosome 1, where this enzyme is encoded. As a result, pregnenolone is not converted into progesterone in the male and female. In this disorder, males develop a vagina and show varying degrees of feminization, including breast development at puberty. Plasma testosterone levels are low and Delta 5 pathway precursors are elevated. Females exhibit some varying degrees of masculinization. The urine contains no Delta 4 steroids for those individuals with the complete deficiency.

3.3.4 17α-Hydroxylase/17, 20-Lyase Deficiency

These two defects are attributed to an error on chromosome 10, where the DNA code for P450$_{17ٿ}$ resides. Both of these enzymes are mediated by the 17α P450 enzyme. A deficiency in the activity of these two enzymes alters the conversion of progesterone to androstenedione in the male, and this alters the conversion of both pregnenolone (Delta 5 pathway) and progesterone (Delta 4 pathway) to androstenedione in the female.

In the 17α-hydroxylase deficiency, males exhibit defective masculinization that can range from partial to complete pseudohermaphroditism and breast enlargement. In females, the individual appears as a prepubescent woman. The 46,XX individual does not exhibit secondary sex characteristics, e.g., no sexual hair, and is amenorrheic. Urinary 17-ketosteroids are low.

In the 17, 20-lyase deficiency, the male exhibits varying degrees of pseudohermaphroditism but can show some masculinization at puberty. No information regarding the physical appearance of the female was found.

3.3.5 17β-Hydroxysteroid Dehydrogenase Deficiency

This defect is the most common of the alterations to the hormone steroidogenic pathway. This enzyme converts androstenedione into testosterone in both the male and female. The 46,XY individual has a female appearance, which includes a vagina, as well as abdominal testes. At puberty, masculinization occurs. Penile and breast enlargement and facial/body hair development occurs. Plasma testosterone levels are low to normal.

3.3.6 5α-Reductase Deficiency

This enzyme converts testosterone to dihydrotestosterone in peripheral tissues. In this defect, abnormal masculinization is localized during embryogenesis to the urogenital sinus and the external genitals, an effect mediated by dihydrotestosterone but not testosterone. Testosterone and estradiol levels are normal, because aromatase catalyzes the conversion of the former to the latter. This deficiency is characterized in the male by a blind vaginal pouch, testis, no enlarged breasts, no internal female genitals, and masculinization at puberty.

3.3.7 Aromatase Deficiency

Aromatase catalyzes the conversion of testosterone to estradiol in the peripheral tissues. This deficiency causes disorders of bone maturation in the male and sexual development in the female.

3.3.8 21-Hydroxylase or 11β-Hydroxylase Deficiency

The disorder that results from the deficiencies of these enzymes is referred to as congenital adrenal hyperplasia. The enzyme 21-hyroxylase uses a steroidogenic hormone, 17-hydroxyprogesterone, as a substrate for the production of an intermediate in the glucucorticoid pathway, 11-deoxycortisol. Obviously, this defect is ***not*** due to a direct disorder of the steroidogenic pathway; rather this defect is a secondary disorder due to the decreased formation of cortisol, which results in a compensatory increase in ACTH. The effect of increased ACTH not only increases the secretion of cortisol, but it also increases the production of androgenic hormones. In the male, this results in premature masculinization, i.e., early maturation of the penis and secondary sex characteristics. The male may or may not be able to exhibit spermatogenesis, depending on a negative or positive effect of adrenal androgens on the release of GnRH. In the female, masculinization is apparent at birth. The female genitals are abnormal in appearance. At puberty, the female does not exhibit normal female sexual development or menstrual period. Plasma progesterone and 17-hydroxyprogesterone are increased.

The enzyme 11β-hydroxylase is also part of the glucocorticoid pathway. It converts 11-deoxycortisol to cortisol. A defect in this enzyme results in phenotypic patterns similar to those described above for 21-hydroxylase.

3.4 Steroidogenesis: Toxic Effects of Substances

Exposure to substances that are not endogenous to the body can lead to chemical reactions that alter the outcome of biochemical pathways. Altering any one step in the steroidogenic pathway for the production of reproductive hormones has the potential to cause toxicity. In general, the disruption of gonadal steroidogenesis could result in an increased concentration of one or more steroids, a decreased concentration of one or more steroids, and/or new steroid synthesis products. Toxic responses to the reproductive system can result in such adverse effects as abnormal sexual and physical development, diminished fertility or sterility, and cancer, to name just a few (Kelce and Wilson, 1997; Gray et al., 1999). The focus of this section is to provide examples of substances that alter specific steps in the steroidogenic pathway as well as to provide a summary table of substances known to produce direct effects on the steroidogenic pathway (Table 3-1).

Table 3-1. Substances and Conditions That Directly Alter Steroidogenesis [a]

Site of Chemical Action	Reference
cyclic-AMP Second Messenger System	
Bisphenol A/octyphenols	Nikula et al., 1999
Nitrate/nitric oxide	Panesar, 1999
Glucocorticoids	Orr et al., 1994
Indomethacin	Lopez-Ruiz et al., 1992
Chloroquine	Lopez-Ruiz et al., 1992
EDS	Klinefelter et al., 1991
DBA	Goldman and Murr, 2002
Nicotine	Patterson et al., 1990
Cotinine	Patterson et al., 1990
Tylosin	Meisel et al., 1993
Gossypol	Pearce et al., 1986
Lindane	Ronco et al., 2001
StAR Protein	
Barbiturates	Gocze et al., 1999
Lindane	Walsh et al., 2000a
DBA	Goldman and Murr, 2002
Dimethoate	Walsh et al., 2000a
Diethylumbelliferyl phosphate	Choi et al., 1995
DMSO	Stocco et al., 1995
$P450_{scc}$	
Lead	Huang et al., 2002
Ketoconazole	Kan et al., 1985
Mibolerone	Fanjul et al., 1989
Aminoglutethimide	Uzgiris et al., 1977
Taxol	Rainey et al., 1985
Cis-platinum	Maines et al., 1990
Vitamin A deficiency	Jayaram et al., 1973
3β-HSD	
Lead	Huang et al., 2002
Daidzein/genistein/biochanin A	Ohno et al., 2002
Lithium chloride	Ghosh et al., 1991
Mibolerone	Fanjul et al., 1989
Danazol (ethinyltestosterone)	Barbieri et al., 1977
Cyproterone acetate	Lambert et al., 1987

Table 3-1. Continued

Site of Chemical Action	Reference
Ethionine	Goldberg et al., 1969
Cyanoketone (WIN-19578)	Goldman et al., 1965
Mitomycin C	Deb et al., 1980
Aflatoxin	Verma and Nair, 2002
P450c17 (17α-hydroxylase/$C_{17\text{-}20}$ lyase)	
Ethanol (17α-hydroxylase)	Murono, 1984
Bromocriptine	Kovacevic and Sarac, 1993
Mibolerone	Fanjul et al., 1989
Danazol	Barbieri et al., 1977
Cyproterone acetate	Ayub and Levell, 1987
Cyclosporin A	Seethalakshmi et al., 1992
Nicotine	Kasson and Hsueh, 1985
Flutamide	Ayub and Levell, 1987
17KSR	
Cotinine	Yeh et al., 1989
Danazol	Barbieri et al., 1977
Cyclosporin A	Kasson and Hsueh, 1985
Lithium chloride	Ghosh et al., 1991
5α-Reductase	
Finasteride	Morris, 1996
Aromatase	
Aminoglutethimide	Johnston, 1997
MEHP	Thomas, 1996
Fenarimol	Vinggaard et al., 2000
Fadrazole	Yue and Brodie, 1997
Letrozole	Bhatnagar et al., 2001
Anastrozole	Bhatnagar et al., 2001
Arimidex	Johnston, 1997
Flavenoids	Saarinen et al., 2001
Prochloraz	Andersen et al., 2002
Enconazole/miconazole/ketoconazole	Doody et al., 1990
Imizolil	Doody et al., 1990
4-hydroxyandrostenedione	Doody et al., 1990
10-propargylestr-4-ene-3,17-dione	Doody et al., 1990

[a] Does not include substances that alter LH or FSH receptor binding.

Table 3-1. Continued

3.4.1 Chemical Inhibition of Cholesterol Side Chain Cleavage ($P450_{scc}$)

The first step in the conversion of cholesterol to steroid hormones involves the enzyme $P450_{scc}$. Substances that inhibit this enzyme include aminoglutethimide (Dexter et al., 1967), 3-methoxybenzidine, cyanoketone, estrogens, azastine, and danazol. Aminoglutethimide will be used to describe the toxicity that results from this type of steroidogenic pathway inhibition.

Aminoglutethimide was prescribed as an anticonvulsant in conjunction with Dilantin (diphenylhydantoin sodium) and Meberal (mephobarbital) (LaMaire et al., 1972). Aminoglutethimide was withdrawn from the market in the early 1970s due to serious side effects. Aminoglutethimide treatment caused gonadal enlargement in rats, which was attributed to cholesterol accumulation (Goldman, 1970). In humans, the drug was directly or indirectly linked to the virilization of a young woman (Cash et al., 1969) and associated with ovarian dysfunction and pseudohermaphroditism in infant females (LeMaire et al., 1972).

Aminoglutethimide also inhibits other enzymes that can have an indirect effect on steroidogenic hormones. It has been shown to inhibit 11β-hydroxylase in adrenal cells cultured *in vitro* (Goldman, 1970). This glucocorticoid pathway enzyme converts 11-deoxycortisol to cortisol. Inhibition of this enzyme channels steroids into androgenic pathway(s) as a result of the partial block at $P450_{scc}$. More than one author has suggested the existence of additional "alternate pathways" in the adrenal or gonads that would force the synthesis of alternate steroid products from the accumulating cholesterol (Burstein et al., 1971; Gual et al., 1962). Finally, aminoglutethimide interferes with thyroxine synthesis (Rallison et al., 1967) and possibly with the metabolism of steroid hormones (Horky et al., 1969; 1971).

3.4.2 Chemical Inhibition of Aromatase

Aromatase is the enzyme complex that converts androgens into estrogens. More specifically, aromatase converts testosterone to estradiol in the male and female, as well as converting androstenedione to estrone in the female. Numerous substances are known to inhibit aromatase activity, and reviews on this subject are available (Brueggemier, 1994; Johnston, 1997; Brodie et al., 1999). Examples of aromatase inhibitors include MEHP (monoethylhexylphthalate); fenarimol; substituted analogs of androstenedione, e.g., C-10 substituted (19R-10β-oxiranyl-), or C-4 substituted (OH-, formestone), or C-7 substituted

(7α-SC_6H_4-p-NH_2-), or 2β, 19 bridged (2β, 19-methylene-), fadrazole, letrozole, and arimidex, to name just a few.

The reproductive effects of inhibiting aromatase activity are dramatic. At birth, females exhibit pseudohermaphroditism and as adults are amenorrheic, have small breasts, and can develop cystic ovaries. In males, the epiphyseal plates do not calcify, which results in elongated bones and tall stature, as well as osteopenia and sterility (Simpson et al., 2002).

3.4.3 Multiple-Site Chemical Inhibitors of Steroidogenic Pathway Enzymes

Several substances are recognized for inhibiting more than one of the enzymes of the steroidogenic pathway. The chemical danazol inhibits 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase, thereby having direct inhibitory effects on the production of steroidogenic hormones. In addition, this chemical inhibits the glucocorticoid enzymes 11β- and 21-hydroxylase, which indirectly affects the synthesis of steroidogenic hormones as previously described. Another chemical that affects multiple sites is spironolactone. This chemical inhibits 17α-hydroxylase of the steroidogenic pathway and 21-hydroxylase of the glucocorticoid pathway. Ketoconozole, an imidazole used as an antifungal agent, inhibits $P450_{SCC}$ and C17, 20-lyase (Morris, 1996).

3.5 Conclusion

The hormones of the reproductive system are synthesized in the steroidogenic pathway. The operation of this pathway is critical to the endocrine system, as well as sexual function and development. Any chemical interference to steroidogenesis, e.g., altering enzymatic activity or hormone production, altering precursor availability, interfering with control mechanisms, etc., can cause adverse effects to the reproductive system. It is for these reasons that an assay is needed to identify substances that could produce toxicity by altering the steroidogenic pathway – an assay that can identify substances that inhibit or stimulate steroid hormone production. Such an assay should identify alterations at the biochemical level – not the physiological level.

Thus, since the goal of this review paper is to lay the foundation for selecting an assay for screening substances that are disruptors of the endocrine system, the next section provides background information about methods currently used to measure steroidogenesis in mammals.

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4.0 MEASUREMENT OF STEROIDOGENESIS

The first objective of this section is to provide a thorough and comprehensive review of the methods used to measure gonadal steroidogenesis in mammalian systems. The various methods can be distinguished by the varying degree that the organism, systems, organs, tissues, and cells remain intact. Five methodologies are used for measuring steroidogenic activity:

- The whole animal (*in vivo*),
- A combination of the whole animal and isolated organ(s) (*ex vivo*),
- Isolated and cultured whole or sections/minced organs, i.e., testis/ovary (*in vitro*),
- Isolated and cultured cells from the testis/ovary (*in vitro*), and
- Cell lines (*in vitro).*

This section will survey and investigate the status of each method for its capacity to measure changes that identify substances that are direct stimulators or inhibitors of the steroidogenic pathway for sex steroids. More specifically, this section examines those methods that measure the effect(s) of a substance, which has as its site(s) of action the gonadal steroidogenic enzymes or other intracellular biochemical components of the gonadal steroidogenic pathway; beginning after the membrane-bound receptor; thereby, excluding methods that identify the receptor as the site of action and continuing through each step of the steroidogenic pathway until the production of the end-hormone, e.g., testosterone (male) and estradiol/estrone (female). Thus, the information presented for each method includes:

- Scope of the method (or particular type of assay or test),
- A description of the method (or particular type of assay or test),
- Specific experimental design considerations,
- Representative studies and data from the literature,
- Distinguishing features of the method (or particular type of assay or test), and
- Concluding remarks.

The second objective of this section is to provide a basis for selecting the most promising method to be used as a screen to identify substances that have inhibitory or stimulatory effects on the production of steroidogenic hormones. The criteria for selecting the optimal screening method are presented in Section 4.6. The detailed information presented in this section should help reviewers evaluate the various methods used for measuring steroidogenesis and assess the one method recommended for the screening tool.

Experiments recounted in the literature were prioritized for inclusion in the DRP. Those that showed the method could be used to detect sterodogenic alteration were preferred over those that used the method for studying biochemical or physiological mechanisms; however, these latter types of studies often provide significant information about the usefulness of the method as a screening tool. In this vein, every attempt was made to identify information and gather data about method parameters that would be useful for evaluating a given method as the screen to identify substances that interfere with the production of steroidogenic hormones.

4.1 Whole Animal Methods (*In Vivo*)

4.1.1 General Assays

Intact animals can be used to measure androgenic and estrogenic activity. *In vivo* methods were the earliest papers cited in the literature; dating back more than 70 years (Allen and Doisy, 1924; Hershberger et al., 1953). Yet, *in vivo* studies still represent a useful method for measuring estrogenicity and androgenicity as demonstrated by more recent investigators (Gray and Ostby, 1998; Kelce et al., 1997). *In vivo* methods take advantage of the animal's innate hormone-directed tissue development and maintenance responses and uses these responses to identify whether administered substances alter the normal response. For this method to work, the animal's inherent capacity to produce the hormones that stimulate the tissue response must be removed. Since this inherent capacity resides in the gonads, *in vivo* studies often involve an ovariectomy or castration. Otherwise, the hormones from the gonadal organs would interfere with the response being measured by the substance being tested. Alternatively, immature or juvenile animals have been used. In this way *in vivo* methods are useful for identifying substances that disrupt the endocrine system.

In vivo studies used for evaluating substances for estrogenicity or androgenicity are not necessarily useful tools for evaluating a direct effect on steroidogenesis. As mentioned above, two widely used bioassays, i.e., uterotropic and Hershberger, generally use gonadectomized animals, thereby removing the organs where steroidogenesis occurs. There is, however, an *in vivo* method that uses the fully intact animal and measures a direct effect of a substance on steroidogenesis. The endocrine challenge test (ECT) is a bioassay that can be used to evaluate the gonodal response to a substance by measuring the steroid hormone production and release following trophic hormone stimulation.

4.1.2 Endocrine Challenge Test (ECT)

ECT is a recently developed *in vivo* method for measuring changes in steroid hormone production. Athough the ECT is an accepted diagnostic method in human and veterinary medicine, only in the past decade, has it been incorporated into endocrine toxicology experiments (Fail et al., 1994; 1995; 1996a, b, c; Anderson et al., 1992).

4.1.2.1 Scope of the Test. In the ECT, the intact mature animal is challenged with GnRH or an LH- or FSH-like substance that stimulates a hormonal response. Serial blood samples are then collected and measured to evaluate whether the substance being tested has androgenic, e.g., increased testosterone, or anti-androgenic e.g., decreased testosterone, activity. The ECT is viewed by some investigators as the best way to detect altered steroidogenesis *in vivo* (EDSTAC, 1998). During the past 10 years, ECT has been used to evaluate the functional capacity of the gonads or pituitary (Fail et al., 1992); determine the effects of a substance on GnRH or hCG challenged steroid hormone production (Fail et al., 1994; 1995); assess reproductive toxicity (Fail et al., 1996b; 1996c); and measure thyroid hormone production (Fail et al., 1999).

4.1.2.2 Description of the Test. A flow diagram for the *in vivo* ECT is illustrated in Figure 4-1.

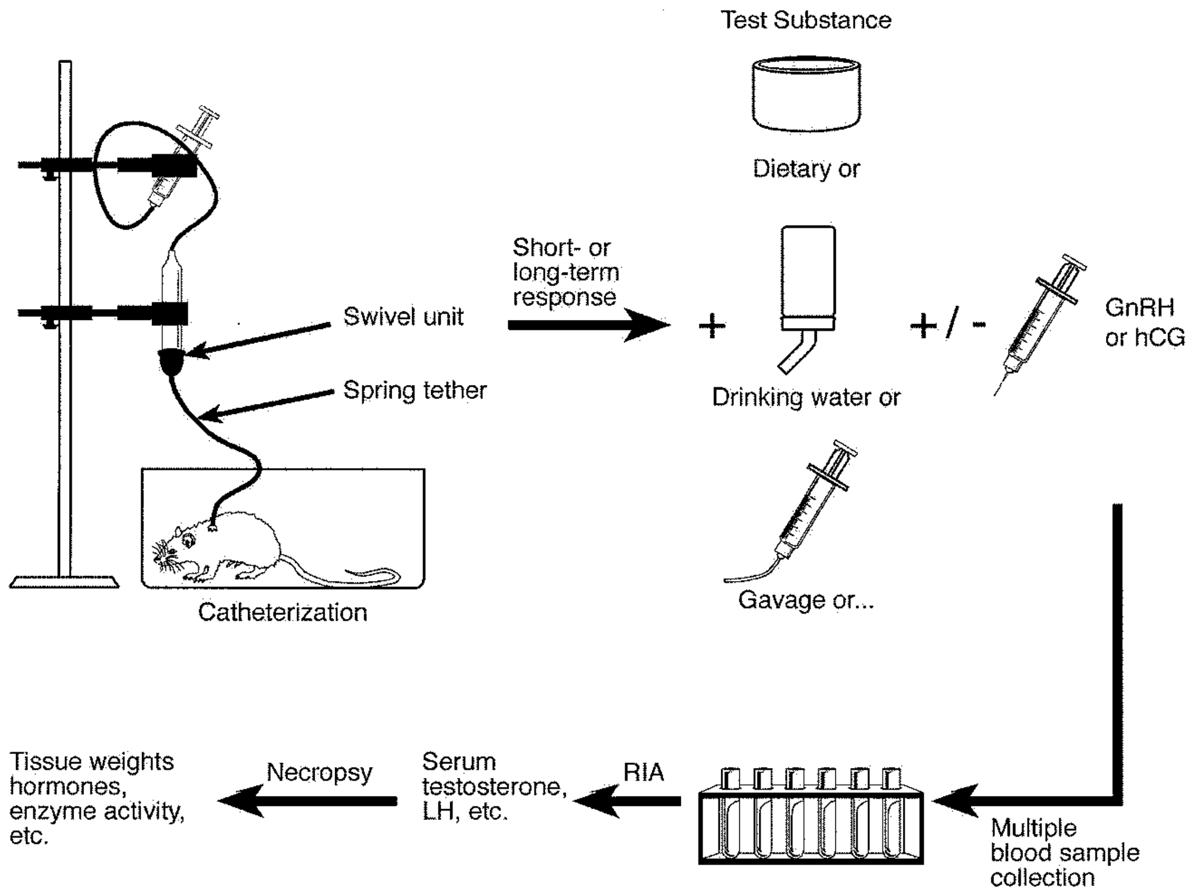


Figure 4-1. Schematic Diagram of the *in vivo* Endocrine Challenge Test

The ECT can be used to test the functionality of hypothalamic, pituitary, or gonadal responses. To test the functionality of the pituitary for LH or FSH release, the animal is given an injection of GnRH. At designated time periods, samples of blood are taken for measurement of FSH and/or LH concentrations, thereby assessing the release of these adenohypophyseal hormones. If the concentrations of FSH and/or LH are altered, then the functionality of the pituitary is compromised, as would be the target organs and glands that are affected by FSH and LH. In a similar manner and of more significance for this DRP, gonadal steroidogenesis can be tested by injecting an animal with FSH or LH. Alternative stimulants to FSH and LH are pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The primary stimulant used is hCG. Steroidogenesis is stimulated in the gonads by hCG, thereby allowing one to measure the effect of a substance on steroid hormone production.

4.1.2.3 Experimental Design Information. The ECT has unique aspects in regard to the approach used to identify steroidogenic inhibitory substances. Other assays have single point endpoints at necropsy, whereas the ECT collects samples over time. In this way subtle effects can be detected (Gray et al., 1997; Fail and Anderson 2002). However, the collection of multiple specimens, i.e., blood samples, can be difficult and require expertise. Techniques used include tail vein sampling, tail vein treatments and/or jugular catheterization (Fail et al., 1995; Fail and Anderson, 2002).

A number of different endpoints have been used with the ETC. The endpoints with the most application for evaluating a direct effect on steroidogenic hormone production are plasma testosterone (males) or estradiol (females), before (basal) and after LH stimulation (Fail et al., 1995; 1996b). Other endpoints include epididymis weight, caudal sperm count, testicular sperm heads, and sperm motility (Fail et al., 1995; 1996b).

4.1.2.4 Representative Studies from the Literature. The hCG-stimulated plasma testosterone response has been characterized. Fail et al. (1996a) treated adult male Sprague-Dawley rats with hCG by tail vein injection at dosages of 0, 0.2, 0.4, 0.8, or 8 IU/100 g BW. Blood and testicular samples were collected at specified time intervals after dosing, and testosterone concentrations were measured by RIA. Significant changes in testosterone were observed for 1 to 6 hours after dosing in both testicular and blood samples. Whole testicular concentrations of testosterone were four times higher than plasma testosterone (Table 4-1).

Table 4-1. Expanded Data Summary for *In Vivo* Preliminary Studies: Defining Dose and Time Responses in Adult Male S-D Rat Plasma and Testicular Testosterone Following a Post-hCG Challenge [a]

	Testosterone (ng/ml)				
	hCG (IU/100 g body weight)				
	0	0.2	0.4	0.8	8
Plasma					
1 hour	3.23 ± 1.12	4.12 ± 0.93	9.72 ± 2.76*	14.51 ± 1.37*	24.68 ± 2.15*
3 hours	1.57 ± 0.44	8.22 ± 1.64*H	10.10 ± 0.68*	15.27 ± 2.47*	13.32 ± 0.81*H
6 hours	3.33 ± 0.84	4.11 ± 0.44	7.31 ± 0.80*	12.01 ± 1.04*	10.91 ± 0.22*H
Testicular Homogenate					
1 hour	14.83 ± 4.07	26.61 ± 8.36	48.01 ± 8.66*	81.69 ± 7.65*	113.82 ± 3.56*
3 hours	8.13 ± 3.59	36.92 ± 7.72*	42.60 ± 4.77*	65.19 ± 8.64*	73.74 ± 7.01*H
6 hours	16.11 ± 4.61	17.62 ± 0.89	32.66 ± 3.58*	77.96 ± 12.14*	150.04±12.83*H

[a] Values are means ± standard error of the means; n=6.
* Significantly different from control (0 IU; within sacrifice time); Dunnett's; $p<0.05$. (rows)
H Significantly different from 1 hour time point (within hCG dose); Dunnett's; $p<0.05$. (columns)

Source: Fail et al. (1996a)

A study using ECT for determining whether boric acid had androgenic or anti-androgenic

activity was conducted by Fail and coworkers (Fail et al., 1992; 1998; Anderson et al., 1992). Data from this study are shown in Figure 4-2.

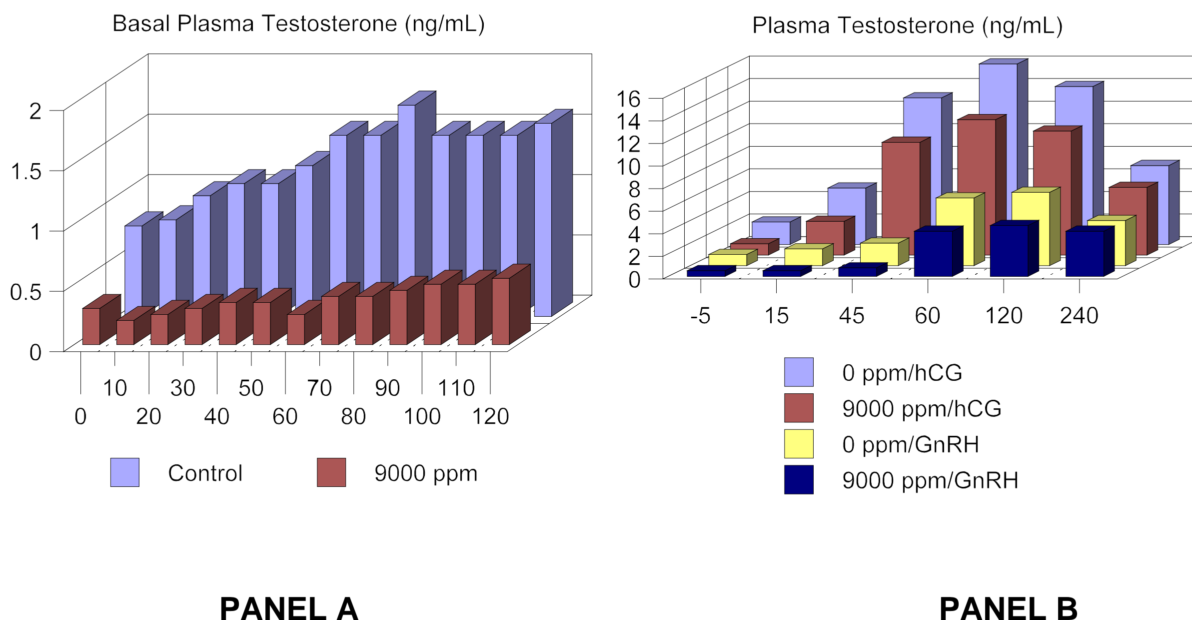


Figure 4-2. Example Data from an ECT Assay

Notes: Sprague-Dawley rats, cannulated to collect serial blood samples (every 10 minutes for 2 hours) for serum hormone analysis, were treated with boric acid in the feed at a concentration of 0 or 9000 ppm for 2, 9, or 14 days. Gonadal response to trophic stimulation was tested by administering GnRH, 25 ng, iv or hCG, 2.5 IU, iv. Basal serum testosterone was decreased 3- to 5-fold at all collection time points (Panel A). Even after challenge with GnRH or hCG, serum testosterone remained lower at most time points (Panel B). Serum FSH was increased about 2-fold for the boric acid group on Day 14 but not at Days 2 or 9. Serum FSH concentrations increased at all time points in response to GnRH stimulation (data not shown). The investigators concluded that boric acid compromised steroidogenesis and the ability of the testis to respond to gonadotropin stimulation.

Source: Fail et al., 1998

Other studies in the literature that use the ECT for evaluating the androgenicity of a substance are summarized in Table 4-2.

Table 4-2. Representative Studies Using the ECT Assay

Animal	Substance Tested/ Stimulant	Measured Response	Reference
Adult male Swiss-Webster mice	boric acid in dosed-feed @ 9000 ppm for 8 weeks / hCG, 15 IU, im	↓basal and hCG-stimulated Serum testosterone	Grizzle et al., 1989
Male Long-Evans Hooded rats (3 - 14 weeks of age)	methoxychlor @ 200 mg/kg/day, gavage, for 11 weeks **/** GnRH, 100 ng, iv or hCG, 2.5 IU, iv	↓ basal plasma testosterone and LH ↓GnRH or hCG-stimulated plasma testosterone ↑ GnRH-stimulated plasma LH	Fail et al., 1994
Male Long-Evans Hooded rats (3 - 14 weeks of age)	vinclozolin @ 100 mg/kg/day, gavage, for 11 weeks / CRH, 100 ng, iv	↑ basal plasma testosterone and LH but no effect on basal plasma ACTH, or corticosterone ↓ CRH-stimulated plasma ACTH No effect on CRH-stimulated plasma corticosterone	Fail et al., 1995
Male Long-Evans Hooded rats (3-14 weeks of age)	methoxychlor @ 200 mg/kg/day, gavage, for 11 weeks / GnRH, 100 ng, iv or hCG, 2.5 IU, iv	↓ basal plasma testosterone Response to hCG was blocked and/or delayed ↑ plasma LH response to GnRH	Fail et al., 1996a

4.1.2.5 Distinguishing Features of the Method.

Stability Primarily dependent on catheter patency or viability of tail veins to allow for serial collection of blood samples. (Animals can be euthanized at each time point – a cross sectional design – but this decreases the power of variation). Sample number and volume are critical when repeated samples are collected in short time periods. Animal growth can affect catheter placement. Heparanization of animal can occur as a result of leakage from catheter. Clot formation can occur, which occludes the catheter. Assay is easily used over a period of a few days and has been used as long as 2 months.

Standardization Assay has not been standardized. Standardization would involve a procedure for purityu, challenge dosage, method of sample collection, times for collection, types of samples to be collected, and analysis methods. Procedures require skill and experience, thereby making standardization between laboratories difficult. Other factors for consideration include circadian rhythms and effects of acute stress (Gray et al., 1997).

Sensitivity	Increased when collecting basal and challenge response. Increased over other assays in that repeated measurements of hormone levels are possible within an animal rather than one measurement at necropsy or use of multiple animals. False negatives could occur if the substance being tested induces negative metabolism of the hormone being measured in the plasma. If metabolic induction occurs, then an increase in the plasma hormone concentration (caused by the test substances) would not be observed.
Specificity	Stress may reduce testosterone levels and increase prolactin and corticosterone, which may interfere with results. Hormone circadian rhythms can also affect assay results (EDSTAC, 1998). Use of cannulated animals and a tether apparatus allows samples to be collected without handling the animal, thereby reducing stress and precluding the need for anesthesia (Fail et al., 1992).
Metabolic Activity	Yes. Test substances that require metabolic activation to produce an effect would be identified in this assay. Metabolic induction of the hormone could also occur giving the false negative results described above (see sensitivity).
Equipment	Animal facilities. Standard and specialized laboratory equipment. Use of permanent ports provide use (up to 2 months).
Training	Animal husbandry, dosing, and necropsy skills. Canula and port insertion surgery, cannula sampling, and tail vein sampling are all specialized skills. Cannulation requires surgical training and sterile technique.
Sample Scheduling	Multiple sampling, e.g., every 10 minutes for 2 hours, causes method to be labor intensive. Time to perform study using this method is measured in several days to a couple of weeks, primarily the time it takes to conduct the in-life phase and collect and analyze the samples.
Animal Usage	Whole animal assays use relatively more animals than some *in vitro* assays, e.g., sectioned testis, or cell line assays. However, of the whole animal assays, ECT allows data to be collected over time from the same animal, thereby reducing the number of animals used relative to other types of whole animal assays.

4.1.2.6 Conclusion. The ECT is a relatively new test for toxicity evaluation and/or evaluating steroidogenesis, as well as the androgenic/anti-androgenic or estrogenic/anti-estrogenic effects of a substance after the animal is challenged with a substance that stimulates hormone-producing organs. ECT requires a high degree of specialized training and laboratory skills. While it may be useful for identifying the anti-pituitary or anti-gonadal effects of a substance, the technical difficulty of performing the test may preclude its use as a screening tool.

4.2 Combination of Whole Animal and Isolated Organs Method (*ex vivo*)

4.2.1 Scope of the Method

The *ex vivo* method is a combination of *in vivo* and *in vitro* procedures. More specifically, study designs using the *ex vivo* method include administration of a substance to the whole animal based on a certain dosing regimen and, at the conclusion of dosing, removal of one or both testes or ovaries and culturing them for further treatment and testing. The *ex vivo* method not only allows the effect of a toxicant on the hypothalamic-pituitary-gonadal axis to be assessed, but it also permits evaluation of any change in the capability of the gonad to produce steroid hormones.

The *ex vivo* method is readily accepted and widely used. Investigators find it a robust method for evaluating substances for their capacity to alter steroid hormone production and secretion as well as characterize the mechanism of action of a substance. In short, is has been used to evaluate reproductive toxicity (Berman and Laskey, 1993) and physiological processes (Bambino and Hsueh, 1981) in cycling, pregnant, or hypophysectomized rats (Laskey and Berman, 1993; Bambino et al., 1980; Piasek and Laskey, 1994). The wide appeal of the *ex vivo* method seems to be that it combines the various *in vivo* dosing regimens with all the *in vitro* organ function assays and procedures. Further, it allows for metabolism of substances, thereby detecting possible active metabolites derived from active or inactive parent compounds, e.g., vinclozolin.

4.2.2 Description of the Assay

A flow diagram of an *ex vivo* method is illustrated in Figure 4-3. The *ex vivo* method first involves treating immature or adult animals according to a selected dosing regimen. Route and duration of exposure are not constrained. In addition to the acute exposures, as described for the *in vivo* methods, exposures for *ex vivo* studies can actually begin as early as gestation and continue for as long as the life expectancy of the animal being tested. During the in-life exposure period, blood samples can be collected at specified times for serum hormone analysis.

After the exposure period is completed, the animal is killed, at which time final necropsy specimens can be collected, and the testis or ovary isolated. The gonads are then processed according to the type of *in vitro* preparation that was selected to assess the toxicant's effect. [The different types of *in vitro* preparations, e.g., whole organ, sections, isolated/cultured cells, etc., are described in sections 4.3 and 4.4.] The *in vitro* preparation is treated with or without a stimulant, e.g., hCG or LH, in order to evaluate whether the toxicant perturbs gonadal function. In addition, the activity of steroidogenic enzymes can be assessed by adding different substrates. This assessment indicates which enzynme(s) is/are affected based on whether intermediate and/or end-hormone production occurs (Gray et al., 1997).

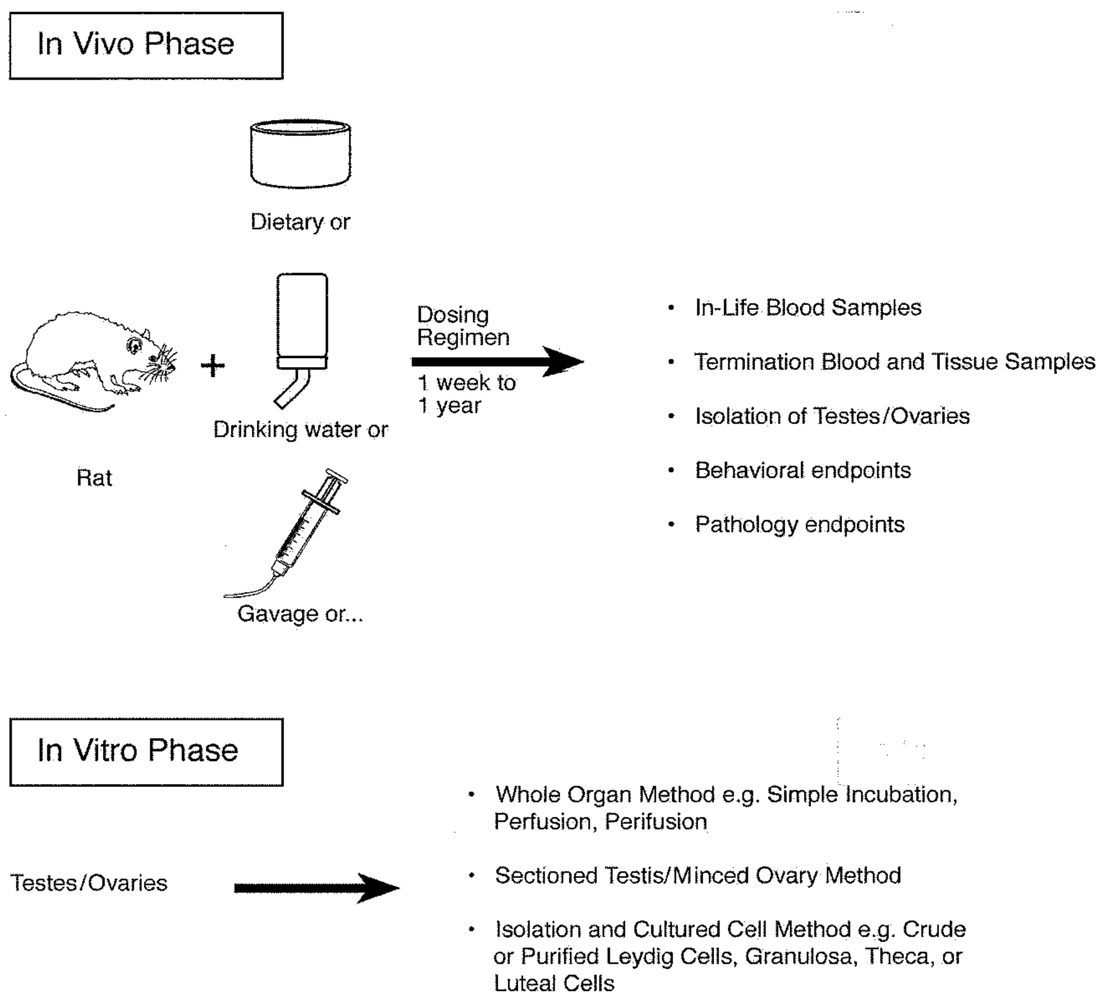


Figure 4-3. Schematic Diagram of *ex vivo* Method

4.2.3 Experimental Design Information

The *ex vivo* method can use a wide variety of animal models. All stages of animal development can be used. Ovariectomized or castrated animals cannot be used, since removal of the gonads would preclude conducting the second (*in vitro*) stage of the assay. For the female, the pregnant rat can be used and it lends itself to assessment of estrogen production better than the cycling rat (Gray et al., 1997). A pregnant rat has more predictable hormone levels during gestation, although measurement of a given hormone is dependent on the stage of gestation, e.g., estradiol concentrations are quite low over the first 8 to 9 days. In contrast, the cycling rat has hormone levels that fluctuate considerably during the 4 to 5 day estrus cycle. For this reason, the stages of estrus must be carefully determined in order to assess ovarian hormone production. Therefore, the cycle has been characterized based on the histopathology of cells and keyed to hormone changes. Laskey and Berman (1993) used a vaginal lavage technique and

observed three classes or stages, based on the kinds of cells present:

- Estrus—Only cornified epithelial cells
- Diestrus—Only leukocytes and cornified and a few nucleated epithelial cells
- Proestrus—cornified and nucleated epithelial cells.

Thus, by sampling the rats during the pre-study phase, animals with regular cycles that are clearly classified can be identified to reduce the variability in study results. Also, necropsy would be performed at a specific stage, e.g., diestrus (or proestrus).

The developmental stage of the male is an important experimental design consideration. The Leydig cells of the testis are where the enzymes concerning the steroidogenic pathway are located, and the responsiveness of these cells to endogenous endocrine and paracrine factors, as well as hormone production rates, change during an animal's life time. Stoker, et al. (2000), have provided a thorough review about pubertal development in the male rat, including a list of substances known to alter male reproductive development (endocrine disruptors).

Payne et al. (1996) has also reviewed the changes that occur in mammalian Leydig cells during the fetal, prepubescent, adolescent, and adult stages. Briefly, during the fetal stage, the testis contains progenitor Leydig cells (maximal production by gestation day, or GD, 21). These cells produce testosterone (maximum production GD18 to GD20) and androsterone (produced up through GD 25). From then until GD 35 to 40, immature Leydig cells predominate (maximum production by GD 35). These cells produce testosterone and 3α-androstanediol. From GD35 to GD90, Insulin Growth Factor (IGF-1) is believed to stimulate the immature Leydig cells into mature Leydig cells, a process that is completed by GD 90. This development of the Leydig cell is also regulated by LH, which has been reported to occur on GDs 25, 33, 35 (surge), 40, and 60 (surge). It is from these elegant studies and reviews that seemingly disparate results are better explained and understood. For example, Molenaar et al. (1985) showed that EDS was toxic to Leydig cells from adult rats, but Risbridger et al. (1989) observed no degeneration by EDS when testes from 10- or 20-day-old rats were tested. Kelce et al. (1991) exposed adult and immature rat Leydig cells (purified) to EDS using both *in vivo* administration and *in vitro* testicular perfusion and reported that the reduced sensitivity could be attributed to an intrinsic factor within the immature Leydig cell. This intrinsic capacity of the fetal Leydig cells has yet to be fully characterized. Nevertheless, these studies demonstrate the importance of giving careful consideration to the age and developmental stage of the testis and Leydig cells when designing a study to identify substances that can alter the hormonal products (qualitative and quantitative) of steroidogenesis.

Experimental design flexibility varies widely for the *ex vivo* method; first for the *in vivo* phase and, second, for the *in vitro* phase. Variations of the *in vivo* phase include different dosing regimens and routes of administration chosen to evaluate a given kind of exposure. Various endpoints can be utilized such as behavioral parameters, cytology (vaginal smears), blood and tissue collection, as well as different types of test groups, e.g., exposed and unexposed F_1. The *in vitro* phase can be varied to involve any procedure based upon the best endpoint for evaluating a particular aspect of steroid production and secretion. The different types of *in vitro* procedures

are discussed in subsections 4.3 and 4.4.

4.2.4 Representative Studies from the Literature

The *ex vivo* method has been employed to evaluate and, ultimately, identify a test substance that can alter steroidogenesis (e.g., Fail et al., 1996c). In this study, adult female Long-Evans rats were treated with methoxychlor by gavage at dosages of 50 or 200 mg/kg/day for 18 weeks. After the specified exposure period, the ovaries were isolated and baseline values for progesterone, testosterone, and estradiol were obtained. Next, the ovaries were stimulated by treatment with 100 IU of hCG (0.5 mL of media). Ovarian weight was significantly increased by methoxychlor treatment. The production of ovarian progesterone and estradiol were significantly increased at all time points, as was testosterone production at the 2-hour time point for the high methoxychlor dosage (Table 4-3).

Other studies that have employed the *ex vivo* method for evaluating the effect of a substance on steroidogenesis are summarized in Table 4-4. There are numerous examples in the literature that describe investigations that used the *ex vivo* method to assess substances for altering steroid hormone production and secretion—those shown in the table represent an attempt to illustrate a fair sample of all these studies.

Table 4-3. Example of *Ex Vivo* Study Results: The Effects of Daily Administration of Methoxychlor on Ovarian Hormone Production in Long-Evans Rats

Parameter	Methoxychlor (mg/kg/day, po)		
	0	50	200
Ovarian Wt. (mg)	56 ± 2	43 ± 3**	23 ± 2**
Progesterone (ng/ovary/hr.)			
Baseline	731 ± 78.7 (12)	498 ± 64.2 (12)	62 ± 29.4 (12)**
hCG + 1 hr.	816 ± 63.6 (12)	684 ± 84.0 (12)	569 ± 71.7 (12)*
hCG + 2 hrs.	814 ± 39.6 (12)	773 ± 93.1 (12)	120 ± 83.7 (12)**
Testosterone (ng/ovary/hr.)			
Baseline	2.8 ± 1.31 (12)	1.7 ± 0.54 (12)	2.1 ± 0.36 (12)
hCG + 1 hr.	4.0 ± 1.81 (12)	4.3 ± 1.47 (12)	5.3 ± 0.94 (12)
hCG + 2 hrs.	5.9 ± 2.82 (12)	6.94 ± 2.4 (12)	8.6 ± 1.65 (12)*
Estradiol (pg/ovary/hr.)			
Baseline	3.5 ± 1.91 (12)	5.8 ± 2.08 (12)	5.2 ± 1.25 (12)*
hCG + 1 hr.	4.6 ± 2.35 (12)	11.7 ± 5.06 (12)	8.6 ± 2.12 (12)**
hCG + 2 hrs.	6.3 ± 3.18 (12)	10.6 ± 3.55 (12)	12.5 ± 3.43 (12)**

[a] Values are mean ± SEM. Units are indicated for each parameter.

* $P<0.05$; *$P<0.01$; ANOVA was performed on log transformed data.

Table 4-4. Representative Studies of the *Ex Vivo* Method

Animal (*in vivo)*	Substance Tested	Preparation (*in vitro)*	Measured Response	Reference
Adult male CD rat	ammonium perfluorooctanoate (C8) @ 0 or 25 mg/kg/day for 14 days in dosed-feed	Leydig cells; stimulated by 1 IU hCG	↑testosterone in C8-treated rats after hCG	Biegel et al., 1995
Adult male SD rat	lead acetate @ 8 mg/kg/day, 5 days per week, for 5 weeks by IP injection	Leydig cells; stimulated by 50 mIU hCG	↓ basal & hCG stimulated testosterone	Thoreux-Manlay et al., 1995
Adult male SD rat	chloroethylmethane-sulfonate (CEMS) @ 6, 9, 12.5, 19, or 25 mg/kg/day for 4 days by IP injection	Sectioned testes (1/4); stimulated by 100 mIU hCG	↓ basal testosterone @ 19 and 25 mg/kg/day ↓ hCG stimulated testosterone @ ≥ 9 mg/kg/day	Klinefelter et al., 1994
Adult female SD rat	bis(2-diethylhexyl)-phthalate (DEHP) @ 0 or 1500 mg/kg/day for 10 days by gavage	Minced ovaries; stimulated by 100 mIU hCG	↑basal and hCG-stimulated testosterone and estradiol (diestrus) ↓Basal and hCG-stimulated estradiol (estrus)	Laskey and Berman, 1993
Pregnant Holtzman female rats	methoxychlor @ 25, 50, 100, 250, or 500 mg/kg/day for 8 days by gavage	Whole ovaries & minced ovaries; stimulated by 100 mIU hCG	↓ ovarian estradiol & testosterone secretion @ 250 or 500 mg/kg/day; no effect on progesterone ↓ serum progesterone @ ≥ 50 mg/kg/day and serum LH @ 500 mg/kg/day; no effect on serum estradiol, FSH or prolactin no effect on ovarian weight	Cummings & Laskey, 1993
Adult male NMRI mice	Tylosin @10 or 100 μg/kg or 10 or 100 mg/kg for 8 days in drinking water	Crude and purified Leydig cells; stimulated by 3.3 to 25 ng hLH	↓ testosterone production with/without hLH	Meisel et al., 1993
Adult male SD rats	ethane dimethane-sulfonate (EDS) @ 25, 50, 75, or 100 mg/kg, IP, 3 or 24 hours before necropsy	Crude Leydig cells; stimulated by 100 mIU hCG	↓testosterone production after 24 hours @ 75 or 100 mg/kg with hCG stimulation	Klinefelter et al., 1991
Adult male SD rats	TCDD @ 100 μg/kg, gavage, single administration 7 days before necropsy	Isolated testicular perfusion; stimulated by 50 to 4050 mIU hCG	↓basal and hCG-stimulated testosterone	Kleeman et al., 1990

Table 4-4. Continued

Animal (*in vivo)*	Substance Tested	Preparation (*in vitro)*	Measured Response	Reference
Immature hypophy-sectomized male SD rats	dexamethasone @ 10 µg/day, injection, for 5 days; FSH, 4 µg/day R5020 (synthetic progestin) @ 500 µg/day, injection, for 5 days	Testicular cell suspension; stimulated 500 ng hCG	↓basal and hCG-stimulated androstanediol no effect on basal or hCG-stimulated androstanediol	Bambino and Hsueh, 1981
Adult male OFA rats	20-438 (anti-spermatogenic) @ 50 mg/kg, gavage, single administration	Sectioned testis; incubated with ^{14}C-pregnenolone	↓testosterone and androstenedione ↑progesterone, 17α-hydroxyprogesterone, and estradiol	Gurtler and Donatsch, 1979

4.2.5 Distinguishing Features of *Ex Vivo* Methods

Stability The stability of the *ex vivo* method is primarily limited by the *in vitro* procedure. Since the *in vivo* phase involves primarily animal husbandry and dosing, the first phase of an *ex vivo* study is regarded as having high stability. During the second phase, when the organ is isolated, incubated and cultured, the stability of the assay is vulnerable. The stability of *in vitro* preparations are described in subsections 4.3 and 4.4.

Standardization The *ex vivo* method is not standardized; to do so would involve consideration of numerous experimental design specifications. For example, the *in vivo* phase would involve selecting a dosing regimen, in-life phase, sample collection, necropsy time, etc., and the *in vitro* phase would require standardization of the procedure selected for inclusion in the *ex vivo* design.

Sensitivity Considered to be a very sensitive method since the *ex vivo* method combines an *in vivo* method, e.g., uterotropic or Hershberger assay, with any of a number of *in vitro* procedures.

Specificity See *in vivo* and *in vitro* specificity claims.

Metabolic Activity Method has full metabolic activation capacity. The substance being tested can be metabolized or excreted, thereby producing a rebound effect rather than a decrease.

Equipment, Training, and Sample Scheduling	Each of these can vary from relatively simple to labor-intensive and technically difficult, depending on the type of *in vitro* approach selected (4.3 to 4.6) for use in the *ex vivo* experimental design. For example, an acute dosing regimen combined with a sectioned testis procedure requires general endocrine and laboratory training and equipment, use of sterile techniques, a CO_2 incubator, and hormone analysis equipment. By contrast, if acute dosing is combined with testicular perfusion with hormone, enzyme, and mRNA analysis, then the equipment and training are highly specialized and labor-intensive.
Animal Usage	Whole animal assays use relatively more animals than some *in vitro* assays, e.g., sectioned testis, or all line assays. However, of the whole animal assays, *ex vivo* assays that include *in vitro* phases involving sectioned testis or Leydig cell isolation and culture, there is a reduction in the number of animals used since one animal generates a number of different samples for testing.

4.2.6 Conclusion

The *ex vivo* method is widely used to study the effects of toxicants on steroid production and secretion, as well as to investigate physiological and biochemical processes of the endocrine system. The *ex vivo* method combines an initial *in vivo* phase with a following *in vitro* phase. This method combines the advantages of using a whole animal for exposure to the toxicant – e.g., internal dosage, metabolic activation – with the advantages of isolating the organ for collecting samples and characterizing organ function – e.g., direct measurement of steroid production and secretion, with and without stimulation.

4.3 Isolated Organ Methods (*in vitro*)

The organs where steroidogenesis occurs can be removed from the animal and kept viable, thereby providing an isolated organ method for assessing substances as toxicants of the steroidogenic pathway. These organs, once isolated, can be used whole or further processed into sections or minced organ preparations. Each of these preparations is described and the studies using these methods are reviewed in this section. Regardless of the type of preparation, these preparations allow the effect of a given toxicant to be measured without the influence of other organs or systems, as well as other physiological factors. Although the organ has been removed from the animal, the integrity and interrelationship of the cells and tissues within the organ remain intact. Furthermore, these preparations retain the cellular and biochemical pathways that involve the receptor and second messenger. Klinefelter and Kelce (1996) note that an *in vitro* (perfused organ) method was used to discover a direct relationship among the following parameters: testosterone production, with Leydig cell volume, with smooth endoplasmic reticulum volume, with peroxisome volume, and with steroidogenic P450 enzyme activity, and all were regulated by LH. Thus, *in vitro* methods are reasonable candidates for consideration in this search for the most promising method that could be used as a screen for substances that alter steroidogenesis.

Several terms may be used to clasify the *in vitro* studies included in this section. In the

literature, the terms "whole," "sectioned," and "minced" are used to indicate the way in which the organ is processed. If the method uses **the whole organ**, then after the organ is removed and placed in media, no further processing occurs. In contrast, the whole organ can be further processed into sections, or even more finely processed, i.e., minced.

Testes are often sectioned, but ovaries are rarely, if ever, sectioned. The consistency of the ovary does not readily permit sectioning. A **sectioned organ** is also commonly referred to in the literature as a slice or slab. For the testes, an organ section is generally understood to refer to an organ that has been cut such that each section constitutes 1/8 to ½ the size of the whole organ, i.e., ~ 50 to 250 mg (rat testis). **Minced organ** generally refers an organ that has been cut with a scissors, knife, or razor blade into very small sizes, i.e., 1 to 50 mg (rat testis). The literature also uses the generic term "piece" to describe any organ that has been processed such that it is less than the whole. The terms "whole," "sectioned," and "minced" appear to be the most descriptive, and will be used for purposes of this review.

4.3.1 Whole Testis/Whole Ovary Methods

4.3.1.1 Scope of the Method. A whole organ *in vitro* method uses the whole ovary or whole testis after removal from an untreated animal. The organ is placed into an artificial environment that as closely as possible simulates *in situ* conditions, in order to test substances for estrogenicity or androgencity. These whole organ *in vitro* methods allow toxicant exposure of the steroidogenic cells while maintained in their normal cytoarchitectural environment. In addition, *in vitro* methods are not affected by neuronal influence. These assays allow for study of hormonal and/or toxicant influence on gonadal steroid hormone production over several hours.

Whole organ methods have been used to investigate a variety of endocrine functions. The simple whole organ incubation procedure has been used to investigate steroidogenic responses to treatment with LH or cAMP (Leung and Armstrong, 1979). The perifused ovary has been used to study follicular growth (Peluso, 1988), steroidogenesis (Soendoro et al., 1992a, 1993), ovulation (Peluso, 1990), and function of the corpus luteum (Nulsen et al., 1991). The isolated perfused ovary has been used to study ovulation and the mechanism of ovulation (Koos et al., 1984), oocyte maturation (Brannstrom et al., 1987), and ovarian physiology (Brannstrom and Flaherty, 1995). The perfused testis provides a method to simulate biosynthesis and secretion of testicular steroid hormones (Chubb and Ewing, 1979a; 1979c) and to detect inhibitors of testosterone biosynthesis (Chubb and Ewing, 1979b).

4.3.1.2 Description of the Method. Three techniques can be used to describe variations of the *in vitro* whole organ method: simple incubation, perifusion, and perfusion. The procedure used to combine the organ and the medium is what distinguishes these techniques. In incubation, the organ is placed in and remains in a medium; in perifusion, the medium passes over and around the organ; and in perfusion, the medium passes through the vasculature of the organ, as described in more detail below.

The media surrounding the tissue can be static or dynamic. In the **static approach**, used for simple incubations, steroids accumulate in a "tissue culture system" over a specified period of time. This approach may (1) use periodic media replacement as samples are collected for

analysis or (2) leave the media undisturbed. If the media are left undisturbed, then the analyte being measured will accumulate. In both situations, the analyte being measured in the media increases over time. However, the rate of production can change over time due to negative feedback effects of accumulating steroid hormones, decreased precursor concentrations, changes in cAMP, etc. By contrast, a **dynamic approach**, used for perifusion and perfusion, allows the tissue to be in a "stream" of media. The medium is continually pumped past or through the tissue. In this approach, the feedback effects are minimized and, therefore, the tissue and steroid production more closely represent the tissue's environment in a biological organism.

The *in vitro* system used to bathe the removed organ can be closed, half-open, or open. In a **closed system**, no medium is replaced and one sample is collected. In this kind of system, product accumulation occurs. A **half-open system** has medium replaced at certain time intervals; samples are collected when the medium is replaced; product accumulation occurs; and the organ is altered each time the medium is replaced. In an **open system** (also called a superfusion system), the medium is continually replaced and samples can be collected at any desired frequency without affecting the organ. The use of one system over another is based on choosing between finding a technically simple assay that minimizes the amount of external interference with the incubation (closed system is best) and being able to get samples at various time points, realizing that the preparation will be altered by doing so (half-open system). The open system can be used to circumvent interference with the organ while, at the same time, allowing for multiple samples to be collected; however, this system is technically more difficult to set up and operate, which requires more labor over time and decreases the number of organs that can be evaluated.

Simple Whole Organ Incubation

The simple incubation technique involves removal of the testis or ovary and incubation of the organ in medium for testing. For the whole testis (Deb et al., 1980), the animal is anesthetized or euthanized and the testes removed. The testes are incubated in Kreb-Ringer-bicarbonate solution (pH 7.6) at 37°C and an atmosphere of 95 percent O_2 and 5 percent CO_2. The testes are incubated with or without the substance being tested, as well as with or without stimulant, e.g., LH. For the whole ovary, a procedure has been used to assess the effect of a toxicant on ovarian steroidogenic function with and without stimulation (Berman and Laskey, 1993; Laskey and Berman, 1993; Laskey et al., 1995; Piasek and Laskey, 1999). As with the testis, the ovarian method is relatively simple. The whole ovary culture for evaluating steroid production (non-stimulated) involves removing the ovary from an anesthetized or euthanized animal and incubating the ovary at 34°C and 5 percent CO_2 for 1 hour with slow shaking (the vials are slowly agitated and the duration can range from 1 to 24 hours). Finally, the vials are centrifuged and the supernatant decanted and stored frozen for later analysis.

Perifusion

The perifusion system (medium passing over and around the tissue in a chamber) is the most technically difficult of the three whole organ methods, but mimicks *in vivo* conditions and allows ovarian function to take place over several days. Perifusion keeps the organ viable long enough to characterize more complicated developmental processes, e.g., corpus luteum formation. The perifusion system for the ovary is described in detail by Peluso and Pappalarda

(1993). The perifusion apparatus is shown schematically in Figure 4-4. Perifusion systems may involve the use of a computer-controlled apparatus to regulate the release of the test substance in a pulsatile pattern (Soendoro et al., 1992a).

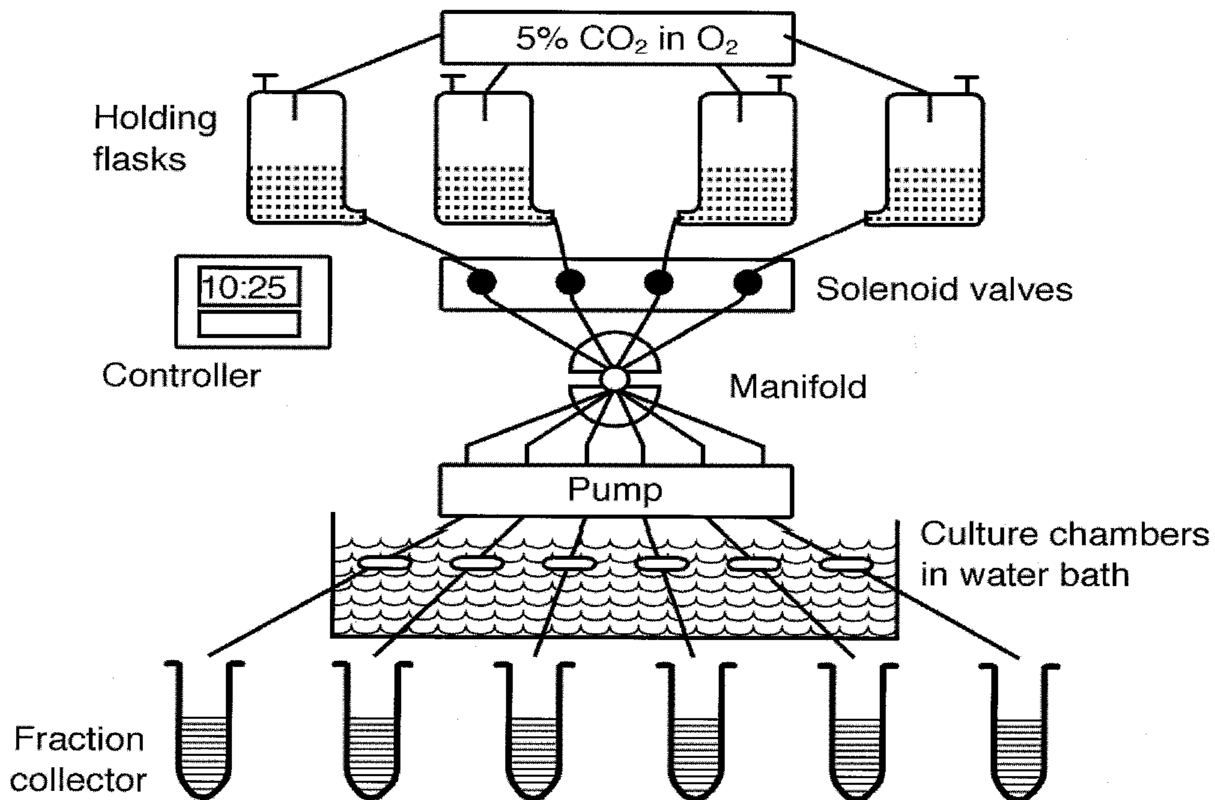


Figure 4-4. Schematic Diagram of the Perifusion Apparatus

Source: Peluso and Pappalarda (1993)

The ovarian perifusion procedure requires a pre-study set-up check. Prior to conducting the study the perifusion system is sterilized and tested for optimal operation, e.g., rates of perifusate media flow need to be equal for all chambers. Verification of the assembly operation is extensive, e.g., autoclaved components, elaborate tubing set-up, medium preparation (laminar flow hood), and aseptic technique required for set-up. Once the perifusion system is set up, immature rat ovaries are collected from anesthetized rats and placed in heparinized medium. The ovaries are incubated at room temperature for a few minutes (allowing microvasculature to dilate and reducing blood content and blood clot formation). The ovaries are placed into the perifusion chamber, the chamber is connected to the perifusion system, and the system started. The perifusate gas tension (95% O_2 and 5% CO_2), temperature (37°C), pH (7.4), and flow rate (6 mL/hr) are carefully maintained. The preparation is viable for a few days. A test for detecting toxic effects on ovarian function involves adding a toxicant to the perifusion system. The response of the ovary is determined by removing samples of perifusate for analysis.

Perfusion

The perfusion system (medium passing through the vasculature) generally involves a laparotomy to cannulate the aorta and vena cava, as well as to isolate and remove the gonad. A perfusion apparatus is connected to the vasculature, and the medium is perfused through the organ in a chamber. The medium is oxygenated, the pH adjusted, the temperature maintained, the perfusion pressure controlled, and the flow rate maintained. Perfusion of the isolated testis or ovary is also a technically difficult procedure, but one that closely mirrors physiological conditions.

The perfused rat testis procedure was developed for the rabbit by Vandemark and Ewing (1963) and modified for the rat by Chubb and Ewing (1979a). An illustration of the perfusion apparatus is shown in Figure 4-5.

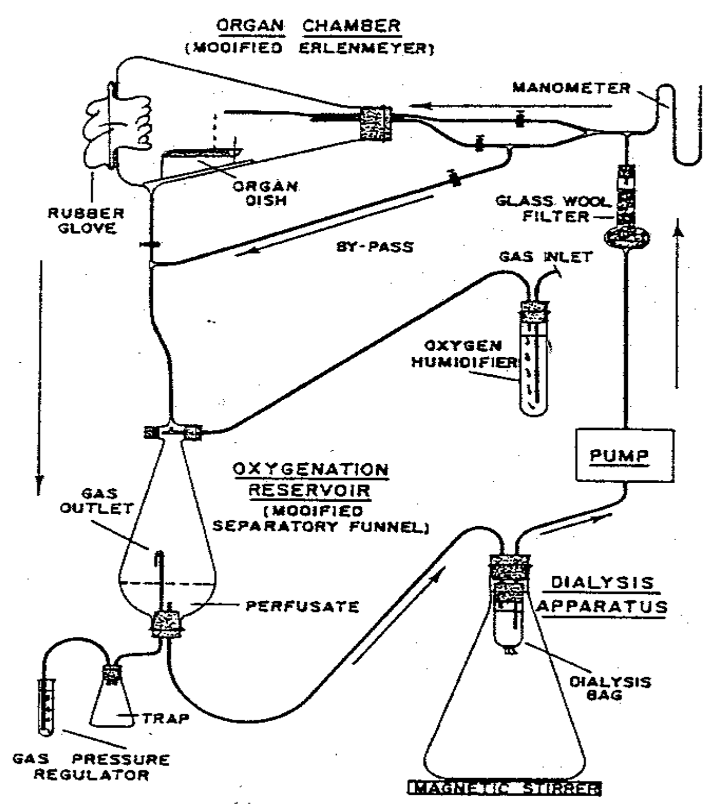


Figure 4-5. Schematic Diagram of a Testicular Perfusion Apparatus

Source: Vandemark and Ewing (1963)

Briefly, the apparatus is sterilized and then assembled. The system must be air-free to prevent bubbles from entering the perfusate and blocking flow. A testis is removed, the spermatic artery cannulated, and the organ flushed of blood with a perfusate solution. The testis is placed in the organ chamber and perfusion initiated. After a preliminary perfusion for approximately 1 hour, the temperature (37°C), rate of flow (20 mL/hr), pressure, and pH (7.4) are monitored and maintained. The perfusion can be maintained for 6 to 10 hours. The testis is maximally stimulated with LH (100 ng/mL of perfusion media). Samples are collected from the perfusate for analysis.

The perfused rat ovary procedure was described by Koos et al. (1984) and Brannstrom et al. (1987). An illustration of the ovarian perfusion apparatus is shown in Figure 4-6. Briefly, the procedure depicted in Figure 4-6 involved anesthetizing the rat and removing the ovary with its arterial supply and venous drainage vessels intact. The ovarian vasculature is cannulated and the assembly placed in a 37°C sodium chloride solution. The ovary is pre-perfused manually with a syringe to remove blood from the preparation. During this time, the ovary is carefully examined to ensure that there is no leakage and the perfusate flows out only from the venous vasculature. Next, the ovary assembly is connected to the perfusion apparatus (Figure 4-6). This recirculating perfusion system pumps medium from the oxygenator-reservoir to the bubble trap and directly back to the reservoir through the shunt line. From the bubble trap, the medium is pumped through the arterial catheter to the ovary, which rests immersed in media in the perfusion chamber. The medium is oxygenated (95 percent O_2 and 5 percent CO_2), the temperature (37°C) and pH (7.4) maintained, perfusion pressure monitored (55 and 80 mmHg), and the flow rate carefully adjusted (0.6 to 0.9 mL/min). The preparation can be maintained for approximately 20 hours. Samples are removed from the perfusate for analysis.

4.3.1.3 Experimental Design Information. The simple incubation assay is technically simple, easy to set up, and can be rapidly used to assess several substances and concentrations simultaneously. The experimental design issues associated with this assay have been described, e.g., age of animal, stage of development, medium, incubation times, etc. In addition, since most investigators are interested in the mechanism and site of action of a toxicant, the simple incubation assay has not been frequently used (to avoid feedback of accumulating steroids), thereby limiting the number of citations available for inclusion in this review paper. This, however, does not preclude it from further consideration as a screen tool for steroidogenesis.

Ovarian maturation, to the extent that normal steroidogenesis occurs, requires a precise sequence of hormonal stimuli. The *in vivo* pattern that brings this about can be mimicked using **perifusion** (Soendoro et al., 1992a). The design of this procedure requires mathematical calculations between the following parameters: the hormone concentration in the holding tank (C_i) and the perifusion chamber (C_f), the rate (R) of delivery, the initial volume (V) of medium, and time. The formula that integrates these relationships with the hormone concentration (C) at any given time (t) is: $C(t) = C_f + (C_i - C_f)\, e^{-Rt/V}$. In practice, these variables are controlled with a microprocessor and solenoid valves. The numerous calculations needed to simulate a complex hormonal gonadotropin release pattern is facilitated with a computer software program or spreadsheet (Peluso and Pappalarda, 1993). In addition to controlling the gonadotropin release, selection of a release pattern is important. The gonadotropin release pattern can cause

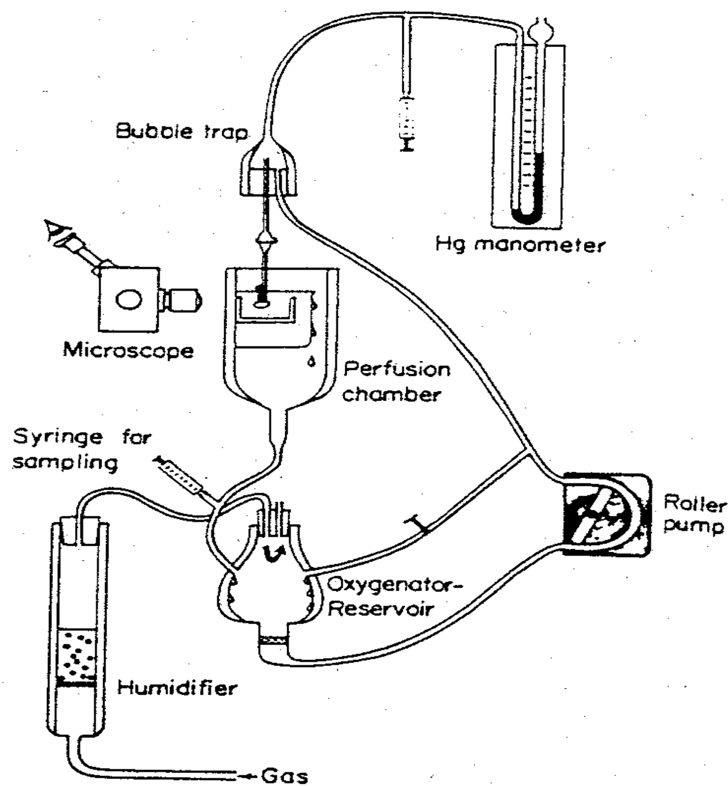


Figure 4-6. Schematic Diagram of an Ovarian Perfusion Apparatus

Source: Brannstrom et al. (1987)

differences in production and secretion of the steroid hormones (Soendoro et al., 1992a; 1993). Futhermore, the developmental status of the ovary affects the response. An ovary from a mature rat (PMSG-treated) responds with a slow and sustained increase in estradiol output, whereas the ovary from an immature rat responds with an acute, transient increase in estradiol. Developmental differences were also noted to affect testosterone and progesterone secretion patterns (Soendoro et al., 1992a). Finally, the size, shape, and/or surface area of the ovary preparation that is exposed results in different estradiol, testosterone, and progesterone profiles under gondadotropin stimulation (Soendoro et al., 1992b).

The **perfused rat ovary** assay requires special consideration of a number of issues, some of which are unique to this assay (Koos et al., 1984; Brannstrom et al., 1987; Brannstorm and Flaherty, 1985; Dharmarajan et al., 1993; Zimmerman et al., 1985). First, regarding the test system, immature rats rather than mature rats are often used because the latter require monitoring the estrous cycle. By using immature rats, the results do not need to be interpreted based on consideration of the endogenous steroidogenic hormone pattern. Second, the perfused rat ovary assay produces a response that is supported by *in vivo* results for such parameters as ovulation

(approximately half of the *in vivo* rate); LH- or PMSG-stimulated ovulation; and steroidogenic production and secretion of estradiol and androstenedione. Progesterone production and secretion is measurable but it does not follow a pattern commensurate with *in vivo* patterns. In addition, progesterone levels are somewhat inaccurate due to nonspecific binding with the components of the perfusion apparatus. One other problem is optimization of the perfusion flow rate. The goal is to simulate normal blood flow, but this parameter is critical so that the ovary is not damaged.

4.3.1.4 Representative Studies from the Literature. Perfusion of the whole testis has been shown to identify substances that alter steroidogenesis. Kelce et al. (1991) used the perfused testis technique to measure the effect of ethane dimethanesulfonate (EDS) on testosterone production. Figure 4-7 summarizes some of the results of this study.

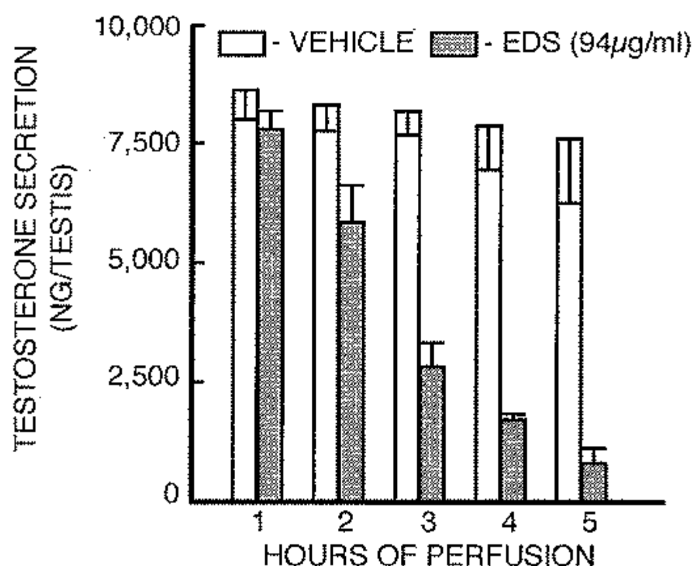


Figure 4-7. Example Data from Study Using a Perfused Testis Method

Notes: *Testes were obtained from adult SD rats and perfused as described above. The testis were perfused with artificial medium containing ovine luteinizing hormone (100 ng/mL) and either vehicle (DMSO, 0.2 percent) or 94 µg of EDS (the ED_{50} concentration for purified Leydig cells and the approximate concentration following an in vivo exposure of 85 mg/kg). Samples of the testicular venous effluent were measured for testosterone concentration at selected time points during the 5 hour perfusion. Testes perfused with the media and DMSO maintained a high rate of LH-stimulated testosterone production. EDS treatment reduced testosterone production and secretion by approximately 10 percent of control by the fifth hour of testing.*

Source: Kelce et al., 1991; Figure 2.

Examples of experimental studies that used isolated whole testis or whole ovary methods for measuring steroidogenesis are summarized in Table 4-5. The table shows that while the perfusion and perifusion procedures are useful for investigating physiological and biochemical processes in the testis or ovary that require long observation periods, the use of these procedures is somewhat limited because simpler procedures can be used to test a substance for altering steroidogenic hormone production and secretion. Note that the Kleeman et al. (1990) study is actually an *ex vivo* study. It was included in the table because it is a good example of using the perfusion procedure in the *in vitro* phase to assess a substance for an effect on steroidogenesis.

Table 4-5. Representative Studies Using Whole Testis/Whole Ovary Methods

Animal/ Procedure	Substance Tested/Stimulant	Measured Response	Reference
Adult male SD rats/Perfused testis	2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin @ 100 µg/Kg, gavage, single administration and terminated after 7 days (*ex vivo* - see text) / hCG @ 50 to 4050 mIU/mL	↓basal and hCG-stimulated testosterone ↓secretion of pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, and androstenedione	Kleeman et al., 1990
Fetal Wistar Rat testes collected @ gestation day 15.5/Perfused testis	aminoglutethimide @ 2 mM/LH @ 100 ng/mL cyproterone acetate @ 1 µg/mL/LH @ 100 ng/mL hydroxyflutamide @ 1 µg/mL/LH @ 100 ng/mL	↓basal and LH-stimulated testosterone	Gangnerau & Picon, 1987
Adult male rats/Whole testis	mitomycin C @ 50, 100 or 200 µgm/mL	↓Δ^5-3β-HSD and glucose- 6-phosphate dehydrogenase activity	Deb et al., 1980
Adult male SD rat/Perfused testis	aminoglutehimide@ 30 to 150 µM SU-10603 @ 1 to 40 µM Medrogestone @ 1 to 40 µM	↓testosterone; inhibition of pregnenolone biosynthesis ↓testosterone; ↑pregnenolone and progesterone; inhibition of 17α-hydroxylase and C-17, C-20-lyase ↓testosterone and inhibition of $\Delta^{5\text{-}4}$ isomerase	Chubb and Ewing, 1979b

4.3.1.5 Distinguishing Features of Whole Organ Methods.

Stability

The simple incubation assay remains viable for at least 6 hours (Deb et al., 1980).

The perifused ovary allows the organ to be viable for a few days based on steroidogenic function, histology, mitogenic activity, and oxygen consumption (Peluso and Pappalarda, 1993).

The isolated perfused ovary is viable for approximately 20 hours based on capacity for the ovary to ovulate when stimulated with LH or PMSG, histology, and steroidogenic hormone production and secretion (Koos et al., 1984).

Standardization

The simple incubation assay has not been standardized. It would be relatively easy to standardize either by (a) optimization testing or (b) by consensus, based on information from researchers with extensive experience using the assay.

The perifused ovary procedure has not been standardized and has a number of critical factors that would require standardization, e.g., specified release patterns and concentrations of hormones from the holding flasks (can be controlled by a microprocessor, solenoid valves, and a computer software program that computes the calculations necessary to simulate hormonal release patterns), the perifusion apparatus, perifusate type and conditions, isolation and removal of ovary, and organ viability checks to name just a few.

The isolated perfused ovary assay is not standardized. This assay would also require standardization of several critical design considerations. Many of the factors are similar to the ones listed above for perifusion.

Sensitivity

Perifusion has been reported to produce lower amounts of steroids per follicle than *in vitro* static perfusion. The disparity has been attributed to the greater medium volume per follicle in perifusion (Hedin et al., 1983) or enhancing factors released by the cell (Campbell, 1982).

Mature ovaries responded more slowly to PMSG-stimulation than did the immature ovary (Soendoro et al., 1992a).

The isolated perfused ovary assay is compromised with regard to measuring progesterone, because this hormone undergoes non-specific binding to the components of the perfusion apparatus.

Testosterone production was decreased 1 to 95 percent when 15 different inhibitors of steroidogenesis were tested at 30 μM using the perfused testis assay (Chubb and Ewing, 1979b).

Specificity Perfusion and perifusion of the ovary respond to LH and PMSG by producing and secreting steroidogenic hormones in a manner similar to *in vivo* responses. Using perifusion, stimulation of steroid release from the ovary occurred above a threshold of 3 to 8 mIU/mL of LH and 4 to 10 mIU/mL of FSH and the slope of the increase was important (Soendoro et al., 1992a).

α-Melanotropin modulates reproductive function and, in the prepubertal female rat ovary, it increased the production and secretion of progesterone, but not estradiol (Durando and Celis, 1998).

Metabolic Activity None.

Equipment Perifusion and perfusion procedures require standard and specialized equipment. Examples of the specialized laboratory set-up are shown in Figures 4-7, 4-8, and 4-9. Perifusion in particular requires a microprocessor, solenoid valve set-up, and a computer in order to achieve accurate and precise control of the perifusion patterns of the gonadotropins.

Training Perifusion and perfusion procedures require standard and specialized training. The assay is labor-intensive and requires a well-trained study team in order to perform the assay consistently from study to study. Perifusion in particular requires additional training in order to operate the system in such a way as to mimic *in vivo* gonadotropin release patterns, i.e., pulses, surges, or steady state.

Sample Scheduling The isolated perfused ovary assay, from the time of organ isolation until the apparatus is set up and operational, is approximately 2 hours. For the experienced laboratory team, two ovaries can be isolated and perfused for study in a day (Koos et al., 1984). This would severely limit the number of substances that could be tested in a given period of time.

Animal Usage Whole organ assays are similar to whole animal assays, which result in an assay that uses relatively more animals than other assays. Since each animal contributes two gonads and each gonad generates one set of data, a reduction in the number of animals used is limited.

4.3.1.6 Conclusion. Isolated whole organ methods are useful for identifying substances that alter steroidogenesis. The different procedures that use isolated whole organs vary greatly in their degree of technical difficulty. The simplest procedure is isolation of the testis or ovary followed by incubation with a stimulant and/or substance for testing. However, the literature infrequently cites this as a method used to identify substances that alter steroidogenesis. The other procedures, perfusion and perifusion, are very labor-intensive and seem to have better application for characterizing physiological and/or biochemical processes that take long periods of time to investigate.

4.3.2 Testicular Sections or Minced Ovary Methods

4.3.2.1 Scope of the Method. The testicular section and minced ovary methods use untreated animals as a source for the organs used. After removal of the whole organ from an anesthetized or euthanized animal, the testes or ovaries are further processed into smaller sizes for use as sections (testis) or for mincing (ovary). A method to prepare the organs for measuring steroidogenesis was worked out in detail for the ovaries by Laskey et al. (1991, 1994) and in the testes by Sikka et al. (1985). The testicular section and minced ovary methods are reliable assays in that they are relatively easy to conduct, the cellular architecture is preserved, different species can be used, and the assay can be conducted relatively rapidly (Gray et al., 1997). The primary limitation of these methods is the absence of metabolism for those substances that require metabolic activation.

4.3.2.2 Description of the Method. A flow diagram for the isolated testicular section method is illustrated in Figure 4-8.

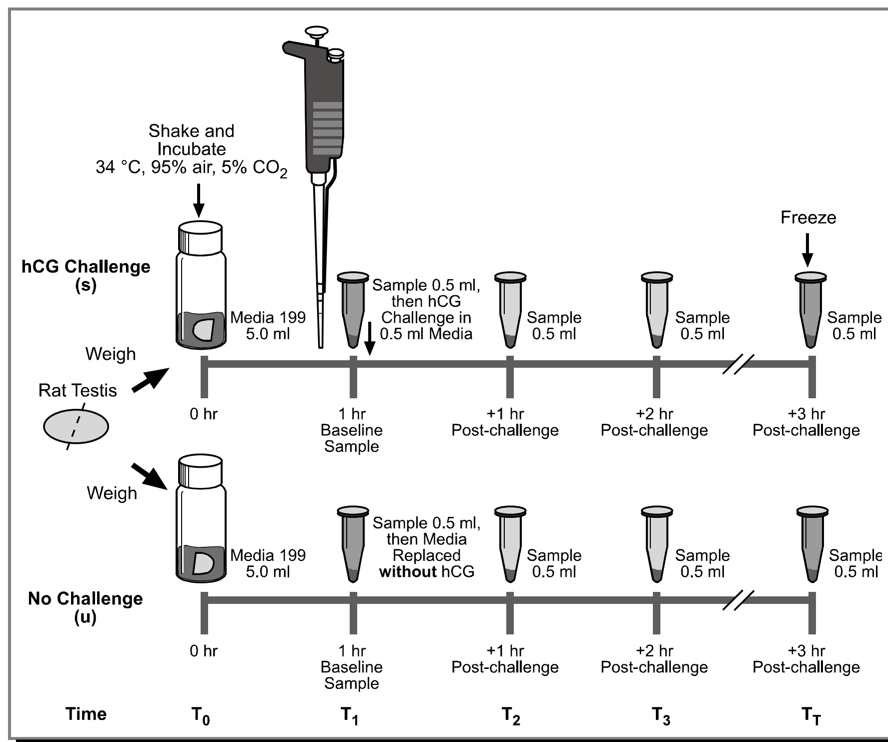


Figure 4-8. Technical Flow Illustration of the Testicular Steroidogenesis Assay

The **testis section procedure** involves euthanizing the animal and removing the testes. The testes are decapsulated, weighed, and sectioned. One testis is separated into 2 to 8 longitudinal sections. Each section is placed in a vial containing media alone or media plus toxicant**.** The medium that has been used in such studies is modified medium 199 with 0.1 percent bovine serum albumin (BSA), 8.5 mM sodium bicarbonate, 8.8 mM HEPES and 0.0025 percent soybean trypsin inhibitor at pH 7.4. The vials containing the testicular sections and media are incubated at 34°C on a shaker in 5 percent CO_2/95 percent air. After the first period of incubation, e.g., 1 hour, an aliquot of media is collected. This sample is the baseline or secretion sample. Next, one half of the replicates are challenged with a stimulant, e.g., hCG, and the other half are not, which provides information regarding the toxicant's effect when the organ sample is stimulated to initiate steroidogenesis and when it is functioning at a basal level. Additional samples are collected from the vials after various incubation periods, e.g., 1, 2, and 3 hours. The media samples are then frozen for later analysis or analyzed immediately for selected hormones using an appropriate method, which is usually RIA.

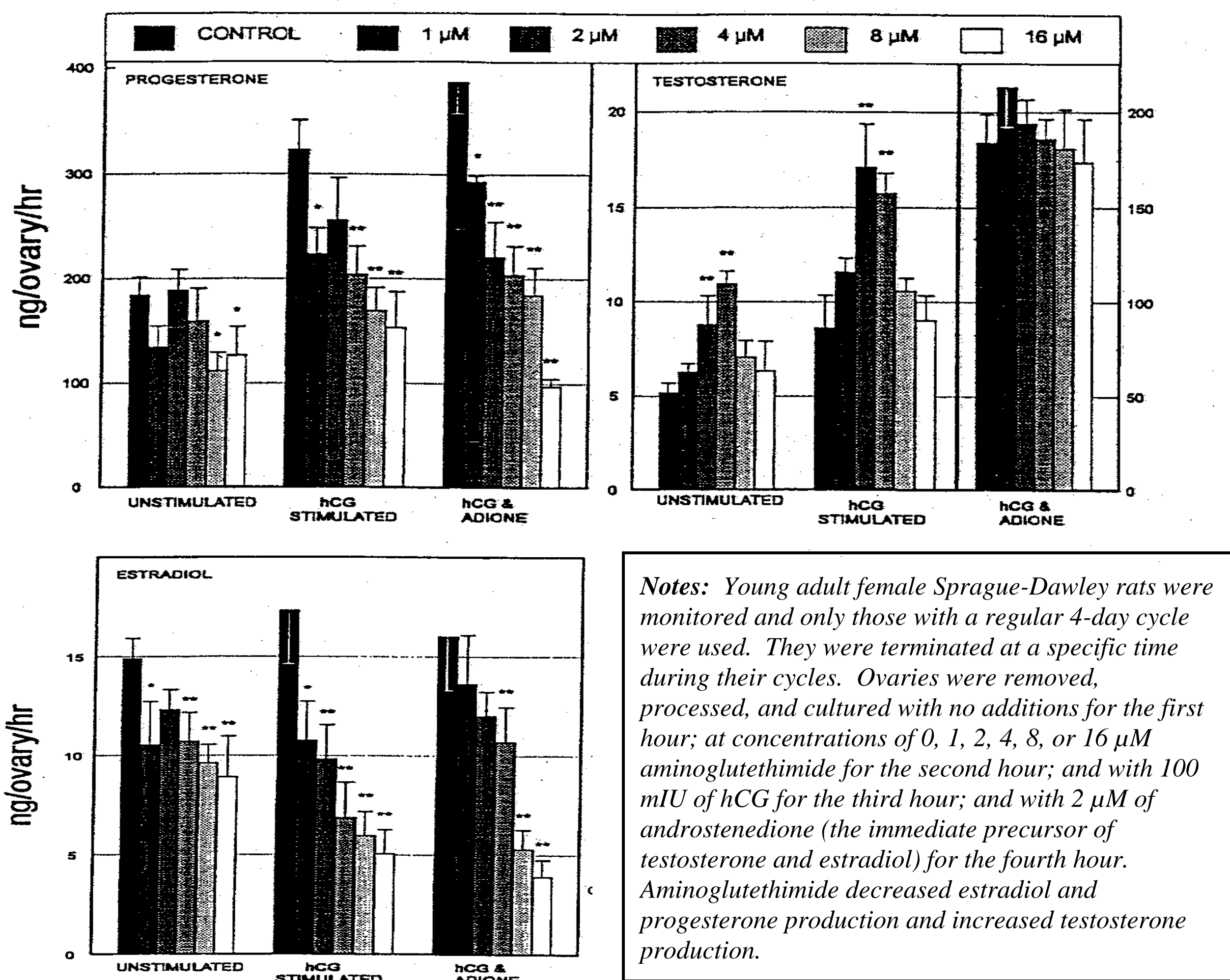
The **minced ovary procedure** involves euthanization of the animal and removal of the ovaries (Laskey et al., 1995). Euthanization must be carefully controlled to ensure that animals are in the same stage of their estrus cycle. The ovaries are trimmed of fat and other extraneous tissue, weighed, and placed in media (as previously described). Each ovary is minced with scissors while in the media. The vial containing the minced ovary and media (with and without toxicant) are incubated for one hour at 34°C in a 5 percent CO_2 environment. The vials are centrifuged and the supernatant decanted and stored frozen for later analysis (baseline). Media containing hCG is added to half the minced ovary samples (stimulated and non-stimulated). The minced ovary samples are resuspended in media, incubated for an additional hour, centrifuged, and the supernatant decanted and stored frozen for later analysis. This process is repeated for sample collection at 2 and 3 hours after stimulation. The media samples are analyzed for selected hormones using an appropriate analytical method.

4.3.2.3 Experimental Design Information. Various incubation conditions are used for *in vitro* methods. Gurtler and Donatsch (1979) incubated ^{14}C-pregnenolone with sectioned testis in order to measure the conversion of this steroidogenic pathway substrate into testosterone, androstenedione, and other hormones. They incubated the testis sections for 2 hours in Krebs-Ringer bicarbonate solution (pH 7.4; 95 percent O_2/5 percent CO_2; at 30°C) with 3 µmoles of NADPH and NADP, 20 µmoles glucose, 40 µmoles glucose-6-phosphate and 5 IU glucose-6-phosphate dehydrogenase. Incubation conditions that have been used for minced ovaries (Piasek and Laskey, 1999) include incubation (34°C, 5 percent CO_2) for up to four hours in M-199 culture medium supplemented with sodium bicarbonate, HEPES, BSA, and soybean trypsin inhibitor.

Careful consideration must be given to the developmental stage of the animal when designing a study for organ collection. The use of immature versus mature animals was described previously (Section 4.3.1.3 - Experimental Design Information for Whole Organ). With regard to immature rats from 2 to 5 weeks of age, an evaluation of ovarian steroid production using the minced ovary procedure indicated that 3- and 4-week-old animals were favorable (Laskey and Berman, 1993). Ovaries from the 2-week old animals produce low estradiol, progesterone, and testosterone levels, whereas 5-week-old animals could begin cycling, which would produce more steroids. Similar consideration must be exercised for

studying ovarian steroidogenesis in cycling versus pregnant animals. For example, Piasek and Laskey (1999) used proestrus rats and pregnant rats at gestation days 6 and 16. Proestrus was selected because it is when maximal steroid production and secretion occurs. Gestation day 6, the time following implantation, is the onset for increased progesterone production and gestation day 16, the time following organogenesis, is when the second peak of progesterone production occurs.

4.3.2.4 Representative Studies from the Literature. The minced ovary method described above was used by Laskey et al. (1995) to assess toxicant effects on the steroidogenic pathway in the rat. The results of their investigation are illustrated in Figure 4-9.



Notes: Young adult female Sprague-Dawley rats were monitored and only those with a regular 4-day cycle were used. They were terminated at a specific time during their cycles. Ovaries were removed, processed, and cultured with no additions for the first hour; at concentrations of 0, 1, 2, 4, 8, or 16 µM aminoglutethimide for the second hour; and with 100 mIU of hCG for the third hour; and with 2 µM of androstenedione (the immediate precursor of testosterone and estradiol) for the fourth hour. Aminoglutethimide decreased estradiol and progesterone production and increased testosterone production.

Figure 4-9. Example Data Using the Minced Ovary Method

Source: Laskey et al., Figure 6; copyright (1995), with permission from Elsevier Science.

Examples of experimental studies from the literature that used isolated testes sections or minced ovary for measuring steroidogenesis are summarized in Table 4-6. The studies in this table used untreated animals prior to collecting the testis sections or minced ovary. It is important to note that the testis section and minced ovary preparations are also used in conjunction with treated animals as described above in the discussion of *ex vivo* methods.

Table 4-6. Representative Studies Using the Isolated Testis Sections or Minced Ovary Method

Animal/Type of Preparation	Treatment & Stimulant	Measured Response	Reference
Adult female SD rats (@ Proestrus and early [GD 6] or late [GD 16] pregnancy)/ Minced Ovary	Cadmium chloride @ 100, 500, 1000, 1500, or 2000 μM/ 100 mIU hCG	↓progesterone in proestrus and pregnant rats ↓testosterone in proestrus and pregnant rats ↓to no effect on estradiol in proestrus and pregnant rats	Piasek and Laskey, 1999
Adult male Long-Evans rats/Testes Sections (1/4)	Ethane dimethanesulfonate @ 0, 3, 10, 32, 100, 320, 1000, or 3200 μg/mL media/ ovine LH (100 ng/mL)	↓ testosterone production	Gray et al., 1995
Male Long-Evans Hooded rats (3-25 weeks of age)/ Testes Sections (1/4)	Vinclozolin @ 5 to 100 mg/kg/day, gavage, for 22 weeks/hCG, 50 IU	↑ basal and hCG-stimulated testosterone @ 15 and 100 mg/kg/day	Fail et al., 1995
Male Long-Evans Hooded rats (3-14 weeks of age)/ Testes Sections (1/4)	Methoxychlor @ 50 or 200 mg/kg/day, gavage, for 11 weeks/hCG, 50 IU	↓ basal testosterone production no effect on HCG-stimulated testosterone production	Fail et al., 1994
Adult male SD rat/Testes sections(1/4)	Ethane dimethanesulfonate @ 0, 500, or 3000 μM/ 100 mIU/mL hCG	↓ testosterone production	Laskey et al., 1994
Adult female SD rat/Minced Ovary	Phenolsulfonthalein @ 20 mg/L	↓progesterone production some alteration to estradiol and testosterone production	Berman and Laskey, 1993
Female rat/ Minced ovary	Methoxychlor & hCG	Progesterone, estradiol, & testosterone	Cummings and Laskey, 1993
Adult female Golden Hamsters/ Quartered Ovary	Aromatase Inhibitors: testolactone @ 10, 100, or 1000 μM /LH @ 100 ng/mL CGS 16949A @ 0.004 to 400 μM/ LH @ 100 ng/mL 4-hydroxy-androstenedione @ 0.33 to 330 μM/ LH @ 100 ng/mL aminoglutethimide @ 3, 30, or 300 μM/ LH @ 100 ng/mL	↓estradiol and estrone; no effect on progesterone or testosterone ↓estradiol and estrone; ↑testosterone; no effect on progesterone ↓estradiol, estrone, and progesterone; no effect on testosterone ↓estradiol, estrone, testosterone, and progesterone	Hausler et al., 1989

Table 4-6. Continued

Animal/Type of Preparation	Treatment & Stimulant	Measured Response	Reference
Adult male OFA rat/Testis Sections (~1/4)	^{14}C-pregnenolone (50 mCi/mmol; 200 nCi - a tracer amount)	~70 and 15 percent of the ^{14}C-radioactivity was testosterone and androstenedione	Gurtler and Donatsch, 1979

4.3.3 Distinguishing Features of Whole Organ and Testis Section/Minced Ovary Methods

Stability Whole ovaries maintained in media without hCG show no histological evidence of necrosis after at least 4 hours (Laskey and Berman, 1993). Untreated quartered testes maintain a steady rate of testosterone production for at least 5 hours with no decrease in production or secretion (Laskey et al., 1994). Minced ovaries with maximal stimulating amounts of hCG maintain steroid production for at least 3 hours in culture (Laskey et al., 1995).

Standardization Methods are not standardized. Standardization would involve selecting animal species/strain, organ collection and preparation, incubation conditions and periods, media, toxicant and stimulant schedules, sample collection schedules, and analysis method. Standardization of the minced ovarian method is more complex due to the dynamic changes that take place during the estrous cycle (Laskey and Berman, 1993; Laskey et al., 1995). Toxicants may have different effects based on the stage of the cycle when the ovary is removed Also, additional procedures are required when designing a study using juvenile female rats to ensure that they are still immature.

Sensitivity These methods provide a technique for evaluating a wide range of toxicant concentrations. Testable low-level concentrations are generally in the µM range and high concentration levels are limited by the solubility of the substance in the carrier and media.

Specificity Preparations of the parenchyma (whole or sectioned) have maximal-stimulating testosterone production that is within approximately 10-fold of the physiological levels (Klinefelter and Kelce, 1996). The selectivity of the *in vitro* method was evaluated by Hausler, et al. (1989), using aromatase inhibitors and evaluating the production and secretion of estradiol, estrone, progesterone and testosterone (see table for results).

Metabolic Activity Little to no metabolism.

Equipment Animal facilities. Standard and endocrine laboratory equipment, e.g., balance. Necropsy equipment. Specialty equipment needed is an incubator and -70°C freezer.

Training A simple assay that requires some practice but can be readily learned by a general laboratory technician. RIA method requires the most training.

Sample Scheduling Methods can be set up and conducted, with RIA samples analyzed, within 1 to 2 days.

Animal Usage The process of sectioning or mincing the organ reduces the number of animals used relative to whole animal or whole organ assays but not to the extent made possible by isolated cell or cell line assays.

4.3.4 Conclusion

The organ section/minced method is not technically difficult and takes relatively little time to conduct. In addition, the method maintains the cytoarchitecture of the organ. The organ preparations remain stable for a sufficient period of time to identify changes in steroid hormone production and appear to be sensitive and specific enough based on the information collected to date. However, one limitation of these preparations is the absence of metabolism pathways for activation of pro-xenobiotics.

4.4 Isolated and Cultured Cell Method *(in vitro)*

The steroidogenic pathway is found in specific cells in the ovary and testis. As described in Section 3, steroidogenesis occurs in the ovarian follicle cells or in cells within the ovarian follicle like the theca interna, granulosa, and corpus luteum. In the testis, the steroidogenic pathway is found in the Leydig cell, with the exception of that part of the pathway where testosterone is converted to dihhydrotestosterone by 5α-reductase or where testosterone is converted to estradiol by aromatase. For the male, these conversions occur to a greater extent in peripheral tissues than in the gonad itself. Isolation of the steroid-producing cells provides more direct access to the biochemical pathway for testing substances for estrogenicity or

androgenicity. The isolated and cultured cell method can be used to evaluate the potential of a direct effect of a substance on the steroidogenic pathway and for identifying the site of action in the pathway.

The testicular and ovarian cell isolates are enriched with the cells that synthesize the steroid hormones. The cells are isolated to different levels of separation. Generally, the cells are described as being in either a crude or a purified preparation. A **crude** preparation includes much of the cellular debris and tissue remnants that remain as a result of processing the organ into cells but the cells are not subjected to additional clean-up steps. A crude preparation of Leydig cells, also called an interstitial cell preparation, generally has approximately 15 percent Leydig cells. In contrast, a purified preparation involves additional clean-up steps. A **purified** preparation is obtained through procedures such as column separation, centrifugal elutriation, histological separation, or biochemical centrifugation to separate the desired cells from other debris. A purified preparation is commonly understood to be approximately 95 percent isolated cells.

Once the cells are isolated, they must also be cultured. Culturing the cells involves placing the cells in an environment that will maintain their viability and extend their longevity. The optimization and maintenance of the cells in culture media requires attention to specific procedures (Klinefelter et al., 1993; Treinen, 1993), which are described in further detail below.

A wide variety of cell preparations have been used for studying the steroidogenic capacity of isolated testicular and ovarian cells. Testicular preparations include fragments of testicular parenchyma, dispersed testicular interstitial cells, purified Leydig cells, and tumor Leydig cells (Klinefelter and Kelce, 1996). It is important to remember that these cell preparations will not take into account the interaction among surrounding cells found within the organ. For example, the isolated purified Leydig cell preparation will not include Sertoli cells, and the granulosa cell preparation will not include many theca cells. Nor will the paracrine factors of the organs be of influence in the responses being measured in these cell preparations. This isolation is both an advantage and a disadvantage. In the following subsections, the crude (interstitial cell preparation) and purified Leydig cell preparations and granulosa cell preparation will be used as examples of the isolated and cultured cell *in vitro* method.

4.4.1 Isolated Leydig Cell Culture Method

4.4.1.1 Scope of the Method. Isolated and cultured Leydig cells provide an *in vitro* method for assessing the androgenic or anti-androgenic effects of a substance. While the isolation and culturing of these cells is a multi-step process, isolated Leydig cells are used to minimize factors, e.g., interstitial or germ cells, that can compromise the response of the substance being tested on steroid production; as a model for understanding mechanistic effects of a toxicant; and evaluating the reversibility of toxicant effects. Extensive reviews about Leydig cells include those by Klinefelter et al. (1993) and Payne et al. (1996). From these reviews, information was evaluated and extracted based on its usefulness for evaluating this procedure as a possible screen for substances to be tested for steroidogenic-altering effects.

Purified Leydig cell preparations have been used extensively to investigate the mechanism and site of action of substances on steroid hormone production and secretion (see

summary table below), as well as to investigate the mechanism of Leydig cell tumorigenesis (Biegel et al., 1995); steroidogenic cytochrome enzyme activity using immunohistochemistry techniques and ultrastructural alterations using electron microscopy (Thoreux-Manlay et al., 1995); comparison of species responses (Laskey et al., 1994); and the mechanism of action of toxicants at the cellular, biochemical, and physiological levels (Klinefelter et al., 1994; Bambino and Hsueh, 1981).

4.4.1.2 Description of the Method. The interstitial cell preparation (crude Leydig cell preparation) is simpler and faster than the purified preparations. The procedure reported by Laskey and Phelps (1991) involves anesthetizing or euthanizing the animal and then removing and decapsulating the testes. The testes are incubated in media containing collagenase for 10 minutes at 37°C with gentle shaking. The cells are then washed with fresh media, filtered (50 µM nylon mesh), centrifuged, and resuspended in fresh media to the desired number of cells per unit volume.

For the purified Leydig cell preparation, the procedure is substantially more laborious. A flow diagram for the isolation of a purified Leydig cell preparation is shown in Figure 4-10a, with expanded details shown in Figures 4-10b and 4-10c.

Isolation and maintenance of Leydig cells involves a multi-step procedure:

- Collagenase dispersion of testicular interstitial cells
- Separation of smaller cells by centrifugal elutriation
- Purification of cells by density gradient centrifugation
- Culturing of purified cells (Klinefelter et al., 1993).

Each of these steps is described briefly in the following paragraphs.

Collagenase Dispersion. This step provides enzymatic separation of the Leydig cells within the testis. The testes from untreated, adult rats are quickly removed and placed in ice-cold medium, e.g., M199D. The testicular artery is catheterized and the testis is perfused with collagenase. The perfusion both removes red blood cells from the organ and initiates the cellular dispersion process. After perfusion, the testis is decapsulated and the parenchyma collected into centrifuge tubes containing media. The tubes are incubated at 37°C for several minutes to initiate intravascular enzymatic digestion. Collagenase dispersion is terminated by adding fresh media. The dispersed cells are filtered through doubled 100 µm nylon mesh, centrifuged, and the pellet resuspended in fresh media. The purity of the cell preparation is approximately 15 percent after this step.

Centrifugal Elutriation. This step separates Leydig cells from other cells and debris based on cell size. The elutriation system (peristaltic pump, loading tube, bubble trap/loading chamber, and centrifuge with elutriator rotor) is filled with media and residual air removed to prevent back pressure and altered buffer flow velocity in the chamber. The media flow rate is optimized for collection of dissociated Leydig cells. The harvested cell suspensions are pooled, placed in a loading tube, set in the elutriator, passed through the system, and collected in a separation chamber. The purity of the cell preparation is approximately 25 percent after this step. The other cell types included with the Leydig cells at this stage include macrophages,

pachytene spermatocytes and multinucleated germ cells.

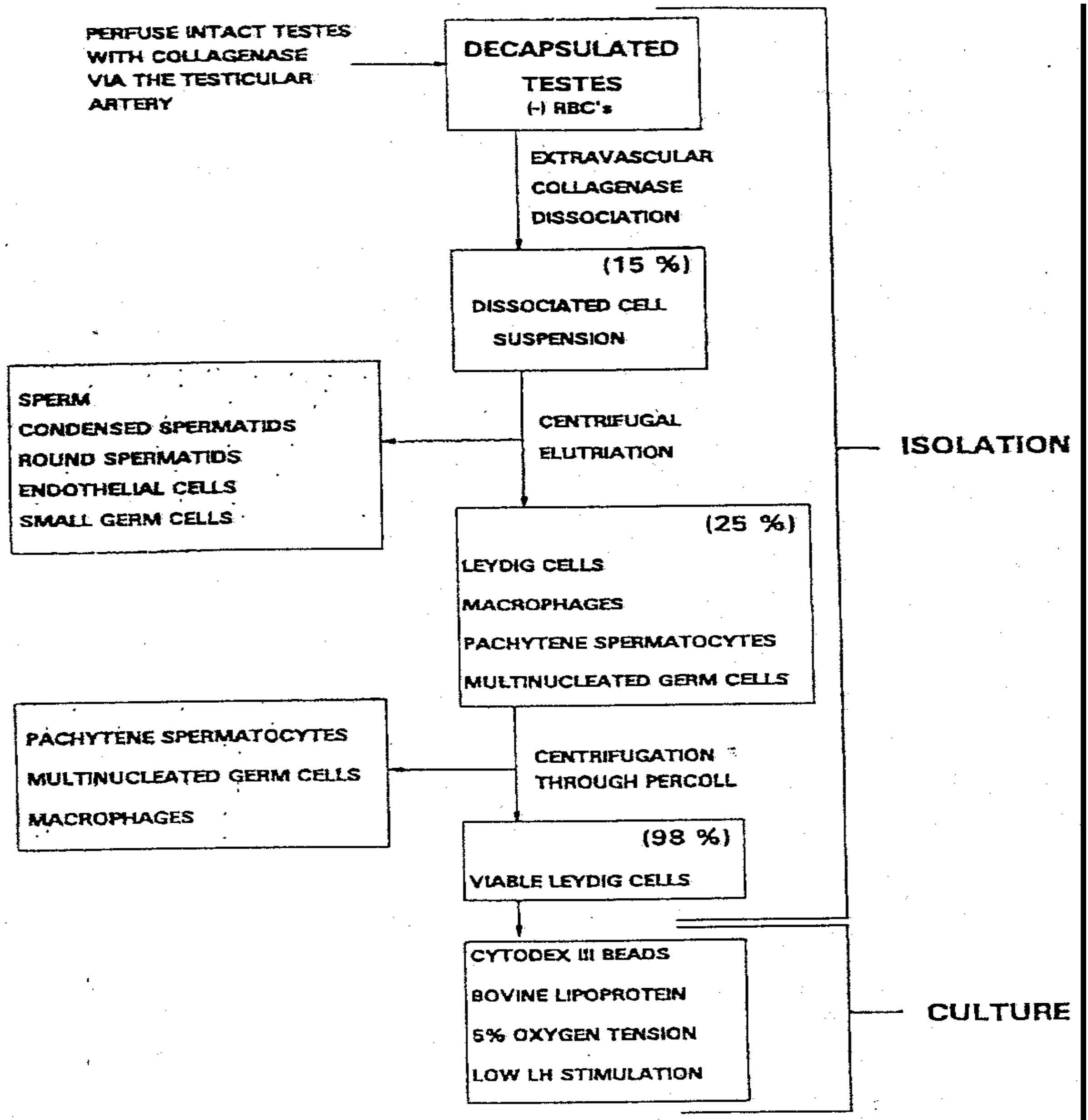


Figure 4-10a. Schematic Diagram of Isolated and Cultured Leydig Cell Method

Source: Klinefelter et al. 1993

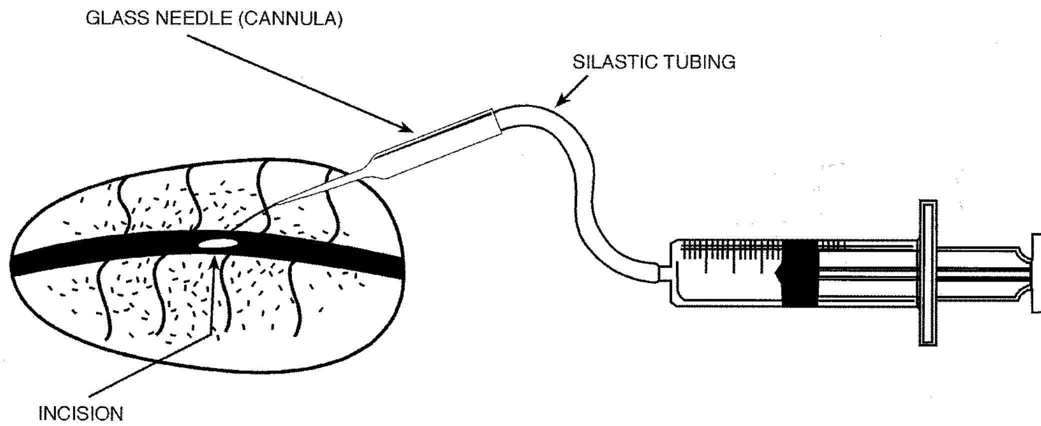


Figure 4-10b. Testicular Perfusion Detail

Source: Klinefelter et al. (1993)

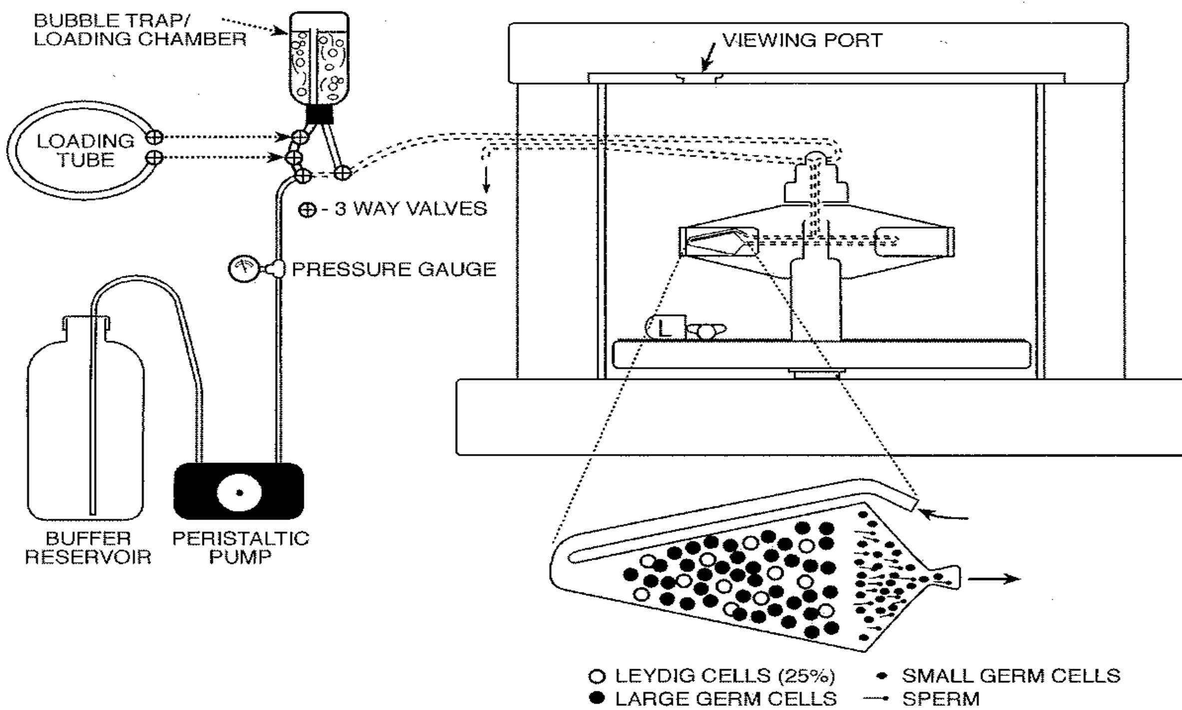


Figure 4-10c. Elutriation System Detail

Source: Klinefelter et al. (1993)

Density Gradient Centrifugation. This step separates Leydig cells from the other cells based on the bouyant densities of the cells. The cell preparation from the previous step are centrifuged and the pellets are resuspended in isotonic Percoll for Percoll density gradient centrifugation. The cell suspensions are centrifuged and the fractions divided. The fraction heavier than 1.068 g/mL contains the purified Leydig cells that are intact and steroidogenically active. The purity of the cell preparation is approximately 98 percent after this step.

Culturing. This step provides an environment that maintains Leydig cell viability for several days. The purified Leydig cell preparation is cultured on Cytodex 3 beads, a porous microcarrier. The beads are coated with a type I denatured collagen, which is used as the substratum for cellular contact. The cells are suspended in media, e.g., DMEM/F12, that contains fetal bovine serum (the source for fibronectin) and incubated at 34°C. The Leydig cell preparation is divided for a yield of approximately 10^6 cells/0.2 mL. The cells are poured into a culture dish, bovine lipoprotein added, and additional media added to bring the final Leydig cell culture volume to 2.0 mL with a cell concentration of 0.5 x 10^6 cells/mL. The final Leydig cell concentration is critical for optimal testing and must be determined from run to run and lab to lab. The cell cultures are maintained at 34°C in 5 percent CO_2 in air overnight. Additional changes of media are required to keep the cells viable for several days.

4.4.1.3 Experimental Design Information. The purity of the Leydig cell preparation must be high to reduce factors that can confound assessment of steroidogenesis. Red blood cells are removed by perfusion of the testis with media. Perfusion with collagenase also increases Leydig cell yield and steroidogenic Leydig cell response (Klinefelter et al., 1993). Sperm cells are an additional confounding factor, and these cells are removed through centrifugal elutriation. This step also removes smaller and damaged Leydig cells, which is important because steroidogenic activity is altered in the presence of such cells (Klinefelter et al., 1987; Abayasekara et al., 1991). In addition, elutriation removes macrophages. Testicular macrophages release substances that stimulate and inhibit Leydig cell function (Hutson, 1998; Nes et al., 2000).

The number of Leydig cells used by investigators for a single incubation can vary greatly among investigators. Laskey and Phelps (1991) used an interstitial cell preparation (15 percent Leydig cells) that had 160,000 cells/mL (Laskey and Phelps, 1991). A semi-purifed preparation (70 percent Leydig cells) was used with only 10,000 cells/mL (Thoreaux-Manley et al., 1995). The number of cells per unit volume from purified Leydig cell preparations (purities of ≥95 percent Leydig cells) has ranged from as low as approximately 20,000 cells/mL (Klinefelter et al., 1991; 1994) and 50,000 cells/well (Biegel et al., 1995) to as high as 200,000 cells/mL (Kelce et al., 1991). All of these preparations were able to show that the substance being tested altered steroid production and secretion.

Various methods have been used to optimize Leydig cell recovery and purity. Cell preparation purity values of ≥90 percent Leydig cells can be obtained with enzymatic dissociation and density gradient separation (Browne et al., 1990); and 98 percent has been achieved using the multi-step procedure described above (Klinefleter et al., 1993). Clean-up steps that improve the purity above 95 percent, such as additional steps to remove unwanted cells like the macrophages, do not appear to improve Leydig cell responsiveness (Dirami et al., 1991). Recently, the multi-step procedure was modified by substituting unit-gravity sedimentation for

filtering during the collagenase dispersion step (Salva et al., 2001). This modification did not increase cell purity but did result in a higher concentration of Leydig cell clusters and a higher recovery of greater numbers of Leydig cells.

Cell viability is important to evaluate in an *in vitro* procedure, and several techniques have been used to ensure that the Leydig cells remain intact. Many investigators actually identify the Leydig cells in the preparation using a histochemical staining technique. Klinefelter, et al. (1987), described how to stain for 3β-HSD activity, and many investigators now use this technique to demonstrate viability. Other techniques that actually assess cell viability (rather than identify the cell type) include using Trypan blue, which is excluded by intact cells, histological examination of the cells (Klinefelter et al., 1991), quantification of [^{35}S]-methionine incorporation into proteins synthesized *de novo* (Kelce et al., 1991), and a colorometric assay using tetrazolium salt MTT, which is reduced by succinate dehydrogenase (a mitochondrial enzyme) to formazan (Thoreaux-Manley et al., 1995).

Procedures used to culture the cells can affect cell viability. Important factors include screening each lot of collagenase for activity, ensuring that the enzyme concentration is kept relatively constant for all incubations, and using an optimal dispersion technique (Molenaar et al., 1985) . In addition, extended culture time is optimized by adding bovine lipoprotein to Leydig cell cultures and using a porous substrate (Cytodex 3 beads) for cellular attachment (Klinefelter et al., 1993). Other factors that extend viability include reduced oxygen and submaximal LH stimulation (Klinefelter and Ewing, 1989).

An important experimental design consideration is the type and age of the animal used to prepare the isolated and cultured cell preparation. For information regarding this topic, the reader is directed to the experimental design considerations described for the in-life aspects of the *ex vivo* method (Section 4.2.3), where this information is presented.

Investigators have used a variety of Leydig cell incubation procedures. For example, Biegel et al. (1995) incubated the purified Leydig cells for 2 hours with (treatment) or without (control) the substance being tested, added the stimulant (2 IU hCG), and incubated the cellular preparation an additional 3 hours before discontinuing the incubation and collecting media samples for steroid hormone analysis. Other investigators have mixed the cells, stimulant, and substance being tested all at once and, after a 3-hour incubation, removed the media for steroid hormone analysis (Klinefelter et al., 1991; 1994). Much longer incubation periods have also been utilized. Thoreaux-Manlay et al. (1995) incubated the cells, stimulant, and substance being tested and, after 4 hours, removed the media for analysis. The media were replenished and the cell preparation incubated for an additional 20 hours and, once again, the media samples collected and the media replenished. A final sampling was made 24 hours later (48 hours from initiating the incubation). These examples demonstrate the flexibility of the isolated Leydig cell method and how it can be modified to test for special characteristics of the substance being tested. In addition, the different procedures provide insight into the difficulty of standardizing such a procedure such that it could be used by multiple laboratories and/or at different times (see below for additional issues to be addressed concerning standardization of this procedure).

4.4.1.4 Representative Studies from the Literature. Laskey and Phelps (1991) demonstrated the usefulness of the interstitial cell preparation (15 percent Leydig cells) to

characterize the mechanism and sites of action of a toxicant. Figure 4-11 summarizes the effects of various cations on testosterone production and secretion using a crude Leydig cell preparation.

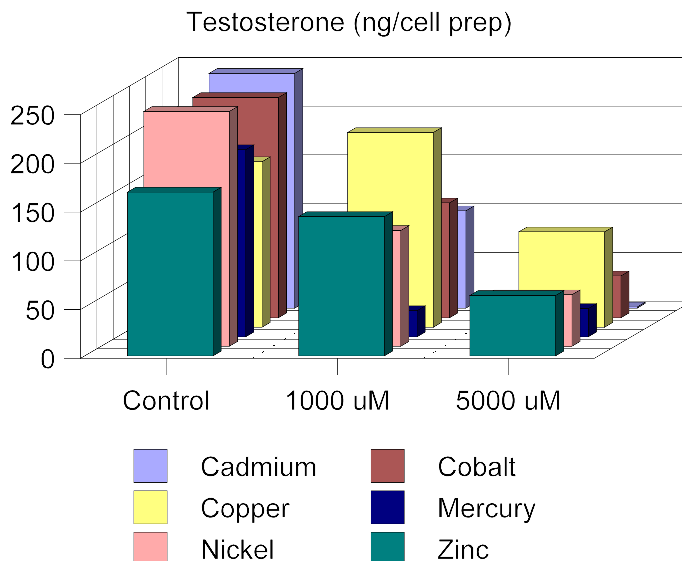


Figure 4-11. Effect of Metal Cation Treatment on hCG-Stimulated Testosterone Production using an *In Vitro* Interstitial Cell Preparation

Notes: A Leydig cell preparation that was prepared from adult SD rats was used to determine the toxic effects of several metal cations. The Leydig cells were stimulated to produce testosterone by using hCG (simulates LH), cAMP (tests for post LH receptor defects), 20α-hydroxycholesterol (a substrate for $P450_{SCC}$), or pregnenolone (a substrate for the isomerase/3β–hydroxysteroid dehydrogenase enzyme). The metal cations, at concentrations ranging from 1 to 5000 µM, were incubated with the Leydig cells for 3 hours in the absence or presence of these stimulating agents or substrates. There was no effect on cell viability. A dose-response depression with hCG- and cAMP-stimulated testosterone production was observed for cadmium, cobalt, copper, mercury, nickel, and zinc. In addition, cadmium, cobalt, nickel, and zinc caused a significant increase in testosterone production when the substrates were used.

Source: Laskey and Phelps, 1991, page 300.

From the results of this investigation, Laskey and Phelps developed a schematic representation of the sites of cationic stimulatory or inhibitory action. Their illustration is provided in Figure 4-12.

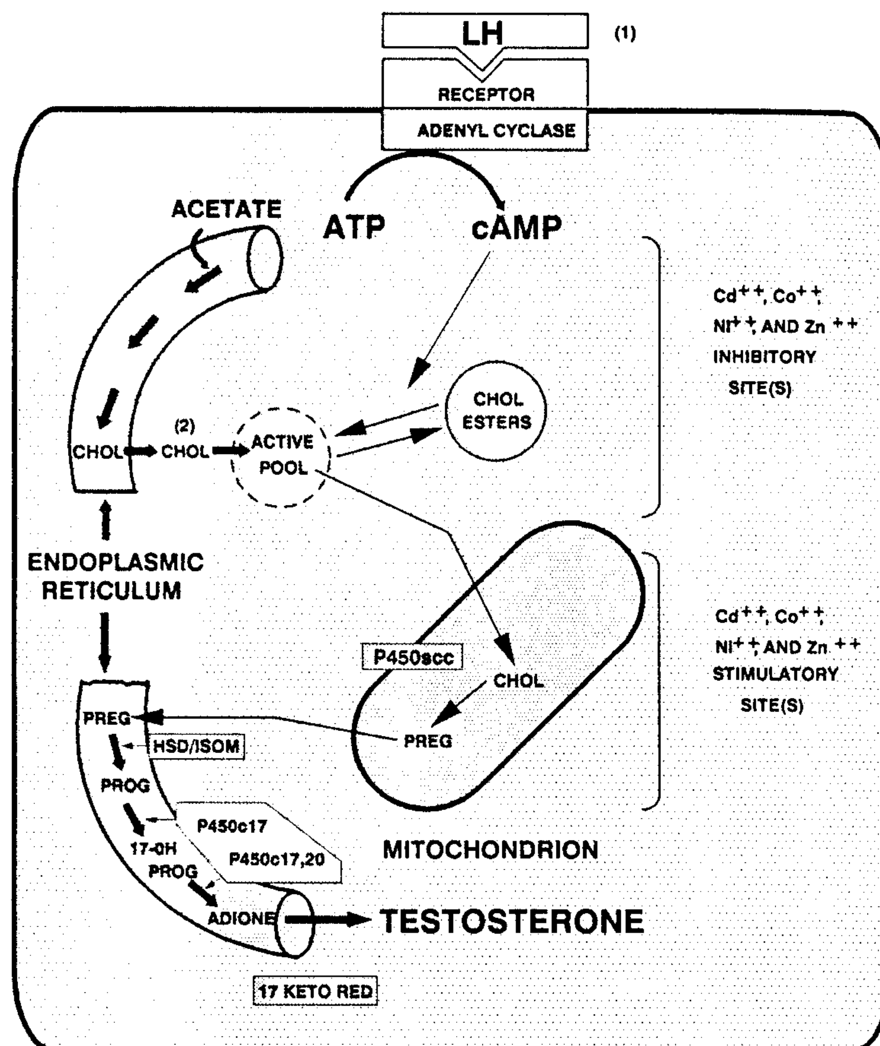


Figure 4-12. Schematic Illustration of Steroidogenesis in the Leydig Cell and the Sites of Metal Cation Toxicity

Notes: *This study investigated the toxicity of metal cations on the responsiveness of an interstitial cell preparation to produce and secrete testosterone. In addition, it characterized the cellular site(s) of action of some of the cations by determining their effect on hCG or cAMP stimulation, as well as the effect on various steroidogenic pathway enzymes by adding various specific substrates e.g., 20α-hydroxycholesterol (HCHOL), pregnenolone (PREG), progesterone (PROG), 17α-hydroxyprogesterone (HPROG) and androstenedione (ADIONE).*

Source: Laskey and Phelps, 1991, page 304.

Purified Leydig cells have been used often to characterize toxicant effects on steroidogenic hormone production and secretion. Examples of experimental studies that used isolated and cultured Leydig cell preparations for measuring steroidogenesis are summarized in Table 4-7.

Table 4-7. Representative Studies of the *In Vitro* Isolated and Cultured Leydig Cells (Purified)

Animal	Treatment/ Stimulant	Response	Reference
Adult male CD rats	ammonium perfluoro-octanoate @ 100 to 1000 μM/2 IU hCG	↓hCG-stimulated testosterone production (IC_{50} approx. 200 μM) cytotoxicity ≥ 750 μM ↓estradiol (0-12 hrs) and ↑basal and hCG-stimulated estradiol production	Biegel et al., 1995
Adult male SD rats	lead acetate @ 100, 250, or 500 μM / 50 mIU/mL hCG	↓hCG-stimulated testosterone and progesterone production ↓Immunohistochemical staining for P450c17, $P450_{SCC}$, 3β-HSD distended smooth ER with fewer anastomoses	Thoreux-Manlay et al., 1995
Adult male SD rats	chloroethylmethane-sulfonate (CEMS) @ 3 to 10,000 μM/100 mIU/mL hCG ethane dimethane-sulfonate (EDS) @ 3 to 10,000 μM/100 mIU/mL hCG	↓testosterone production @ ≥3000 μM (EC_{50} = 2200 μM) ↓testosterone production @ ≥300 μM (EC_{50} = 445 μM)	Klinefelter et al., 1994
Adult male SD rats	EDS @ 100, 200 or 500 μM/100 mIU hCG EDS/5 mM db-cAMP EDS/5 μM 20α-hydroxycholesterol EDS/2 μM pregnenolone	↓hCG-stimulated testosterone production (EC_{50} = 370 μM) ↓db-cAMP-stimulated testosterone production (EC_{50} = 370 μM) Maintained testosterone production Maintained testosterone production	Klinefelter et al., 1991
Adult male SD rats	EDS @ 10 to 10,000 μg/mL /100 ng/mL LH	↓LH-stimulated testosterone production (EC_{50} = 94 μg/mL)	Kelce et al., 1991

4.4.1.5. Distinguishing Features of Isolated and Cultured Leydig Cell Methods.

Stability

The Leydig cell preparation is incubated for 4 to 6 hours but has remained stable for as long as 48 hours (Biegel et al., 1995; Thoreux-Manlay et al., 1995). The stability of the cell preparation is evaluated by measuring whether the hCG continues to stimulate steroid hormone production and secretion. For example, a study using Leydig cell cultures that did not produce a linear amount of pregnenolone over a 24-hour period gave different results than those obtained in a 3-hour culture with linear production of pregnenolone (Rommerts et al., 1988; Klinefelter et al., 1991).

Standardization

Leydig cell isolation requires screening and specifying collagenase concentration and the duration of incubation in the collagenase (by lot), because these are critical factors that affect the number of viable Leydig cells obtained in the preparation. Other factors that require standardization include percent cell viability, maximal hCG stimulation of steroid hormone production, ratio of hCG-stimulated over hCG-unstimulated steroid hormone production, and linearity of steroid hormone production over the incubation period. Many parameters require standardization in each phase of the Leydig cell preparation and clean-up, e.g., media flow rate during elutriation as the dissociated Leydig cells are harvested, concentration of cells during loading, dilution volume of cells in the loading chamber/bubble trap, and the loading flow rate. In addition to technical issues, there are experimental design issues that affect whether the comparisons between and among experiments conducted between and among laboratories at different times can be made, e.g., block design (Laskey and Phelps, 1991).

Sensitivity

Leydig cell preparations that are less than 95 percent purified include debris and other cells that reduce the method's sensitivity (increased variance). Leydig cell viability can affect sensitivity of the assay and, therefore, requires incorporation of a viability assay in the method. The method allows for assessment of substances at the µM level. Leydig cell preparations have been described as more sensitive than the *in vivo* method (Klinefelter et al., 1994). It can appear that the Leydig cell preparation does not respond when in actuality the substance being tested, e.g., lead acetate, has a lag time (Thoreux-Manlay et al., 1995). Also, the preparation allows the potency of different substances to be assessed, e.g., EDS vs. CEMS (Klinefelter et al., 1994). Finally, the purified Leydig cell preparation will not take into account interactions between this cell type and surrounding cells, e.g., Sertoli cells, and other paracrine factors in the whole testicle.

Specificity

Purified Leydig cells respond to standard stimulators at standard concentrations, e.g., LH (0.1 and 1 ng), 22(R)-hydroxy-cholesterol (0.2 ng), 8-bromoadenosine 3', 5' - cyclic monophosphate (0.1 mM)

(Salva et al., 2001). Negative controls have a response that is similar to the vehicle control with or without hCG stimulation, e.g., sodium acetate vs. medium (Thoreux-Manlay et al., 1995).

Metabolic Activity None.

Equipment Leydig cell preparations require specialized equipment. For example, the elutriation system can require customized manufacturing, limited availability, and high costs.

Training A high level of training and experience are needed to isolate the Leydig cells and ready the cell preparation for testing. In addition, training and experience are needed to maintain cell viability and to evaluate the cells for continued testing.

Sample Scheduling Leydig cell isolation utilizes about six animals, 12 testis, per experiment (Klinefelter et al., 1993). A full day is needed for cell preparation. Evaluating cell viability, testing with toxicants, and measuring selected endpoints requires additional time.

Animal Usage The isolation of cells for use as an assay significantly reduces the number of animals used because a number of runs can be conducted from a single set of gonads. The isolated cell assay uses the lowest number animals of the assays that use animals to obtain gonadal organs.

4.4.1.6 Conclusion. The isolated Leydig cell preparation is a sensitive and effective procedure for evaluating the effect of a substance on steroid hormone production and secretion, as well as to investigate the mechanism of action of a substance. However, the method requires extended time, relatively high costs, and laborious effort to isolate and purify the Leydig cells. Thus, the procedure needs to be carefully evaluated with respect to the objectives and characteristics of the screen that is desired for identifying substances that alter steroidogenesis.

4.4.2 Isolated Granulosa Cell Culture

4.4.2.1 Scope of the Method. The isolated granulosa cell culture procedure can be used to evaluate substances for their effect on steroid hormone production and secretion by using immature female rats, thereby removing endogenous influence of the hypothalamic and pituitary hormones, and treating them with a stimulant, e.g., estradiol or DES, for several days (usually five consecutive days), which exogenously induces development of the ovary. The ovary is removed and the granulosa cells are isolated in order for the substance being tested to be incubated directly with the granulosa cells and measurement of any alteration on steroidogenesis. Use of granulosa cells has been extensively reviewed by Hsueh et al. (1984) and Erickson (1983).

Isolated granulosa cells have been used to study the effects of substances on FSH-dependent processes such as steroid hormone production and secretion, pathways involving c-AMP and LH receptor induction (Treinen, 1993); ovulation and the mechanism of substances

that alters or inhibits ovulation (Milne et al., 1987) and biochemical, cellular, and physiological pathways (Leung and Armstrong, 1979), to name a few.

4.4.2.2 Description of the Method. Like the isolated Leydig cell method, preparation of isolated and cultured granulosa cells is a labor-intensive procedure (Treinen, 1993). Briefly, to prepare rat granulosa cell cultures, 18- to 25-day old female rats are obtained and prepared for implantation of DES implants. The implants are inserted between the skin and subcutaneous tissue of the anesthetized animal. Animals are allowed to recover until they are used 4 to 5 days later. An alternative to the DES implants is daily subcutaneous injections of estradiol or DES (1 mg) for 5 days prior to being used for culturing. Removal of ovaries for culturing involves euthanizing and exanguinating the animal and removing the ovaries using aeseptic techniques. The extraneous tissues are cut away from the ovary, which is then placed into a sterile beaker containing warmed medium. The process is repeated until all ovaries are collected (up to approximately 50 ovaries per beaker can be processed at a time).

The next steps disperse the granulosa cells from the ovary and prepare the cells for usage. The ovaries are washed and poured onto a flat-bottomed culture plate. Each ovary is teased apart manually with a needle to release the granulosa cells. The medium containing the granulosa cells is transferred to a centrifuge tube, fresh media added, and the suspended cells are centrifuged. The pelleted cells are resuspended in fresh media. An aliquot is removed and checked for viability using the Trypan blue exclusion procedure and a hemocytometer to calculate the number of viable cells in the culture. The cells are then split to obtain approximately 2,500,000 cells/mL and the tubes incubated for 18 to 24 hours to allow the cells to completely attach. Next, the medium is poured off, leaving the granulosa cells in a ring on the bottom of the tube (non-granulosa cells, e.g., theca cells, constitute less than 10 percent of the cells retained). Culture tubes are better than culture plates for steroid production (Carnegie et al., 1988). After 24 hours, the medium is changed at least every 72 hours, antibiotics added, e.g., penicillin and streptomycin, to reduce bacterial contamination. Viability is assessed with Trypan blue exclusion and ATP measurements.

4.4.2.3 Experimental Design Information. The variety of granulosa cell functions that can be assessed requires consideration of the culture conditions and times for endpoints (Treinen, 1993). For example, FSH, cAMP, or some other stimulant can be added initially in order to evaluate the effect of a substance on steroid hormone, e.g., estradiol or progesterone, production and secretion. The cultures of stimulant and substance being tested are often incubated for 36 to 48 hours in order to get a measurable concentration of the steroid hormone being analyzed. Measurement of FSH-dependent cAMP accumulation by a toxicant can be measured within 24 hours of culturing (Treinen et al., 1990). The stiumlant, optimal concentration of stimulant, and time for collection of samples for analysis all must be characterized as has been previously described for a given cell preparation in order to ensure that the cells are viable.

Cell viability is assessed using a variety of procedures. Most granulosa cell cultures are used within 48 hours; however, some investigators have reported that the culture system is viable for over 10 days (Azhar et al., 1988). Viability has been tested using such procedures as intracellular ATP concentrations, protein determinations, and the Trypan blue exclusion and neutral red assays (Treinen, 1993). In addition to assessing cell viability after isolation, the cell

preparation should also be assessed for viability after incubating with the substance being tested, especially at the higher concentrations, to ensure that the cells remain intact and functional during the entire testing period.

The stage at which the granulosa cells are harvested for isolation and culturing can be important. The steroid hormone production and secretion from the granulosa cells differs for each hormone, based on the stage of development of the follicle. Nordenstrom and Johanson (1985) studied the capacity of rat granulosa cells that were isolated from follicles at different stages of maturation to produce various steroid hormones. The changes in steroid hormone production at each of these time periods are illustrated in Figure 4-13.

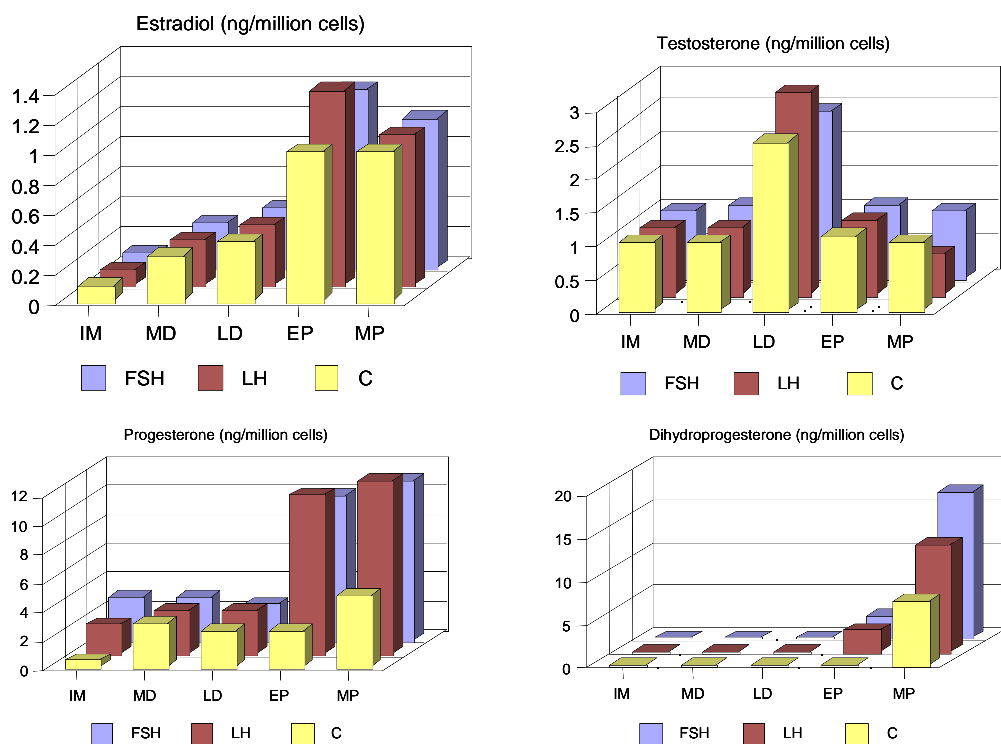


Figure 4-13. Steroid Hormone Production of Rat Granulosa Cells of Follicles at Various Stages of Maturity

Notes: *Accumulation of steroid hormones during 4 hour incubations of rat granulosa cells in the absence (C) or presence of FSH or LH at various estrus stages: IM = immature; MD = mid dioestrus; LD = late dioestrus; EP = early pro-oestrus; MP = mid pro-oestrus. The stages were defined based on the time of sacrifice without a stimulant (control - IM) or after stimulation with PMSG (26 hrs, Mid-dioestrus; 34 hrs, Late dioestrus; 46 hrs, Early pro-oestrus; and 52 hrs, Mid pro-oestrus). During these time points, the follicles ranged in size and type from small, antral follicles to large preovulatory follicles.*

Source: Nordenstrom and Johanson, 1985; page 552.

4.4.2.4 Representative Studies from the Literature

4.4.2.4 Representative Studies from the Literature. Other studies that have been conducted using isolated granulosa cells to evaluate substances for estrogenic or anti-estrogenic effects are summarized in Table 4-8.

Table 4-8. Representative Studies Using the Isolated Granulosa Cell Preparation

Animal	Treatment	Response	Reference
Adult rat (strain unspecfied)	nomegestrol acetate (a synthetic progesterone) @ 0.45, 0.9, or 1.8 mg/L / Stimulated with testosterone (0.5 µM/L)	IC_{50} = 6.85 mg/mL ↓estrogen production @ ≥0.9 mg/mL	Qian et al., 2001
Porcine	cadmium chloride @ 0.2, 10, or 20 ng/mL	↓cell membrane, ER, Golgi apparatus complex integrity; ↑lysosomes and lipid droplets; and ↓progesterone and 17β-estradiol production	Massanyi et al., 2000
Immature hypophysectomized rats	TCDD @ 0.1 to 100 nM / Stimulation with LH or FSH	No effect on estradiol or progesterone	Son et al., 1999
Immature female Alpk/AP Wistar rats (primed with DES)	R151885 (substituted triazole) @ 0.1, 1, or 10 µM / FSH @ 100 ng/mL and testosterone @ 10 µM	↓FSH- and testosterone-stimulated estradiol production; no effect on progesterone	Milne et al., 1987

4.4.2.5 Distinguishing Features of the Isolated Granulosa Cell Culture Method.

Stability Isolated granulosa cell cultures are routinely used for 48 hours, and some investigators have reported cell viability for over 10 days (Azhar et al., 1988).

Standardization This procedure is not standardized and it would be very difficult to do so because of the number of variables. For example, standardization of this method involves many of the same issues that make isolated Leydig cell preparation methods difficult to standardize. Items that are unique to the isolated granulosa cell preparation include the stage of maturity of the follicle and granulosa cells, type and amount of stimulant (DES, estradiol, PMSG), and degree of "contamination" with thecal cells.

Sensitivity The isolation of granulosa cells does not require the use of collagenase or proteolytic enzymes, which can enhance the response of substances, since the membrane-bound receptors and other cellular proteins would not be digested (Treinen, 1993). However, the sensitivity can be compromised in that it does not take into account the interaction that can take place between the surrounding theca interna cells, as well as the oocyte, or

paracrine factors in the whole follicle.

Specificity The granulosa cell preparation steroidogenic response can be antagonized by factors and substances in the serum (Orly et al., 1996; Nothnick and Curry, 1996). Also, residual cells can have an effect. Corpus luteum angiogenic factor, also termed fibroblast growth factor (FGF), inhibits FSH's stimulation of estrogen production and induces LH receptor synthesis. Also, at suboptimal concentrations, FGF enhances the synthesis of progesterone (Baird and Hseuh, 1986).

Metabolic Activity None.

Equipment Standard and specialized laboratory equipment are required. Specialized equipment primarily involves laminar flow hoods for maintaining a sterile work environment when isolating and handling the cells.

Training. Requires general and special laboratory training.

Sample Scheduling Isolation of the granulosa cells takes 1 day, and then the cells are allowed to incubate and attach to the test tube, which takes another 18 to 24 hours. Afterwards, the cell preparations are usually incubated for 48 hours with the stimulant and the substance being tested before samples are collected for analysis. Investigators prefer using the isolated cells within 48 hours of isolation. Thus, the cell preparation is labor-intensive to obtain, and the cells are generally used promptly after collection for best results.

Animal Usage The isolation of cells for use as an assay significantly reduces the number of animals used because a number of runs can be conducted from a single set of gonads. The isolated cell assay uses the lowest number animals of the assays that use animals to obtain gonadal organs.

4.4.2.6 Conclusions. The isolated granulosa cell preparation is a sensitive and effective procedure for evaluating the effect of a substance on steroid hormone production and secretion, as well as to investigate the mechanism of action of a substance. However, as with the Leydig cell methods, extended time, relatively high cost, and laborious effort are needed to isolate and purify the granulosa cells. Thus, the procedure needs to be carefully evaluated with respect to the objectives and characteristics of the screen that is desired for identifying substances that alter steroidogenesis.

4.5 Cell Line Methods (*In Vitro*)

Cell lines can be used to measure the effect of a substance on the steroidogenic pathway. This is made possible by the “immortalization” of Leydig cells from Leydig cell tumors. However, cell lines obtained from tumor cells can be different from normal cells. For example, most Leydig cell lines do not retain their capacity to be stimulated by LH/hCG. The use of cell lines requires the researcher to characterize the cell line and evaluate its capacity to simulate the biochemical pathways found in a nontransformed, differentiated cell. Once the differences are characterized and understood, then a given cell line has been shown to prove useful for screening, identification, and characterization of xenobiotics with endocrine-altering properties. This subsection provides an overview of this method.

4.5.1 Scope of the Method

Cell lines offer another *in vitro* method for studying the effects of substances on the steroidogenic pathway. In regard to steroidogenesis, cell lines have been used to purify and identify proteins that affect steroidogenesis (Boujrad et al., 1995); second messenger activation (Pereira et al., 1987); regulation of receptors (Rebois and Fishman, 1984); gene regulation (Payne et al., 1992); cholesterol transport (Freeman, 1987); paracrine factors that stimulate steroidogenesis (Boujrad et al., 1995); and steroidogenic enzyme characterization (Clark et al., 1994; Walsh et al., 2000b). A review of immortalized Leydig cell lines is provided by Hoelscher and Ascoli (1996).

4.5.2 Description of the Method

Figure 4-14 shows a schematic diagram for a cell line assay.

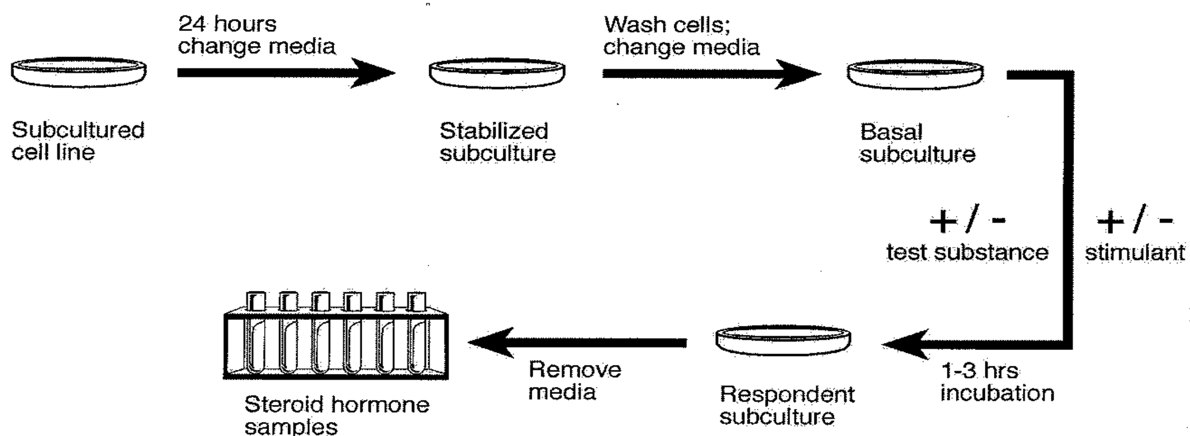


Figure 4-14. Schematic Diagram for a Cell Line Assay

The MA-10 cell line method will be used as an example of the procedure used for this assay (Chaudhary and Stocco, 1989). Briefly, stock cultures of the cell line are grown in tissue culture flasks and, when prepared for use, are subcultured into culture dishes or plates. A medium used for the stock or initial subculture preparation is modified Waymouth's MB752/1 medium containing HEPES (20 mM), sodium bicarbonate (1.2 g/L), and heat-inactivated horse serum (15%) at pH 7.4. After 24 hours, the MA-10 cells are washed with PBS and the serum changed to bovine serum albumin (BSA, 1 mg/mL). Next, the cells are treated with or without stimulant, e.g., hCG or cAMP, and the substance being tested. The cells are incubated, and at various time intervals, samples are removed for steroid hormone analysis.

4.5.3 Experimental Design Information

4.5.3.1 Immortalized Leydig Cell Lines. Several types of cell lines can be used to study the effects of substances on steroidogenesis. Most of the cell lines come from rat or mouse Leydig cell tumors. Some cell lines derived from Leydig tumors and the species/strain from which they were derived are listed below (Hoelscher and Ascoli, 1996).

Cell Line Name	Species/Strain	Source[a]
NWL2, NWL15	Rat/SD	--
R2C	Rat/Wistar-Furth	ATCC
LC540	Rat/F344	--
MA-10	Mouse/C57Bl/6	Dr. M. Ascoli Univ of Iowa, Ames, IA
MLTC-1	Mouse/C57B1/6	ATCC
B-1-A-2	Mouse/BALB/c	--
TM3	Mouse/BALB/c	ATCC
I-10	Mouse/BALB/cJ	ATCC
K-9	Mouse/hybrid MA-10 cells and fresh Leydig cells	--

[a] ATCC = American Type Culture Collection.

The Leydig tumor cell lines do not express the full function of a normal Leydig cell. None of these cell lines express the full steroidogenic pathway found in the Leydig cell. In other words, the synthesis of cholesterol—through the several intermediate substrates, and eventually resulting in testosterone formation—does not occur in these cell lines. In most, the final steroid hormone is progesterone or 20α-hydroxyprogesterone. One exception is the K9 cell line, which is capable of producing testosterone. Unfortunately, K9 cells require frequent and routine subcloning (Finaz et al., 1987). Even though some of the steroidogenic pathway is absent in the Leydig tumor cell lines, that part of the pathway that is present appears to be very similar to the pathway of normal cells. This is especially true of the MA-10 and MLTC-1 cells, as much of their pathway has been characterized (Ascoli, 1981; Rebois, 1982).

The Leydig tumor cell lines also vary with regards to their response to stimulation. The MA-10 and MLTC-1 have receptors that respond to LH or hCG and exhibit cAMP synthesis when stimulated (Ascoli, 1981; Rebois, 1982). In contrast, R2C cells maintain the capacity to synthesize steroid hormones constitutively in a hormone-independent manner (Papadopoulos et al., 1997). The characterization of the receptors, binding, and coupling of the

receptor to second messenger activity are important considerations regarding the use of the cell line for evaluating the effect on steroidogenesis. Such factors have an effect on the sensitivity of the cell line relative to normal cells. For example, Hoelscher and Ascoli (1996) compared the bound receptor occupancy with maximal steroid hormone production for MA-10 cells and normal Leydig cells. The former exhibited maximal stimulation when 60 to 70% of the receptors were occupied, whereas the latter required only about 1% occupancy. Further study demonstrated that the MA-10 and normal Leydig cells had similar hCG binding properties and hCG-induced cAMP stimulation, but differed in regard to the levels of cAMP needed to stimulate steroidogenesis.

Another design consideration is the cell line media. The media used to culture the cells differs from cell line to cell line. However, the media is not a variable in the experimental design; rather, the media for optimal growth and activity is used throughout the experiment. Examples of media used for specific cell lines include: Waymouths MB752/I, 20 mM HEPES, and horse serum for MA-10 cells and RPMI-1640 and fetal calf serum for ML TC-1 cells (Chaudhary and Stocco, 1989; Rebois, 1982). Thus, the culture medium is important for optimal culturing of the cells and should be determined for the particular cell line being used.

4.5.3.2 Adrenocortical Carcinoma Cell Line. Steroidogenesis occurs in the adrenal glands, as well as the gonads. Much of the same pathway found in the gonads, which synthesizes cholesterol into androgens and estrogens, is also found in the adrenal gland, which synthesizes cholesterol into glucocorticoids. As described for the steroidogenic pathway in the testis/ovary (Figure 3-6), the glucocorticoid pathway converts cholesterol to pregnenolone via $P450_{SCC}$ and pregnenolone to progesterone via 3β-HSD and progesterone to 17-hydroxyprogesterone via P450c17. These three enzymes are common to both the androgen/estrogen and glucocorticoid steroidogenic pathways. After formation of 17-hydroxyprogesterone, the glucocorticoid pathway includes two additional reactions involving 21-hydroxylase and 11β-hydroxylase, which results in the formation of 11-deoxycortisol and finally the end-product glucocortico-steroid hormone, cortisol. Thus, except for the last two steps, common enzymes and substrates are found in the steroidogenic pathways of the gonads and adrenal glands.

The commonality of the androgen/estrogen and glucocortiocoid steroidogenic pathways is the basis for considering an adrenocortical carcinoma cell line as an alternative to an immortalized Leydig cell line. One such adrenocortical carcinoma cell line is the human NCI H295 cell line. Although the cell line has not been completely characterized with respect to the complete steroidogenic pathway as described in Section 3, investigators have shown that the cell line includes a cyclic-AMP second messenger system, as well as the steroidogenic enzymes $P450_{SCC}$ and P450c17 (Rodriguez et al., 1997; Fassnacht et al., 2000). These components of the pathway have been altered following treatment of the H295 cells with aminoglutethimide, metyrapone, and etomidate (Fassnacht et al., 2000). Endpoints that have been measured to evaluate the effect of a test substance on steroidogenic function of these cells include 17-hydroxyprogesterone and cortisol.

4.5.3.3 Cell Line Properties. A summary of the properties of the cell lines described above is included in Table 4-9, showing whether the research cited did (✓) or did not (x) confirm the presence of the component in the respective cell line. The properties listed in the table are those components that comprise the steroidogenic pathway found in the testis/ovary and, for the H295 cell line, found in the adrenal glands.

Table 4-9. Summary of Immortalized Cell Line Properties [a]

Component of Steroidogenic Pathway	Cell Line						
	Rat		Mouse				Human
	R2C	LC540	MA-10	mLTC-1	TM3	I-10	H295R
LH and/or hCG Stimulation	X (Stocco, 1992)	--	✓ (Stocco & Chen, 1991)	✓ (Rebois, 1982)	✓ (Taylor et al., 1997)	--	--
	Signal Transduction						
cAMP	X / ✓ (Stocco, 1992; Freeman, 1996)	✓ (Pignataro et al., 1992)	✓ (Stocco & Chen, 1991)	✓ (Manna et al., 2001a)	✓ (Taylor et al., 1997)	✓ (Pignataro et al., 1992)	✓ (Fassnacht et al., 2000)
Ca^{2+}	--	--	✓ (Ramnath et al., 1997)	✓ (Manna et al., 1999)	✓ (Adebanjo et al., 1998)	--	--
Cl^{-}	--	--	✓ (Ramnath et al., 1997)	✓ (Panesar, 1999)	--	--	--
Arachidonic Acid	--	--	✓ (Wang et al., 2000)	--	--	--	--
	Intracellular Cholesterol Storage, Release, and Transport						
cholesterol storage/release	✓ (Freeman, 1996)	--	✓ (Dees et al., 2001)	--	--	--	--
StAR protein	✓ (Stocco et al., 1995)	--	✓ (Stocoo et al., 1995)	✓ (Manna et al., 2001b)	--	--	--
	Enzymes						
P450scc	✓ (Stocco et al., 1995)	✓ (Pignataro et al., 1992)	✓ (Stocco et al., 1995)	--	--	✓ (Pignataro et al., 1992)	✓ (Fassnacht et al., 2000)
3B-HSD	✓ (Teixeira et al., 1999)	--	✓ (Teixeira et al., 1999)	✓ (Manna et al., 2001b)	--	--	--
P450c17	✓ (Teixeira et al., 1999)	--	✓ (Teixeira et al., 1999)	✓ (Manna et al., 2001b)	--	--	✓ (Rodriguez et al., 1997)
17KSR	--	--	--	--	--	--	--

Table 4-9. Continued

Component of Steroidogenic Pathway	Cell Line						
	Rat		Mouse				Human
	R2C	LC540	MA-10	mLTC-1	TM3	I-10	H295R
5α-reductase	--	--	✓ (Rommerts et al., 2001)	--	--	--	--
aromatase	✓ (Doody et al., 1990)	--	--	--	--	--	--
	End-Product Hormones						
progesterone	✓ (Stocco & Chen, 1991)	--	✓ (Rommerts et al., 2001)	✓ (Rebois, et al., 1982)	--	✓ (Taylor et al., 1997)	--
20α-hydroxy-progesterone	--	--	✓ (Gocze & Freeman, 2000)	✓ (Rebois, 1982)	--	--	--
testosterone	--	✓ (Steinberger et al., 1970)	--	--	--	--	--
estradiol	--	✓ (Steinberger et al., 1970)	--	--	--	--	--
17-hydroxy-progesterone	--	--	--	--	--	--	✓ (Fassnacht et al., 2000)
cortisol	--	--	--	--	--	--	✓ (Fassnacht et al., 2000)

[a] No information could be found in the literature for the NWL2, NWL15, and B-1-A-2 cell lines.
✓ Information found, and this component is present in the cell line.
x Information found, and this component was not shown to be present in the cell line.
– No information found.

4.5.4 Representative Studies from the Literature

Chaudhary and Stocco (1989) used the MA-10 cell line to characterize the inhibition of steroidogenesis by ketoconazole. Some of the results are illustrated in Figure 4-15.

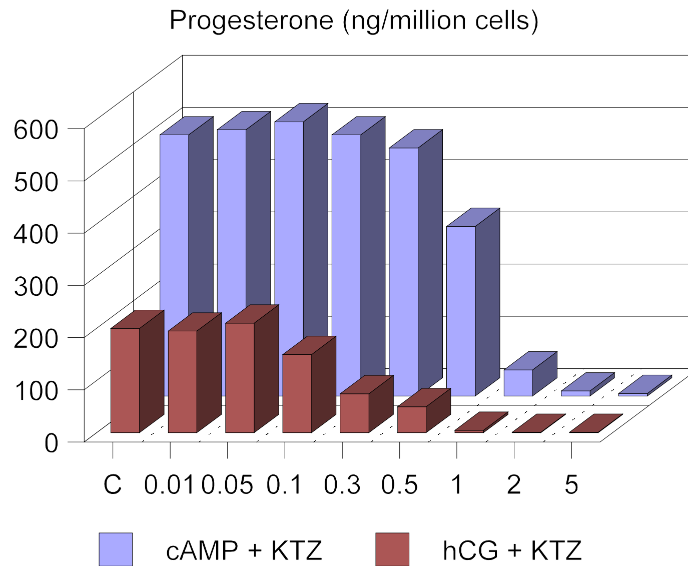


Figure 4-15. Example Data from an MA-10 Cell Line Assay

Note: *The effect of different concentrations (µM) of ketoconazole (KTZ) on hCG- or cAMP-stimulated progesterone production in MA-10 cells was determined during a 3-hour incubation. Cells were grown in 12-well tissue culture platres in Waymouth's medium. The concentration of hCG was 34 ng/mL and of cAMP was 1 mM. Wells without KTZ were used as control (C). Aliquots were removed after three hours from each of the wells and assayed for progesterone by RIA. KTZ inhibited MA-10 cell production of progesterone when KTZ concentrations were ≥0.1* µM *in the hCG-stimulated cells and ≥0.5 µM in the cAMP-stimulated cells.*

Source: Chaudhary and Stocco (1989)

Additional examples of cell lines used in experimental studies for measuring steroidogenesis are summarized in Table 4-10.

4.5.5 Distinguishing Features of the Cell Line Method

<u>Stability</u> Cell lines remain viable for at least 3 to 4 hours, which was the time needed to assess the effect of a substance on steroid hormone production, e.g., ketoconazole using the MA-10 cell line (Chaudhary and Stocco, 1989).

Table 4-10. Representative Studies Using a Cell Line Method

Cell Line	Treatment	Response	Reference
MA-10 cells	carbonyl cyanide m-chlorophenylhydrazone (m-CCCP, protonophore) valinomycin (ionophore)	Inhibited StAR protein & $P450_{SCC}$	King et al., 1999
R2C & H540 Leydig tumor cells	None	Characterized proximal promotor region of mRNA for aromatase	Young and McPhaul, 1997
MA-10 cells	STP (a steroidogenic stimulatory protein isolated from cultured rat Sertoli cell medium) @ 0.01 to 1µg/mL conconavalin A @ 0.01 to 50 µg/mL	↑progesterone @ > 0.01 µg/mL ↑progesterone @ > 1 µg/mL	Boujrad et al., 1995

Standardization A standard cell line assay has not been established. Also, down-regulation of receptors can occur when incubated with LH, hCG, cAMP, epidermal growth factor (EGF), or phorbol esters (Hoelscher and Ascoli, 1996). MA-10 cell progesterone production can be inhibited at µM concentrations of a substance (Chaudhary and Stocco, 1989).

Sensitivity Each cell line must be characterized as to its capacity to be stimulated by LH, hCG, or cAMP. Some cell lines are not stimulated to increase steroid hormone production by these hormones or intracellular second messengers. In addition to these common stimulators, other biochemical messengers, e.g., AlF, a universal activator of G protein, do not initiate intracellular biochemical pathways, which is attributed to receptor density.

Specificity Cholera toxin can increase steroidogenesis in Leydig tumor cell lines (Ascoli, 1981). Other endogenous substances that can stimulate steroidogenesis in MA-10 cells are endothelin-1 and EGF (Hoelscher and Ascoli, 1996).

Metabolic Activity None.

Equipment Standard and specialized laboratory equipment. Specialized laboratory equipment is required for cell line culturing, e.g., laminar flow hood, -80° freezers, liquid nitrogen storage, incubators, etc.

Training Standard and specialized laboratory training. Specialized training involves cell line culturing techniques.

Animal Usage This assay does not use animals.

4.5.6 Conclusion

Leydig tumor cell lines have been used to study the effects of substances on steroid hormone production. These cell lines do not express the complete steroidogenic pathway. Although incomplete, the pathway that is present is similar in many ways to the steroidogenic pathway in the normal Leydig cell. For this reason, it can be used to assess the effect of a substance on steroid hormone production when stimulated with LH, hCG, cAMP, etc.

4.6 Basis for Selection of a Steroidogenic Screening Method

The first objective of this DRP is to determine whether a method can be selected, which could serve as the most promising screen, so as to identify substances that have inhibitory or stimulatory effects on the production of testicular and ovarian steroidogenic hormones. To achieve this end, the methods that are used to identify such substances, examples of data generated by each of the methods, and distinguishing features of each method were reviewed (Subsections 4.1 to 4.5). The information that was presented about each method has been compared with criteria that define an optimal screen. Out of this comparison and critique, one of the methods was selected for recommendation to be used as a screening tool. The following subsections provide the basis for this selection.

Several comparisons were made in order to narrow down the choices and select the most promising assay to be used as the screen. These comparisons include:

- Method type comparisons, i.e., *In Vivo* vs. *Ex Vivo* vs. *In Vitro*
- Gender comparisons, and
- Different method-subtype comparisons, e.g., sectioned testes vs. isolated/cultured Leydig cell preparation.

4.6.1 Method Type Comparisons

The purpose of this subsection is to provide a review of the literature for some of those studies that tested the same substance for its effect on steroidogenesis using two or more of the methods described in the preceding subsections. In this way, a side-by-side comparison of the relative outcome can be made for the various methods and the strengths/weaknesses of each method relative to one another will be made more apparent. Although many different investigators have tested a given substance using the different methods, the information presented here focuses on those studies where the same investigator tested the same substance but used different methods. In so doing, a better comparison of the methods' outcome (rather than the differences between laboratories) can be made. The examples presented below will also identify some of the strengths and weakness of each method and thereby allow some conclusions to be drawn regarding the relative benefits of each method.

4.6.1.1 *Ex Vivo* vs. *In Vitro*. Numerous studies are available in the literature that make it possible to compare the outcome of *ex vivo* and *in vitro* methods. In fact, the natural progression of a scientific investigation involves using both methods in order to evaluate the

response in an intact animal followed by an attempt to characterize the test substance's mechanism and site of action using isolated organs. Klinefelter et al. (1994) used an *ex vivo* method (four-day in-life phase followed by isolation and testing sectioned testis) and an *in vitro* method (purified Leydig cell preparation) to evaluate the effects of chloromethylmethane-sulfonate (CEMS) on steroidogenesis. The results are illustrated in Figure 4-16.

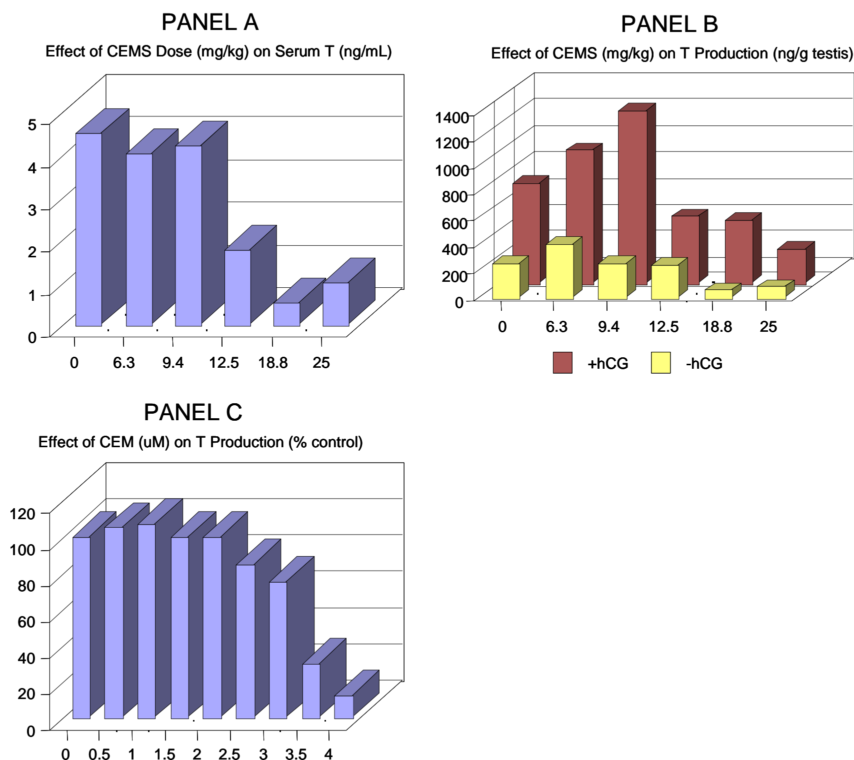


Figure 4-16. Example of *Ex Vivo* vs *In Vitro* Data

Source: Klinefelter et al. (1994)

As can be seen from the figures, a dose-related decrease in serum testosterone was observed at dosages ≥12.5 mg/kg of CEMS (Panel A). Likewise, hCG-unstimulated and hCG-stimulated testosterone production using sectioned testes was reduced at dosages ≥18.8 and 25 mg/kg of CEM, respectively (Panel B). Agreement with these *ex vivo* results was observed when CEMS was incubated with isolated and cultured Leydig cells. In the *in vitro* purified Leydig cell preparation, a dose-dependent decrease in hCG-stimulated testosterone production was measured (Panel C). It was from the significant dose-related decrease in serum testosterone (*ex vivo*) that the investigators deduced that the chemical was affecting steroidogenesis. They confirmed this finding with the *in vitro* method. In addition to characterizing the mechanism and site of action of CEMS, the investigators were able to identify differences in sensitivity. For example, serum testosterone decreased significantly at 12.5 mg/kg, which was a lower dose than that required to significantly decrease testosterone production from the testis (18.8 to 25 mg/kg). The authors concluded that LH-stimulation of Leydig cells is compromised *in vivo*. Finally,

although use of these two methods both resulted in decreased testosterone production, there are times when the two methods produce opposite effects, as described below.

Dissimilarities have also been observed between *in vivo/ex vivo* and *in vitro* results. Phelps and Laskey (1989) used an *ex vivo* method (21-day in-life phase and an interstitial cell preparation) and reported that cadmium inhibited testosterone production when the Leydig cells were stimulated by hCG, cAMP, 20α-hydroxycholesterol, or pregnenolone. But when cadmium was incubated with interstitial cells from untreated rats (*in vitro* method), cadmium stimulated testosterone production (Laskey and Phelps, 1991). The investigators attributed the different responses to 1) changes that could have occurred during the 21-day interval between cadmium treatment and interstitial cell evaluation, 2) cadmium-induced vascular damage in the testis and the subsequent necrosis that could ensue, and 3) varying mechanisms of action for cadmium that become apparent only when using the two different methods. In regard to this last explanation, the investigators speculated that at low concentrations, only one binding site could have been affected which causes stimulation; whereas at higher concentrations, cadmium may bind to a second site, which causes inhibition. Thus, the concentration tested *in vitro* may not be relevant to the concentration at the site of action following an *in vivo* exposure. However, it is through the response of the *in vitro* method that possible dual binding sites and further characterization of metal toxicity were able to be postulated.

4.6.1.2 *In Vivo* vs. *Ex Vivo* vs. *In Vitro*. Investigators have utilized *in vivo*, *ex vivo*, and *in vitro* methods to characterize the steroidogenic effects of a substance. Biegel, et al. (1995), investigated the effect of ammonium perfluorooctanoate (C8) following a 14-day in-life exposure phase (*in vivo*). They also treated the animals with the same dosing regimen and, at termination, isolated and cultured purified Leydig cells (*ex vivo*). Finally, these investigators isolated and cultured purified Leydig cells from untreated animals for incubation with C8 (*in vitro*). Some of the results for this study are illustrated in Figure 4-17.

These results show that C8 produced no effect on serum testosterone but decreased testicular interstitial fluid testosterone based on the *in vivo* method (Panels A and B). Using the *ex vivo* method, C8 increased testosterone production in hCG-stimulated cells (Panel C). Finally, in the *in vitro* experiment, C8 produced a dose-dependent decrease in hCG-stimulated testosterone production (Panel D). By using all three methods, these investigators were able to show that C8 directly inhibits release of testosterone from Leydig cells (*in vitro*). Furthermore, using the *ex vivo* method, they were able to demonstrate that the effect of C8 was reversible. The *in vivo* data supported their hypothesis that estradiol may modulate growth factor expression in the testis.

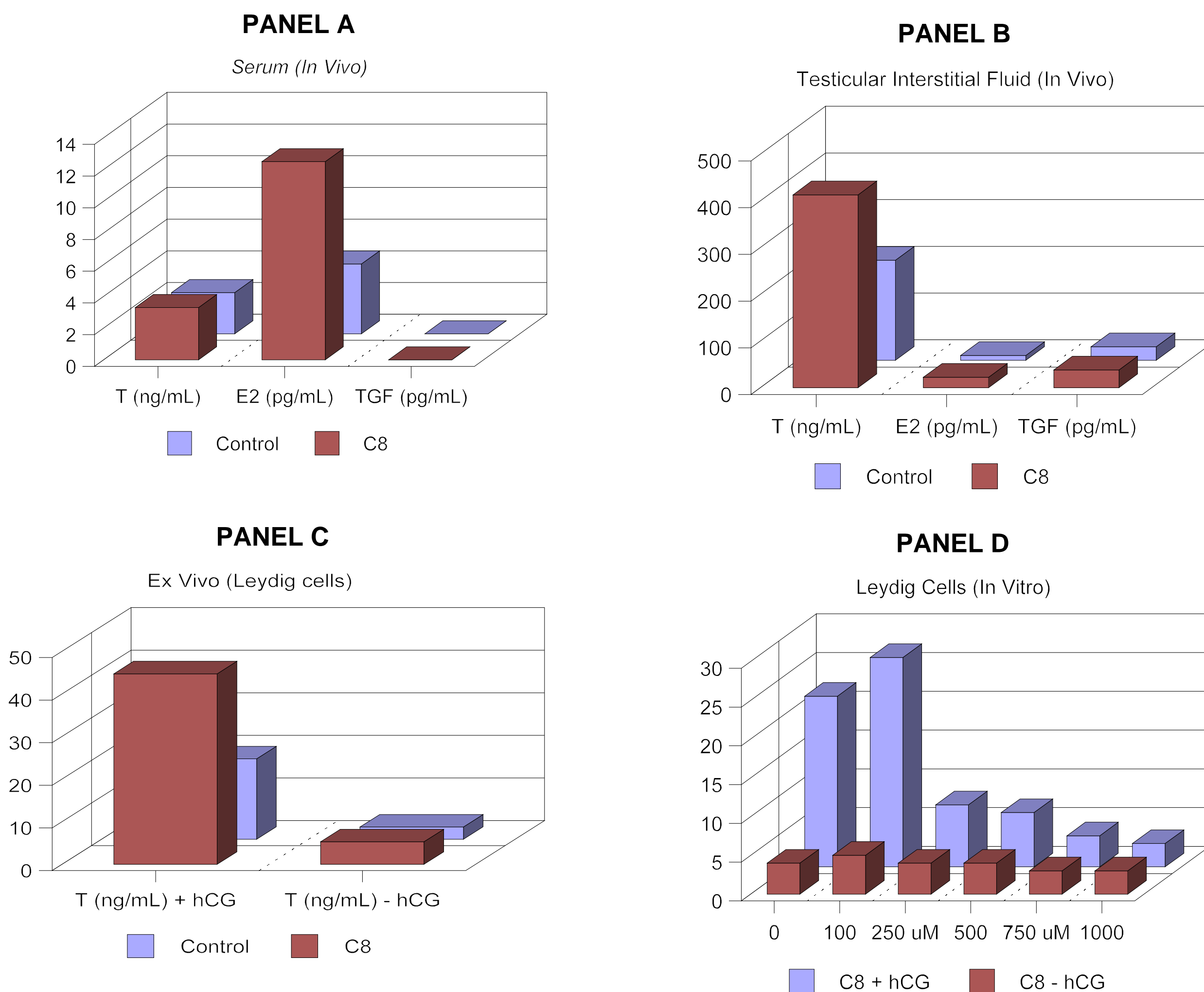


Figure 4-17. *In Vivo* vs *Ex Vivo* vs *In Vitro* Results for C8

Source: Biegel et al. (1995)

4.6.1.3 *In Vitro* vs. *In Vitro*. A comparison of results from different *in vitro* procedures can also be extracted from the literature for a single substance and a given group of investigators. Kelce, Klinefelter, Laskey, et al. have reported on the steroidogenic effects of ethane dimethanesulfonate (EDS) using sectioned testis (Laskey et al., 1994), perfused testis (Kelce et al., 1991), and purified Leydig cells (Kelce et al., 1991; Klinefelter et al, 1991) in the adult rat. The EC_{50} values obtained for EDS by using these different *in vitro* procedures were:

In Vitro Procedure	EC_{50} (µM EDS)
Sectioned Testis	336
Perfused Testis	430
Purified Leydig Cells	370 to 430

The results obtained for EDS using the different procedures were in good agreement with one another regardless of which *in vitro* procedure was utilized. These investigators also made comments regarding the strengths and weaknesses of these procedures. With regard to technical difficulty, the most rapid and easiest procedure was using the sectioned testis. This procedure was the least disruptive of the cytoarchitecture. In addition, direct application and maintenance of the cytoarchitecture was also possible with the perfusion procedure, but it was much more technically difficult. Finally, while direct application and characterization of the mechanism of action was more feasible with the purified Leydig cell preparation, the cytoarchitecture was not maintained, enzyme was needed, which disrupts paracrine interactions, and the procedure was labor-intensive.

4.6.1.4 Conclusions Regarding Method-Type Comparisons. The *in vivo, ex vivo,* and *in vitro* methods all provide a means to evaluate steroid hormone production in the testis or ovary following exposure to a substance. However, while these methods complement each other and together provide more information than any one method alone, each method also has unique attributes that favor characterization of the substance in a way that the other method is not able to do. Distinct advantages can be enumerated for the different types of methods. The following list is a compilation of the attributes of *in vivo* and *in vitro* methods (EDSTAC, 1998). Obviously, the *ex vivo* method includes the attributes from both lists.

In Vivo	*In Vitro*
Accounts for Absorption, Distribution, Metabolism, and Excretion (ADME)	Sensitivity to low concentrations (increases detectability)
Well defined, widely used for long period of time	High specificity
Generally accepted in toxicity testing	Low cost
Endpoints are toxicologically relevant and used in risk assessment	Small amount of test substance required
Evaluates a broad range of mechanisms	Procedure can be automated
Comprehensive evaluation of endocrine system	Can utilize high-throughput assays
Comparative perspective to other endpoints	Can test complex mixtures
	Reduces or replaces animal usage

This list of attributes can easily be associated with the specific methods reviewed earlier in Section 4. As was described in subsection 4.1, the ECT is gaining in favor as a tool to investigate the mechanism and site of action of a substance on disrupting the endocrine system. This assay has a fully functional hypothalamic-pituitary-gonadal axis. In addition, the assay retains complete metabolism capabilities of the organism, thereby allowing for pro-drugs and metabolites to be tested. As for the *in vitro* methods described in subsections 4.3 to 4.5 – e.g., whole organ, sectioned testis/minced ovaries, interstitial cell preparation, cell lines – many meet most of the attributes listed above. However, there are some *in vitro* methods, e.g., perfusion, perifusion, purified Leydig cell preparation, that cannot be automated, are costly, do not lend themselves to high sample throughput, etc. Thus, based on these recognized attributes of the *in vivo* and *in vitro* methods for measuring steroidogenesis, a decision can be made regarding whether one method would be preferred over the other by comparing these attributes to the criteria for a screen.

The characteristics of a screen must be considered in order for the most promising method to be chosen. A screen is not used to establish a dose response, describe the mechanism of action, or determine the adverse response of a substance on development and/or the reproductive system (Gray et al., 1997). Rather, to evaluate a method for its capacity to be a screen, the following list of criteria can be used:

Inexpensive (low cost)	Predictive level of age, sex, and mammalian systems
Degree of representation of the organ function (cytoarchitecture)	Numerous endpoint options
Short time needed to set-up the method	Reduced animal usage
Fast sample throughput	Standard laboratory technical training required
Standard laboratory equipment required	Specific
Sensitive	Minimal inter-/intra-laboratory variability
Stable preparation (viability)	No chemical solubility/permeability limitations
Can be standardized	Acceptable to the scientific community
Metabolic activation	Minimization of false negatives, i.e., absent
"Acceptable level" of false positives (active *in vitro* but not *in vivo)*	Metabolic activation
Quick and easy to perform	

A decision can now be made that reduces the number of possible methods for consideration. Based upon the attributes of *in vivo* and *in vitro* methods and the criteria of a screen, the *in vitro* method is in better agreement with the criteria for a screen. The information presented in the preceding sections supports this conclusion. For example, all of the *in vivo* assays are more labor-intensive, costly, and require more time and special laboratory skills to conduct, than most of the *in vitro* method assays or procedures. **Thus, based on these criteria and their better alignment with the *in vitro* methods, assays using the *in vitro* method will be given further consideration for selecting one as a screen.**

4.6.2 Gender Comparisons

The *in vitro* methods can be adapted to male and/or female animals. For every procedure designed to evaluate testicular function, there is a co-procedure designed to evaluate ovarian function. For example, a perfusion apparatus exists for the testis as well as the ovary. A sectioned testis procedure exists for the male and a minced ovary procedure for the female. However, with respect to selecting a screen method, it may not be necessary to have both a male and a female screen. Is there a basis for selecting a method from one gender over the other, or are methods from both genders required? This subsection attempts to provide a basis for demonstrating that an *in vitro* method using a single gender may be reasonable.

Distinguishing characteristics of the male and female steroidogenic pathways may be useful for excluding a gender from consideration as a screen. One such distinction occurs with regard to location or cell type. In the male, the steroidogenic pathway is located in the Leydig cell of the testis. The initial substrate (cholesterol) to the primary terminal hormone (testosterone), together with the intermediate substrates and enzymes, are in this cell type (Griffin and Wilson, 1994). There are two steps of the pathway not located in the Leydig cell: the conversions of testosterone to DHT by 5α-reductase and to estradiol by aromatase. These reactions occur in peripheral tissues, as was reviewed in Section 3.

In the female, the steroidogenic pathway is in the ovary but distributed in and among different cell types – the follicular cell, which contains the granulosa and theca cells, and the interstitial cells (Carr and Wilson, 1994). The synthesis of various hormones can differ by cell type at various times. In short, while steroidogenic hormone production occurs in most cell types, each cell type can produce varying amounts of a given enzyme and hormone. This results in some cell types producing more of one hormone than another at varying times, which is associated with the estrus cycle. Thus, the cyclicity of the female is another complicating factor in using the ovary for evaluating the effect of a substance on the steroidogenic pathway. Therefore, since the male steroidogenic pathway is also representative of the female steroidogenic pathway, it may be reasonable to consider using the testis to screen for effects of a substance.

Organ isolation and preparation steps must also be given consideration when considering a screen method. Regarding collection of the organ, the testis is a much simpler organ to remove than the ovary. This is important considering the number of organs that would be needed. In addition, the size of the testis is much larger than the ovary, thereby providing more organ mass per animal for screening substances. This is very advantageous, as it would allow more experiments to be conducted with fewer animals.

Thus, based on these considerations, it is reasonable to use only the testis in the *in vitro* method selected as a screen.

4.6.3 Different Method Sub-Types

The *in vitro* methods described for the male include several different assays or procedures. The male *in vitro* methods included the whole or sectioned testis, perfusion, perifusion, isolated crude and purified Leydig cell preparations, and cell lines. Narrowing these assays down to the most promising one to be used as a screen should, at this point, be based on the criteria for a screen as listed above. Therefore, each of these criteria is evaluated for each of the *in vitro* assays used to assess steroidogenesis. This information has been summarized in Table 4-11 presented at the end of Section 4. (The criteria are further described, defined, and discussed in Appendix C).

Comparison of the assay's relative attributes with the criteria for a screen points out apparent differences among the assays (Table 4-11). Regarding **cost**, the least expensive assays are the simple whole testis, sectioned testis, and cell line assays. A cost estimate for running a

steroidogenic assay in sectioned testis is estimated to be less than $12K per test substance

(EDSTAC, 1998).

A second criterion, **time**, should be given high-level consideration. Two aspects of this parameter were evaluated for comparison—time required for the initial set-up and time to conduct a single experiment (includes organ removal, preparation, testing and sample collection). The whole testis simple incubation, sectioned testis, and crude Leydig cell assays require a relatively short investment in time to obtain the supplies and set-up the laboratory. In contrast, the other assays require specialized supplies, which can take weeks to months to obtain (some perifusion equipment requires customized manufacturing) and, once obtained, require additional time to set up and ensure that they are operational. Furthermore, the actual time needed to conduct the assay and the number of testes preparations and substances that can be tested per day favor the whole or sectioned testes, and crude Leydig cell assays. Of these three, the sectioned testes and crude Leydig cell assays use the organ more efficiently than the whole organ preparation, e.g., 4 times more if using quartered sections, while requiring only a small additional time and effort. Finally, the sectioned testis assay uses less time for preparation relative to the crude Leydig cell preparation.

A comparison of the **laboratory parameters** (level of training and type of equipment) was made. The whole testis simple incubation, sectioned testis, and crude Leydig cell assays can be performed by broad-based, experienced, laboratory-trained technicians and in laboratories equipped with standard supplies and instrumentation for general biology experiments. For the perfusion, perifusion, purified Leydig cells, and cell lines, specialized training and equipment are needed, e.g., laminar flow hoods, special apparatus and training to set up and operate the perfusion or perifusion assays.

Standardization of the assays has not occurred. Standardization of the assay selected for screening would need to be performed by optimization or consensus regardless of which assay was chosen. However, the level of difficulty involved in standardizing the assay is important to consider. As described earlier in Section 4 for each method, the assays have varying numbers of variables that must be controlled in order to generate data that can be compared among and between the laboratories. The least difficult assays to standardize are the whole and sectioned testis assays, as they involve the fewest number of procedures and factors to standardize.

Animal usage is replaced by cells if a cell line assay were to be used. This assay offers the best approach to achieving the goal of reducing, refining, or replacing animals for use in toxicity studies. Fewer animals are used in the Leydig cell preparations and the sectioned testes assay than the other assays.

The degree that the organ's **cytoarchitecture** is maintained varies considerably among the different *in vitro* assays. There is little difference in the cytoarchitecture of the organ in the whole testis simple incubation, perifusion, perfusion, and sectioned testis assays. However, the cytoarchitecture of the Leydig cell preparations is extensively altered during the cellular isolation steps that involve collagenase incubation, elutriation, and Percoll density gradient centrifugation. Of course, the cytoarchitecture of the cell line is even further reduced when compared to the organ/cells of the other assays, since cells from cell lines are tumor cells, thereby necessitating consideration of not only the absence of *in situ* intercellular communication

but also intracellular and biochemical pathway differences.

As for **stability**, all of the preparations remain stable for approximately 3 to 6 hours. Stability data were based on the length of time that the preparation would respond to stimulation, e.g., hCG, with a linear increase in steroid hormone production. Some preparations have been reported to be used for 24 to 48 hours, i.e., Leydig cells, perfusion. The studies using the preparation for this prolonged length of time did (Thoreaux-Manlay et al., 1995) or did not (Rommerts et al., 1988) show linear steroid hormone production.

In regard to **sensitivity**, there is little disparity among the assays based on the very limited information available to date that can be used to assess sensitivity. None of the assays introduce factors that compromise the sensitivity of the RIA method to measure a steroid hormone in the medium, perfusate, etc. Measurement of sensitivity from the viewpoint of responding to LH or hCG may distinguish the assays one from another. However, only the cell line assay would be different from the other assays in that some cell lines do not respond to stimulation, e.g., I-10, although others can, e.g., MA-10, MLTC-1.

The **specificity** of the *in vitro* assays is a difficult parameter to assess without experimental data specifically designed to evaluate this parameter. The most relevant information available comes from those studies where the same investigators tested the same substance using different assays (e.g., Kelce, Klinefelter, and Laskey tested EDS using sectioned testis, perfused testis, and purified Leydig cells in the adult rat). In general, all of the methods are able to detect a substance-induced change in steroid hormone production at similar µM concentrations of the test substance.

Metabolic activation is not present to any appreciable extent in the testicular preparations. However, it may be possible to include an S9 fraction with the purified Leydig cell and cell line assays.

Endpoints for the *in vitro* assays primarily involve measuring steroid hormone production and secretion. Based on the literature reviewed for this DRP, the whole testis simple incubation and cell line assays used the fewest endpoints, i.e., enzyme activity and single-point steroid hormone, respectively. The perfused testis assay analyzed the most steroid hormones, i.e., 11 (Chubb and Ewing, 1979b). Purified Leydig cells were used to determine the most different endpoints, i.e., steroid hormones, enzyme activity, and histology (light and electron microscopy). The other assays, sectioned testis and crude Leydig cells, also had several different steroid hormones (5) used as endpoints.

The other parameters that were not listed in the table for comparison were believed to be similar for all the assays, i.e., sample throughput, chemical solubility/permeability, and predictive capacity (age, sex, and species), or there was believed to be insufficient information to comment, i.e., inter-/intra-laboratory variability, acceptance by the scientific community, and frequency of false positives/false negatives.

4.6.4 Recommended Steroidogenic Screen Assay

Based on the information presented above, a recommendation can be made for selecting—from the various methods and assays—the single most promising assay to further pre-validate as a screen for substances that alter steroidogenesis. **The *in vitro* sectioned testis assay is recommended as a screen for measuring disruption of steroidogenesis.** The most salient features of this assay are that it identifies substances that alter steroid hormone production and can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory training. All of these are important features for a screen. In addition, the assay is stable (hours), relatively sensitive and specific, maintains the cytoarchitecture of the organ, uses a reduced number of animals (quartered sections), will be relatively easy to standardize (by optimization or consensus), has well-defined and multiple endpoints. A protocol for the sectioned testis assay is included in Appendix D.

Table 4-11. Comparison Summary of *In Vitro* Methods

Parameter	Whole Testis (simple incubation)	Perfused Testis	Perifused Testis	Sectioned Testis	Isolated & Cultured Leydig Cells (crude)	Isolated & Cultured Leydig Cells (purified)	Cell Lines
Cost	$	$$$	$$$	$	$$	$$$	$
Time: - Initial Set-Up	Day(s)	Week(s)	Week(s)	Day(s)	Day(s)	Week(s)	Week(s)
- Conduct	~30 to 50 testes/day (estimate)	~2 testes/day (deduced; Koos et al., 1984)	~ 2 testes/day (deduced; Koos et al., 1984)	~30 to 50 testes/day (deduced; EDSTAC, 1998)	~12 testes/day (deduced; Klinefelter et al., 1993)	~12 testes/day (Klinefelter et al., 1993)	Un-determined
Lab: - Training	General	Specialized	Specialized	General	General	Specialized	Specialized
- Equipment	General	Specialized	Specialized	General	General	Specialized	Specialized
Standardization (Level of Difficulty)	+	+++	+++	+	++	+++	++
Animal Usage	♂♂♂	♂♂♂	♂♂♂	♂♂	♂	♂	None
Cytoarchitecture	Intact organ	Intact organ	Intact organ	Semi-intact organ	Incomplete organ (with cellular debris)	Incomplete organ	Trans-formed/Un-differentiated cell
Stability (Viability)	6 hours (+) (Deb et al., 1980)	4.5 hours (+) (Chubb and Ewing, 1979b)	no data	5 hours (+) (Laskey et al., 1994)	4-6 hours (Biegel et al., 1995)	48 hours (+) (Thoreux-Manlay et al., 1995)	3 hours (+) (Chaudhary and Stocco 1989)
Sensitivity	no data	15 Inhibitors @ 30 μM → inhibited T from 1 to 95 % (Chubb & Ewing, 1979b)	no data	Detect Δ @ μM conc. (Laskey et al., 1994)	Detect Δ @ μM conc (Laskey and Phelps, 1991)	Detect Δ @ μM conc (Kelce et al, 1991)	Detect Δ @ μM conc (Chaudhary and Stocco, 1989)
Specificity	++	++	++	++	++	+++	+(+)

Table 4-11. Continued

Parameter	Whole Testis (simple incubation)	Perfused Testis	Perifused Testis	Sectioned Testis	Isolated & Cultured Leydig Cells (crude)	Isolated & Cultured Leydig Cells (purified)	Cell Lines
Metabolic Activation	None	None	None	None	None	Add an S9 fraction (evidence is equivocal)	Add an S9 fraction (evidence is equivocal)
Endpoints	Enzyme act. (Deb et al., 1980	Steroid hormones (11) (Chubb & Ewing, 1979b)	Steroid hormones (deduced)	Steroid hormones (5) (Gurler & Donatsch, 1979)	Steroid hormones (5) (Bambino & Hsueh, 1981)	Steroid hormones (4) Enzyme Act. Histology (Kelce et al., 1991; Biegel et al., 1995; Klinefelter et al., 1991)	Steroid hormones (2) (Hoelscher and Ascoli, 1996)

5.0 CANDIDATE PROTOCOL FOR AN *IN VITRO* ASSAY

As previously described in Section 4, several different assays can be used to assess the capacity of a substance to alter steroidogenesis. Based on the criteria for a screen and the attributes of the different types of assays, the *in vitro* sectioned testis assay was selected as the most promising assay for assessing whether the substance being tested changes steroid hormone production and secretion. This assay is simple to set up and conduct, relatively inexpensive, allows substances to be screened rapidly, reduces animal usage, preserves the cytoarchitecture of the testis, uses standard laboratory equipment, requires only general laboratory training, and remains viable for a sufficient length of time. For these reasons, the *in vitro* sectioned testis assay was selected as the screening assay for evaluating substances for steroidogenesis-altering activity, thereby completing the triad of *in vitro* assays as designed to comprise the Tier 1 Screening Battery.

Substances with steroidogenic altering activity have been tested using the sectioned testis assay. Table 5-1 provides example data from previously conducted studies that used this assay to evaluate ethane dimethane sulfonate (EDS) for its capacity to alter testosterone production. The unit of measure used to describe the efficacy of a substance to effect the response is the effective concentration (EC) and the concentration of the substance that produces 50 percent of the maximal response is termed the EC_{50}. Other units of measure that are used to describe the capacity of a substance to obtain 50 percent of the maximal response is the effective dose (ED_{50}) or to inhibit the maximal response by 50 percent is the inhibitory concentration (IC_{50}).

Table 5-1. Sectioned Testis Assay Results for EDS

Response[a]	Reference
EC_{50} = 2250 µM[b]	Gray et al., 1995
EC_{50} = 336 µM	Laskey et al., 1994

a. Effective Concentration of EDS, which produces 50 percent of the maximum hCG-stimulated release of testosterone.

b. The authors reported this value as an IC_{50} of 320 µg/mL. The value in the table was calculated using the molecular weight of EDS (~142 g/mole) and the necessary conversion factors to express the results in similar units as reported by Laskey et al., 1994.

The variation in the results was large. Some variability is attributed to experimental design and data collection differences. Sampling time is an experimental design factor that could account for some of the disparity. The EC_{50} values were determined based on data collected at the 3-hour time point (Gray et al., 1995) versus the 5 hour time point (Laskey et al., 1994).

To assess the extent of consistency of results across studies comparable experimental data were extracted from various studies and compared among one another. Four studies were identified as including data that could be compared - Laskey, et al. (1994), Fail, et al. (1994), Gray, et al. (1995), and Gurtler and Donatsch (1979). Each study utilized different test chemicals, different test chemical concentrations, and even different test chemical concentration units. Thus, only the untreated control groups were compared. Testosterone concentrations (ngT/gm Testes) were assessed at various sampling times. Cumulative standard errors of the mean were calculated under the assumption that the incremental values were independent. For each hour, where more than one study reported a cumulative mean and a cumulative standard error of the mean, a weighted one way analysis of variance test was carried out. The weights were based on the standard errors of the mean. The summary control results from the studies are presented in Table 5-2.

Table 5-2. Cumulative mean concentration and standard error of the mean by hour and study.

Hour	Reference[a]	n	Mean	SEM	F-value	Approx Degr Fr[b]	Approx p-value
0	F	12	130	25.0000	--	--	--
1	L	4	510	45.0000	4.14	(1,5)	0.10
	F	12	400	30.0000			
	Gr	6	216	(c)	--	--	--
2	L	4	1030	63.6396	116.23	(2, 5)	< 0.0005*
	F	12	700	75.0000			
	Gu	5	80	25.0000			
	Gr	6	417	(c)	--	--	--
3	L	4	1550	77.9423	48.91	(2, 5)	0.001
	F	12	925	80.0000			
	Gr	6	595	57.0000			
4	L	4	2100	84.6404	--	--	--
5	L	4	2620	90.8460	--	--	--

* p < 0.005 based on a comparison following a logarithmic transformation

a. F = Fail et al., (1994); Gr = Gray et al., (1995); Gu = Gurthler and Donatsch (1979); and L = Laskey et al., (1994).

b. The degrees of freedom associated with each study is somewhere between n-1 and 2n-1, depending on whether one or two testes per animal were used and the degree of correlation between testes from the same animal. We conservatively assume n-1 degrees of freedom.

c. No SEM reported. Also, not used for ANOVA calculation.

The results of this comparison showed that for each hour for which several studies reported cumulative testosterone concentrations there was wide variation of mean concentrations across studies. In each case there was strong indication of significant statistical differences among studies, particularly after 2 hours and 3 hours.

For each hour where more than one study reported results, the total variance of the cumulative mean values among studies was divided into variance between studies and variance within studies. The variance within studies was estimated as the average of the squares of the within study standard errors of the mean, based on those studies for which standard errors were reported. The variance between studies was estimated as the variance of the mean values among studies minus the variance within studies. Table 5-3 displays the standard deviations among the means between studies and the standard errors of the means within studies after 1, 2, and 3 hours. The standard deviation of the means between studies is approximately 3.8 to 6.8 times the standard errors of the means within studies. This agrees with the results shown in Table 5-2. The variation among study means far exceeds that which is due to within study variation.

Table 5-3. Standard deviations of between studies and within studies components of variance by hour.

Hour	n_{mean}	n_{sem}	Std Dev of Means Between Studies	Std Err of Mean Within Studies
1	3	2	143.5	38.24
2	4	3	400.4	58.59
3	3	3	479.6	72.40

5.1 Flow Diagram of the Sectioned Testis Assay

Figure 5-1, which also appeared in Section 4.3, illustrates the procedure for the sectioned testis assay.

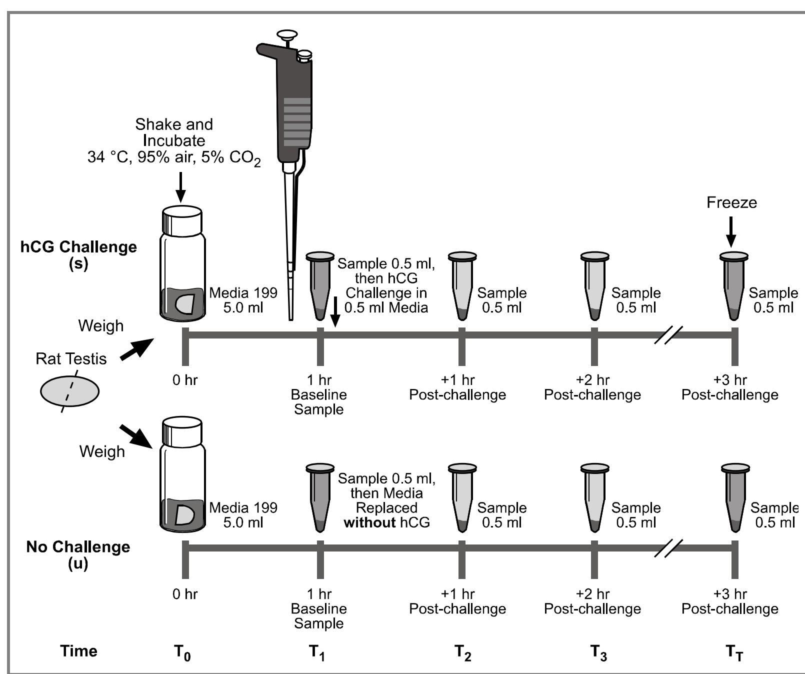


Figure 5-1. Technical Flow Diagram of the Sectioned Testis Assay

5.2 Detailed Description of the Sectioned Testis Assay

The purpose of this subsection is to describe the proposed steroidogenesis assay. A draft protocol is presented in Appendix D.

5.2.1 Species Tested/Removal of Testes

Fortunately, the literature includes sectioned testes assay studies that investigated using the testes from a few different species. Gray et al. (1995) used sectioned testes from rats and hamsters to evaluate EDS and found the rat to be approximately four times more sensitive than the hamster. In addition, Laskey et al. (1994) used the sectioned testis assay to evaluate EDS using rats and rabbits and found the rat to be approximately six times more sensitive to EDS than the rabbit. Based on these studies, the order of sensitivity of the testis to toxicity is rat > hamster > rabbit. Thus, the rat is the recommended species for testing. Furthermore, based on animal husbandry considerations, cost of the animal, and animal availability, the rat is the preferred species to test. The most common strain of rat used is the Sprague-Dawley rat (Crl:CD®[SD] IGS BR) and, since a large data base exists and previous studies have used this rat strain, the SD rat is the recommended strain for testing.

The age of the rat is an important consideration. As described in Section 4, steroidogenic

hormone production in the Leydig cells changes as the cells undergo sexual development. Testes from adult male rats, 10 to 12 weeks of age (250 to 275 g BW), will be used in the assay. Each testis weighs approximately 1 g and, when both are quartered, will provide eight organ sections, each weighing approximately 250 mg. (Although the 250 mg section is the most commonly cited amount of testis used according to the literature, it is not known if smaller amounts could be used; therefore, Section 6 describes a prevalidation experiment that would determine the minimal optimum amount of testis to use.)

Specific animal husbandry conditions will be followed. Upon receipt, animals are quarantined for at least 7 days. During the quarantine period, the animals are housed singly in solid-bottom polycarbonate cages fitted with stainless steel wire lids. Sani-Chips® is used as bedding. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) is made available *ad libitum*. The source of drinking water is the municipal water supply, which is made available *ad libitum* via automatic water delivery systems or water bottles. Animal rooms are maintained on a 12 hours on and 12 hours off light cycle. Target conditions for temperature and relative humidity are 64 to 79°F (18 to 26°C) and 30 to 70 percent, respectively. Room air change rate is 10 to 15 times per hour.

Testes will be collected with adherence to limiting discomfort or injury. Animals are humanely terminated by carbon dioxide inhalation. A possible alternative to termination is to use the testis from animals that are used on studies that require castrated animals, e.g., Hershberger assay. In such studies, animals are anesthetized and the testes removed. If the testes are not being used directly in the study that requires castrated animals, then there may be a way to keep the testes viable long enough to use them for the *in vitro* steroidogenesis assay. Conversely, if fresh testes are determined to be necessary, then after the testes have been removed for the *in vitro* steroidogenesis assay, the animals could be saved and used later on the study that requires castrated animals. This latter procedure may be the most pragmatic as animals require a recovery period after castration in order for hypothalamic and pituitary hormone levels to become stable. Only then can they be used in reproductive toxicity studies.

The assay is initiated by properly removing and preparing the testes. After the animal is anesthetized, the testes are removed by cutting open the scrotum and cutting away connective tissue. The freed testes are cleaned of any residual tissue, e.g. epididymus, fat, etc., and then the whole testis weight is obtained (weighed to the nearest 1 mg). The connective tissue capsule surrounding the testis (tunica albuginea) is removed and cut along the longitudinal axis in order to provide four similar portioned sections. The testis sections are weighed (to the nearest 0.1 mg). Each section is placed in its own scintillation vial that contains media. At this point, a given testicular section is ready for testing.

5.2.2 Test Substance Evaluated

The test substance (chemical, product, test article) to be evaluated will be fully characterized prior to use in the sectioned testis assay. The compound will be identified by name and, if a mixture, information on all components will be provided. The CAS registry number, chemical and/or product class, and supplier/source will also be identified. Physical and chemical

characteristics directly related to test performance, e.g., water and lipid solubility, pH, pK_a, stability of the test material in the test medium at low and high concentrations, and purity and stability of the bulk substance will also be provided.

5.2.3 Method of Exposure

A stock solution of the substance being tested will be dissolved in an appropriate solvent. The solvent will be the medium used to incubate the testicular sections. The medium is modified Medium 199 (GIBCO BRL, Life Technologies, Inc., Grand Island, NY) with 0.1 percent bovine serum albumin, 8.5 mM sodium bicarbonate, 8.8 mM HEPES, and 0.0025 percent soybean trypsin inhibitor, at pH 7.4. Medium 199 without phenol red (an indicator) will be used. In addition, it will be necessary to check the solubility after the hCG and any other components have been added, to ensure that the mixture remains in solution. If a solvent other than the medium is used to prepare the formulation for mixing the substance with the sectioned testis, then the formulation should be checked for solubility in the medium. If the substance being tested is not soluble in the media, then it will need to be dissolved in another solvent for addition to the media. Other solvents that could be considered are ethanol or DMSO, to name a few.

5.2.4 Incubation Concentration Selection Procedures and Number of Replicates

The sectioned testis screening assay will be conducted using three concentrations of the test substance. Examples of the concentrations that could be tested are 5, 50, and 500 µM (final concentration). It is important to note that the objective of a screen is not to characterize a concentration-response relationship between the concentration and the response. Rather, the most important objective is to test a concentration range that identifies a concentration of the substance that will initiate at least a single event expressed as an increase or a decrease in the steroid hormone production. If in the course of identifying a concentration that produces a measurable effect there is also a graded response over the concentration range tested, such a finding represents the advantage of using this particular assay for screening substances. Along those lines, if an effective concentration (EC) response curve is desired, then more concentrations could be tested so that an EC_{50} can be determined. The concentration range for these full concentration-response studies could be performed at concentrations ranging from 1 nM (10^{-9} M) to 1 mM (10^{-3} M), depending on solubility limitations of the substance being tested. Three replicates should be performed at each concentration.

5.2.5 Controls

The assay is performed using vehicle, negative, and positive controls. The basic vehicle control is the medium. A second type of vehicle control would be included if the test substance requires a solvent other than the medium. In such an instance, an additional vehicle control group would be added to evaluate the effect of the additional solvent(s). The vehicle control(s) is/are conducted to identify whether the incubation medium and its components could confound the response being produced by the substance being tested. Theoretically, the medium and/or solvents could increase or decrease hCG-stimulated or non-stimulated steroid hormone production.

The assay also includes testing positive or negative controls. These terms require further explanation and clarification. The unique feature of the sectioned testis assay is that it is performed without hCG (basal or unstimulated steroid hormone production) and with hCG (stimulated steroid hormone production). Looking at just the basal condition to make a point, the term "positive control" could have two different meanings. First, it could mean the substance being used as the positive control increases hCG-stimulated or non-stimulated steroid hormone production or, second, that it inhibits hCG-stimulated or non-stimulated steroid hormone production, thereby having a "positive" effect. In contrast, a negative control would have no effect on hCG-stimulated or non-stimulated hormone production. Thus, these terms will be defined and used as follows:

Positive control - a substance used at a non-cytotoxic concentration that increases or decreases the production of the steroid hormone(s) in the presence and absence of hCG stimulation.

Negative control - a substance that has no effect on the production of the steroid hormone(s) in the presence and absence of hCG stimulation.

In addition, the testicular section is evaluated for its capacity to respond by treating the sections with hCG, thereby demonstrating that the tissue is viable.

Based on these definitions, the substances and their expected affects in the various incubation conditions are:

Condition	**Positive Control**	**Negative Control**
Basal	Aminoglutethimide ($\downarrow$steroid hormone prod.)	Finasteride (no effect - inhibits 5α-reductase
hCG-Stimulated	Aminoglutethimide ($\downarrow$steroid hormone prod.)	Finasteride (no effect)

Each of the controls is tested at a single concentration. The amount of hCG added is 50 to 100 mIU, and the concentrations of aminoglutethimide and finasteride are 250 µM and 10 µM.

5.2.6 Test Conditions

The animals are euthanized and the testes removed, sectioned, and weighed (see 5.2.1). The sections are placed in 15 mL glass scintillation vials containing 5.0 mL of medium (see below). Vials are kept refrigerated (4°C) until the assay is initiated. The various groups and replicates per group are summarized in Table 5-4.

Vials are prepared to correspond with the groups in Table 5-4, except that the hCG is not added to any of the vials at this stage. The vials are placed in the incubator (34°C on a shaker in 5% $C0_2$/95% air) and, after the first period of incubation, i.e., 1 hour, an aliquot of medium (0.5 mL) is collected. The sample is added to a small tube, the tube centrifuged, the sample removed and frozen. This is the baseline secretion sample.

Table 5-4. Test Condition Matrix, Showing Number of Replicates per Group

Treatment Group	+hCG & media	-hCG & media
Vehicle Control(s)		
Medium alone	3	3
Additional solvents (each)	3	3
Negative Control		
1 concentration	3	3
Positive Control		
1 concentration	3	3
Test Substance		
Unknown 1, low conc.	3	3
Unknown 1, mid conc.	3	3
Unknown 1, high conc.	3	3
Unknown 2, low conc.	3	3
Unknown 2, mid conc.	3	3
Unknown 2, high conc.	3	3
Unknown N, low conc.	3	3
Unknown N, mid conc.	3	3
Unknown N, high conc.	3	3

One half of the replicates are then challenged with hCG (0.5 mL) and the other half are given media without hCG (0.5 mL). After the designated time intervals, i.e., 2, 3, and 4 hours, aliquots (0.5 mL) are removed, processed as described above, the samples frozen, and retained for later analysis of the steroid hormone concentration using an RIA method.

5.2.7 Endpoint Measured

The testosterone concentration will be the determined endpoint that is measured in each sample. Testosterone is the only endpoint considered necessary for this assay, since the goal of the assay is only to detect an effect of the test substance on the steroidogenic pathway, and not to identify the site or characterize any aspect of the mechanism of action. Since testosterone is the terminal steroid hormone of steroidogenesis in the testis, any effect by a test substance on the steroidogenic pathway will present itself by affecting the production of testosterone. In addition, by using this major endpoint as the only endpoint, the time, cost, and efficiency of the assay as a screen is maintained. However, as described in Section 6, it would be useful to evaluate whether there are instances when a test substance might alter the production of an intermediate hormone, e.g progesterone, without affecting the production of testosterone. This situation may present itself if there are pathways that allow the stoichiometric relationship between the intermediate hormone and testosterone to be something other than 1:1.

Testosterone will be measured using a radioimmunoassay (RIA) method. A commercially available kit will be used because they have proven to be very reliable in previous experiments. RIA standards of the highest purity will be obtained and prepared. The range of the standards for the testosterone standard curve is 0.07 to 500 ng/mL. As for reporting, the testosterone concentrations, as well as the other assay endpoints, e.g., antibody specificity, cross reactivity, and both intra- and inter-assay variation, will be summarized.

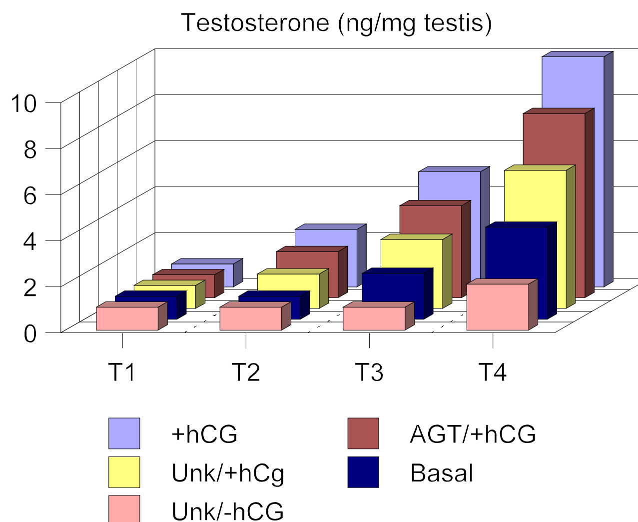
Based on the experimental design described above, the total number of samples to be analyzed for testosterone will be 624 (78 with and 78 without hCG, in duplicate, at four collection intervals).

5.2.8 Relevance of Data Collected and Associated Endpoints

Each testosterone concentration, obtained by RIA (ng/mL), is normalized by the weight of the testicular section and the time collection period. The data are expressed as ng testosterone/mg testis/hr for summarization of data and statistical analyses. A common way to illustrate the results is to plot the testosterone concentration over time for the various test groups. (The illustration below is not meant to accurately reflect initial baseline hormone secretion since the first hour likely includes increased hormone levels following release from damaged cells and equilibration of the organ sections with the culture conditions). Based on the information presented previously, examples of the test groups would include:

- hCG-stimulation
- Positive control (hCG + aminoglutethimide)
- Basal level (without hCG)
- Unknown, with and without hCG

The following illustration shows a possible scenario that could occur as a result of performing the assay with a single unknown (Unk):



Based on this simulation, hCG produced a linear and proportional increase in testosterone production over the entire collection period. The positive control, aminoglutethimide (AGT) and hCG, reduced the hCG-stimulated testosterone production as can be seen by comparing the results to hCG alone. As for the unknown substance, it was tested with and without hCG-stimulation. The unknown substance inhibited hCG-stimulated testosterone production even more than the positive control. Furthermore, when it was tested in the absence of hCG, testosterone production was less than that observed under basal conditions. These simulated data can be used to describe the relevance of the data collected and associated endpoints.

A number of individual and group comparisons can be made from such data sets. Suggested comparisons include:

- For a given group, the concentration at T1 is compared with the following concentrations at T2, T3, and T4. If T1 is a sample taken after equilibration and just prior to addition of a stimulant or test substance, then it could be considered a baseline measurement. If stimulation occurs, then the concentration at T1 would be different from the concentrations at the later time points. If no stimulation occurs or there is inhibition, then the concentration at T1 may be similar to the concentrations at the later time points.

- The baseline value for all groups could be compared. The optimal outcome would be for all groups to have similar baseline values so that effects after adding the stimulant and/or substance being tested could be compared directly. This value could also be used as a quality control standard by determining the degree of variation that occurs in this value under standardized assay conditions, and then use that value and its determined degree of variability as the standard for accepting results from future studies and to ensure comparable work is performed from laboratory to laboratory.

- The slopes of the lines for each group could be determined and differences between groups could be determined by comparing the lines using linear regression analysis. An unknown could stimulate or inhibit testosterone production and this could be evaluated by comparing the slope of the line produced by the unknown to see if it is steeper (stimulant) or flatter (inhibitor) than the line obtained using hCG alone or under basal conditions. The slopes of the lines for the hCG-stimulated and basal condition groups could also be used as quality control standards.

5.2.9 Known False Negatives and False Positives

The assay will almost certainly produce false negative results. As for false negatives, this is most likely to occur for those test substances that require metabolic activation, since the testes do not include pathways for metabolism. Other examples of false negatives involve those instances when a substance evokes an indirect effect on steroidogenesis, e.g., site of action is at the hypothalamus or pituitary gland. Finally, if the effect of the toxicant is delayed for a time greater than the duration of the incubation period, then a false negative result will occur. An example of a delayed effect was observed when lead was tested for its effect on steroidogenesis, which inhibited steroid hormone production 4 hours after initiation of the incubation (Thoreux-Manlay et al., 1995).

There are no known false positive instances to report at this time.

5.2.10 Sensitivity of the Assay and Lowest Level of Detection

The sectioned testis assay has measured hCG-stimulated testosterone production and secretion to be 200 ng/g of testis/hour (Gray et al., 1995). The lowest concentration of a substance tested is in the 1 to 10 µM range. Actual effects on the production of testosterone have been reported for substances at the 50 to 100 µM range.

5.2.11 Statistical Methods

First, a test will be performed on suspected outliers. If examination of pertinent study data do not provide a plausible, technologically sound reason for inclusion of the data flagged as "outlier," the data will be excluded from summarization and analysis and will be designated as outliers. For all statistical tests, $p \leq 0.05$ (one- or two-tailed) will be used as the criterion for significance.

Second, the data are expressed as ng testosterone/mg testes/hour for each replicate in the incubation vial. (Each replicate determination will be the mean of two replicates.) All group data are reduced to mean and standard deviation values. Treatment groups will be compared to the concurrent control group (within time) using either parametric ANOVA under the standard assumptions or robust regression method, which does not assume homogeneity of variance or

normality.

The homogeneity of variance assumption will be examined via Levene's test, which is more robust to the underlying distribution of the data than the traditional Bartlett's test. If Levene's test indicates lack of homogeneity of variance ($p<0.05$), then a log 10 conversion of the data will be made. These values will be tested for normalcy and an ANOVA applied to test all treatment effects. If Levene's test does not reject the hypothesis of homogeneous variances, standard ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in SAS® will be used to evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group to control via Dunnett's Test. A two-tailed test (i.e., Dunnett's test) will be used for all pairwise comparisons to the vehicle control group.

If the assumptions of ANOVA are not met, then robust regression methods will be applied. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for overall treatment group differences, followed by individual tests for exposed vs. control group comparisons (via Wald Chi-square tests), if the overall treatment effect is significant. The presence of linear trends (over the time points) will be analyzed by GLM procedures for homogeneous data or by robust regression methods for nonhomogeneous data. Standard ANOVA methods, as well as Levene's test, are available in the GLM procedure of SAS® and the robust regression methods are available in the REGRESS procedure of SUDAAN®.

5.3 Strengths of the Sectioned Testis Assay

The *in vitro* sectioned testis assay is optimal as a screen because it is reliable, easy to set up, relatively simple to conduct, and has short conduct-time requirements. In addition, the assay preserves the cytoarchitecture of the testis, uses standard laboratory equipment, requires only general laboratory training, and the sections remain viable for a sufficiently long period of time.

The method of exposure allows for an exact concentration to be achieved by dissolving an accurately measured amount of test substance in a particular vehicle and adding it to the testicular preparation. The recommended exposure period is 4 hours, but this can be varied to improve the sensitivity or reduce test substance and/or product degradation.

The complete steroidogenic pathway from signal transduction to end-hormone production is present in the testicular section. This preparation also includes receptors for stimulation, e.g., LH, hCG, as well as the receptor-second messenger system for an evaluation of the receptor-intracellular coupling mechanism.

In some sense, this assay does provide some information about the mechanism of action of a substance in that if it alters gonadal steroid hormone production, then it is reasonable to consider the gonads as a target organ, thereby identifying a site of action at the organ level.

This assay is able to identify substances that either increase or decrease steroid hormone production. Thus, it can identify inhibitors or stimulants of the steroidogenic pathway.

The assay minimizes the number of animals used for study. In addition, if organs from another study are able to be used in the steroidogenesis assay, then the assay will contribute to the goal of reducing, refining, and replacing animals.

The assay lends itself to multiple endpoints, i.e., intermediate hormones. Also, the media can be collected and stored, which allows additional hormones to be analyzed at a later date.

5.4 Weaknesses and/or Limitations of the Sectioned Testis Assay

The primary limitation of the testis section assay is its lack of metabolism. Test substances that require activation will not alter steroid hormone production in this *in vitro* assay, whereas they would in an *in vivo* assay.

The assay, when used as a screen with a single endpoint, does not provide complete information regarding the site or mechanism of action. However, the sectioned testis assay can be used for additional endpoints, if so desired, which will make the assay a more mechanistic type assay.

The assay may be compromised by a substance that is insoluble in an aqueous medium. Based on the solubility of the substance in appropriate solvents, the range of concentrations that can be tested could be limited.

5.5 Test Method Performance and Test Method Reliability

The performance of this assay is assessed as part of the experimental design. First, the sectioned testes are checked for viability by measuring the testosterone production over time before and after the addition of hCG, which stimulates the steroidogenic pathway to synthesize steroid hormones. When the organ preparation remains viable, then hCG will stimulate the testis to produce and secrete testosterone in a linear fashion. The production and secretion of testosterone at a rate of 200 ng/g testis/hour was measured using the sectioned testis assay (Gray et al., 1995). Second, the assay evaluates an active substance by blocking hCG-stimulated testosterone production, if it is an inhibitor of the pathway, or it may induce an increase in non-stimulated testosterone production, if it is an inducer of the pathway. The response curves will be evaluated using statistical tools that compare treated responses to the control.

The endpoint of the assay is reliable. Measurement of testosterone in the media is a very well-established analytical endpoint. The analytical method used is a RIA, which is accurate and precise. Concentrations as low as 0.07 ng/mL are routinely determined.

A limited number of examples exist in the literature for evaluating the sectioned testis

assay as a screen of substances. It is not reasonable with the present information to claim with assurance that the assay will be sufficiently rigorous, sensitive, and reproducible. The assay is, however, the most promising assay from the number of competing assays. Even with the limited number of studies to draw from, these studies show that the assay identifies substances that alter steroid hormone production by altering enzyme activity or substrate availability. Also, this assay has been used by a number of different laboratories, which allows some estimate of the assay variability to be assessed. Assay variability, sensitivity, and reproducibility, will become better understood once additional substances are screened using an assay that has been standardized, either by optimization or consensus of various experimental design considerations.

5.6 Implementation Considerations

5.6.1 Establishment of Assay

The *in vitro* sectioned testis assay was selected as a screen in part because it is relatively simple to set up and conduct. The materials, supplies, and equipment needed to conduct this assay are standard for any biology laboratory. In addition, the training needed to conduct the assay requires skills that are fairly common among general biology laboratory technicians. A general list of the materials, supplies, and equipment needed and skills required are listed below:

Materials and Equipment	Skills
Centrifuge (low and high speed)	General necropsy
Analytical balance	Radioimmunoassay (RIA)
Incubator	Incubation/*in vitro* organ preparation
Shaker/water bath	Animal husbandry
pH meter	
Gamma or scintillation counter(s)	
Glassware, test tubes, vials	
Pipettes, automatic pipettors	

5.6.2 Cost/Time Required

For purposes of estimation, the assay as described here is assumed to be the standard method. The cost of conducting such a study has not been estimated. A sectioned testicular assay was estimated by EDSTAC (1998) to cost approximately $12,000. The actual cost of such a study would be greatly affected by such factors as whether it was conducted in accordance with Good Laboratory Practices (GLPs), the degree of physical and chemical characterization of the substances being tested, number of endpoints and replicates for each time point, number of time points, and amount of statistical analysis, to name a few.

The time required to conduct the assay described here and in Appendix D would be 2 days, which would cover the time between the removal of the testes to the time that the medium samples were collected. Additional time would be needed to analyze the medium samples, summarize the data, interpret the results, and write the report.

5.6.3 Animal Welfare Considerations

Based on the design described here, three test substances would be tested at a time, which would require a total of 10 animals or about 3 animals/substance being tested.

A possible alternative to using animals solely for this assay would be to utilize animals from other studies that require castrated animals. Such a proposition has not been checked to determine whether such a plan is feasible. It remains to be determined how long a testis will remain viable after it has been removed and whether any practical storage conditions can extend its viability. If feasible, then the use of animals for this assay would be less of an issue, as it would involve using discarded organs from other studies already being conducted. Alternatively, the testis could be collected fresh, and the animals used at a later date in the studies that require castrated animals. Often times such studies require a 3 to 5 week recovery period, which if scheduled properly may allow the same animals to be used for both the *in vivo* and *in vitro* studies.

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6.0 DEVELOPMENTAL STATUS OF THE ASSAY AND RECOMMENDATIONS FOR PREVALIDATION STUDIES

6.1 Current Status

The endpoint included in the sectioned testis assay has been evaluated in other studies. However, the protocol itself has not been validated. Pending a final decision on the study design, the protocol would be ready to enter the prevalidation phase.

6.2 Recommendation for Optimization of the Sectioned Testis Assay Protocol

6.2.1 Testicular Preparation Issues

Optimization of the assay could be determined for the amount of testis actually needed to obtain a given level of sensitivity. For example, a single testis from an adult SD rat weighs approximately 1 g. If such a testis were quarter sectioned, then each section would weigh approximately 250 mg, which is the weight of the sample generally described by investigators who have used quartered sections of testis. However, no documentation was found that demonstrates whether smaller sections would give similar results. Thus, it would be advantageous to conduct a study that investigates whether the sensitivity of the preparation is affected by the amount of testicular tissue used and, if so, if there is an optimal and/or threshold amount to use. The weight of the sections to be tested could range from the customary amount used, i.e., 250 mg, down to an amount of tissue that represents a practical minimum, e.g., 5 to 10 mg.

It would also be useful to explore storage and viability of the testis and/or sections. One possible scenario to assist in meeting an objective to reduce, refine, and replace animal usage would be to use testes from animals in a separate study, that requires castration in the experimental design, e.g., Hershberger studies. It may be possible to store testes after removal in such a way that they remain viable, can be shipped to various locations, and are used at a later date for the *in vitro* sectioned testis steroidogenesis assay. A storage condition, stability, and viability study could be designed and tested.

6.2.2 Endpoint Issues

The importance of measuring progesterone could be evaluated. For example, it is possible that the stoichiometric molar relationship for progesterone and testosterone is not 1:1. If such a relationship exists and a substance inhibits progesterone production but the "pool" of progesterone is sufficiently large such that the production of testosterone is not affected, then the assay would not detect an effect on steroidogenesis if only testosterone were measured. However, this effect would be observed if progesterone were measured. This possibility could be determined during the initial experiments used to optimize the assay.

The stability of the media samples could be determined during these initial studies. Since the assay lends itself to multiple endpoints but the assay is most efficient by measuring a single important endpoint, i.e., testosterone, it would be useful to evaluate the length of time that the media could be stored and used at a later date to measure other endpoints, e.g., progesterone, estradiol. This information could easily be obtained by conducting storage stability studies of the media collected from studies used to optimize the experimental design.

Another experimental design factor that could be optimized by experimental determination is the number of collection time points. The current study design includes four time points. Media samples are collected at 1, 2, 3, and 4 hours after the incubation is initiated. The possibility exists that other time points are better suited to characterize the effect of a substance on steroid hormone production. Along those same lines, perhaps fewer time points are equally as useful to measure an effect. Statistical analysis could be used to determine whether concentrations measured at 1 and 4 hours provide no more or no less information than that obtained by measuring samples at four different time points. Such information could be used to reduce extraneous collection and analysis steps.

6.2.3 Stimulation Factor Issues

The initial studies need to optimize the concentration of stimulant added to the testicular preparation. The stimulant planned for use is hCG. The amount of stimulant used is important because it can affect whether a steady proportional increase in steroid hormone production occurs over the entire duration of the incubation period. Also, a measure of the variability of different lots of hCG could be determined during such experiments. This would serve to provide needed information about factors that affect the variability of the assay.

6.3 Recommendation for Sectioned Testis Assay Prevalidation Studies

Prevalidation studies following the ICCVAM validation process should be initiated. Prevalidation studies should include evaluation of six to eight substances to establish the database for the validation studies. It is recommended that the study be performed using test substances with different chemical classifications, as well as varying sites and/or mechanisms of action, which will aid in the development of the prevalidation database for the assay. The recommended positive and negative control test substances were selected based on their sites of action, i.e., aminoglutethimide inhibits $P450_{SCC}$ and finasteride inhibits 5α-hydroxylase, which is not found in the testes. Other test substances of interest that are recommended for testing in the prevalidation studies include:

- bisphenol A (inhibits steroidogenic signal transduction)
- lindane (inhibits signal transduction and the StAR protein)
- ketoconazole (a weak imidazole anti-fungal; inhibits $P450_{SCC}$ and aromatase)
- genistein (a weak phytoestrogen/flavonoid; inhibits 3β-HSD)

- flutamide (inhibits P450c17)
- econazole (a potent imidazole; inhibits aromatase).

6.4 Recommendation for Further Development of Cell Line Methods

In addition to the prevalidation studies for the *in vitro* sectioned testis assay, further characterization and development of the cell lines as screening tool assays is recommended. Based on the information summarized in Section 4.5 (Table 4-10), there are 2 to 3 cell lines that could be studied further for their possible use as assays for testing substances for steroidogenesis altering activity. The recommended cell lines are the MA-10, R2C, and H295R cells. These cell lines are recommended because they represent cell lines from three different species, i.e., mouse, rat, and human, respectively. The Leydig-like steroidogenic properties of the MA-10 cell line have been characterized to the greatest extent and exhibit many of the properties of Leydig cells up through the production of progesterone. In addition, the MA-10 cell line will provide a good standard for comparison as the properties of the other cell lines are more fully investigated, which is also recommended in the prevalidation studies.

Another reason that these cell lines are recommended for further study is that they are readily available; Dr. M Ascoli (University of Iowa, Ames, Iowa) holds the MA-10 cell line and the American Type Culture Collection (ATCC) stocks the R2C and H295R cell lines. As for the H295R cell line, it is unique in that it is an immortalized human cell line and, although it is derived from non-gonadal tissue, it appears to possess many of the properties that would make it a viable tool for testing substances for their effects on both the gonadal and adrenal steroidogenic pathways. Finally, the goal to develop non-animal assays could be further attained if one or more of these cell lines were found to be useful investigative paradigms. For these reasons, it is recommended that consideration be given to further study of cell lines as screening tools for identifying substances with steroidogenic altering activity.

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APPENDIX A:

LITERATURE SEARCH

APPENDIX A

Literature Search

A comprehensive literature search was conducted in support of this DRP. Appendix A describes the initial results from a Dialog search of electronic databases. In addition, as reference sources were obtained and reviewed following this initial screen, additional information was gathered, reviewed, and included in the DRP as appropriate. The Reference section (7.0) presents citations for all works discussed in the DRP.

Databases Searched

Steroidogenesis Search Strategy

Date of Search: 13 April 2001

Database Files

The following files from the database vendor, Dialog®, were searched:

File 155 ***MEDLINE***—Covers virtually every area in the broad field of biomedicine. Coverage is from 1966-present .

File 156 ***TOXLINE***—Information on the toxicological effects of chemicals, drugs, and physical agents on living systems. Coverage is from 1965-2000.

File 144 ***PASCAL***—Provides access to the world's scientific and technical literature in the fundamental disciplines of physics and chemistry; life sciences (including biology, medicine, and psychology), applied sciences and technology, earth sciences, and information sciences. Includes about 450,000 new citations per year. Coverage is from 1973-present.

File 5 ***Biosis Previews***—Comprehensive, worldwide coverage of research in the biological sciences and biomedical sciences. Coverage is from 1969-present from nearly 6,000 primary journals and monographic titles.

File 73 ***EMBASE***—Comprehensive index of the world's literature on human medicine and related disciplines. *EMBASE* provides access to periodical articles from more than 3,300 primary journals from approximately 70 countries. Coverage is from 1973-present.

File 34 ***SciSearch®: A Cited Reference Science Database***—An international, multidisciplinary index to the literature of science, technology, biomedicine, and related disciplines produced by the Institute for Scientific Information® (ISI®). *SciSearch* contains all of the records published in the Science Citation Index® (SCI®), plus additional records from the Current Contents® publications. Coverage is from 1990-present.

Database Search Strategies

English Language Articles
Foreign language articles with English abstracts

Keywords and phrases considered

- steroidogenesis
- testosterone
- dihydrotestosterone
- testis
- ovary
- aromatase
- 5-alpha reductase
- cholesterol
- minced testis
- minced ovary
- P450 isoforms/isozymes
- Leydig cells
- Luteinizing hormone (LH)
- adrenal cortex
- fetal steroidogenesis

Storage of electronic database information in Reference Manager format

Results of the online search:

- The search term "steroidogenesis" was used; the term was limited to appear in at least the titles or descriptors of relevant records; this search set was then combined with the search set on the term "vitro." This resulted in 7008 records.
- This search then combined a search on the terms "method or methods or methodology or assay* or test or tests or testing or protocol* or guideline*." This reduced the set to 887 records. [Note: The asterisk denotes the use of a truncation symbol to gather plurals or alternate endings of terms.]
- The term "testosterone" was added to this second set and limited the results to be in the English Language only. After removing duplicate records, 179 items remained.
- Next, I added the term "dihydrotestosterone" to the second set. After limiting by English Language only and removing duplicate records, 11 items remained.
- Going back to the second set (method or methods…), duplicate records were removed from that set to see how many unique records were there. This was also limited to English Language only. This reduced the set from 887 to 607 records. From this set, a sample of 12 items was retrieved for initial review to determine appropriateness of journals retrieved. Based upon examination of the sample records, it was apparent that the terms "method or methods" were problematic. It was decided to limit those terms to appear in only the descriptors of relevant records. This resulted in 110 items from search Set 2 having the terms "method or methods" appearing in the descriptors.
- The second search was repeated by initially leaving out the terms "method or methods" and then searching them separately with Set 1. This search was

then combined with our previous search, limited by English Language only and removed duplicate records, resulted in 530 records remaining, down from 607 records.

- A sample of records from this set of 530 were then reviewed. After review of this sample, a decision was made to include the concept "procedure*;" in doing so, this search was combined with Set 1, resulting in 111 records retrieved. The term "procedure*" was limited to appear in at least the titles or descriptors of relevant records. After removing duplicate records and limiting to English Language, only 6 records remained.
- Upon investigation of the titles of the 6 records, it was concluded that they were on target. However, during examination of other sample records, the terms, "test, tests, or testing" were also problematic. Articles where the terms "test, tests, or testing" appeared only in the abstract were removed, leaving a total of 420 articles remaining. This set was combined with the 6 from the "procedure" search, making a total of 426 records.
- From this set of 426 records, a online review of approximately 12 articles was conducted to determine whether identified articles met research expectations. It was concluded that this set of articles closely matched the research objectives of this task. However, to ensure adequate coverage of the published literature on steroidogenesis, a slightly different retrieval approach was used to find additional records.
- Another search was conducted on the term "steroidogenesis," limiting this term to appear in at least the titles or descriptors of relevant records, and combining that with the phrases "minced ovary or minced testes or minced testis." After removing duplicate records from the above set of 426, 9 items remained. This set was then combined with the previous set for a grand total of 432 records. All 432 records were downloaded in a tagged format.

Literature Evaluation Process

Criteria for Literature Selection

- Appropriateness of methods for measuring endpoints of interest,
- method clearly described
- appropriate use of controls
- data adequately reported
- appropriate statistical analysis

Literature Review

Summary of the Review Process

Literature describing *in vitro* studies of mammalian reproduction and/or endocrine disruption, and/or applicable steroidogenesis test protocol evaluation was retrieved. Approximately 232 of the 432 references were reviewed for relevancy. The references

were given a number from the reference list. These references were divided into ovarian, testicular, and adrenal steroidogenesis. Twenty-eight papers regarded adrenal steroidogenesis and were not used in this paper. Twenty-two were not in mammals and were not included in the ovarian steroidogenesis descriptions of methods. The ovarian references were divided into categories such as human IVF granulosa cell cultures (32), by species and compounds, and by methods used to determine steroidogenesis. Approximately 11 papers were purely mechanistic in nature. The references were then read by the authors and placed in the following categories:

- **5**—Excellent methods with technical details, highly relevant
- **4**—Good methods, relevant
- **3**—Good for other areas such as background, introduction, references, some relevance
- **2**—Poor for the purpose of this report, little interest
- **1**—Not useful for this report, no interest
- **0**—Not relevant to this report

Each article in each group was then evaluated according to the criteria described. Summaries of the best articles that illustrated the usefulness of an *in vitro* exposure protocol for detection of endocrine-disrupting activity of the test compound are presented below.

Hazard- and risk-based study designs may examine different endpoints, but if these endpoints will be used for risk assessment, they must first be shown to be robust, reproducible, appropriately sensitive, biologically plausible, and relevant to the adverse outcomes of concern. Definitions of the attributes of such endpoints are as follows:

Reproducible: These endpoints must be reliable; the same findings occur under the same conditions within the initial reporting laboratory (intra-laboratory) and among other laboratories (inter-laboratory). If the results from endpoints are not reproducible, they cannot form the basis for future research and are most likely not useful for risk assessment.

Robust: These endpoints must be present after comparable routes of exposure (e.g., whole organism as well as cell or organ culture). Different effects may be observed by different exposure routes, different species, or different neurological controls. The findings from routes unrelated to human or environmental exposures may not be useful for risk assessment. These findings must also be present at the same routes and doses over time.

Sensitive: These endpoints should not be dependent on unique conditions, especially those which are not relevant to the species at risk. These endpoints should not exhibit high variability (insensitive) or be greatly affected by confounders (too sensitive).

Relevant: These endpoints must be biologically plausible and related to adverse effects of interest/concern. If there are no adverse effects at the dose/duration/route evaluated, these endpoints should be predictive of other adverse effects at higher doses, after longer exposure duration, and/or by different routes, etc.

Consistent: These endpoints must occur in the presence of effects in other related, relevant endpoints, if possible, at the same dose, timing, duration, routes of exposure, etc.

The literature was evaluated in terms of how well the study design was described. Studies that were described in such a way that they could be repeated in the authors' laboratory only on the basis of information contained in the manuscript were further evaluated for scientific soundness, and for the likelihood that the results could be reproduced by repeating the experiment.

The literature was evaluated for the use of appropriate controls. In *in vitro* studies, the use of a concurrent control sample (media) that is under the same culture conditions as the treated samples is essential for valid statistical analysis.

Data and results were considered to be adequately reported if the data followed the study design in a logical manner, and all of the animals or samples could be tracked through the description of the results and placed appropriately in their data groups. In addition, it was necessary that the data appear to be consistent and realistic within groups and compared to the concurrent control.

Data analysis was evaluated with regard to the use of appropriate statistical methods, appropriate comparisons with the control group, tightness of data, and appropriate interpretation of the statistical results.

This appendix contains the entire "online" search results in chronological fashion such that one can discern the number of articles indexed for given key word combinations and phrases that were encountered during the online search conducted in April 2001. This section also includes the title and abstract for all articles retrieved.

Summary of Literature Retrieved

The purpose of this section is to numerically categorize and organize the articles (hard copy retrieved articles) for the steroidogenesis reference manager database. The criteria used are species, test species sex, tissue type, chemicals or steroids used, and various components of experimental design. The experimental design is further categorized into those studies involving the investigation of a certain chemical on the steroidogenesis process, along with those studies that simply investigate the general biological function of the steroidogenesis process. Many of the papers in this database explore several different species, tissues, chemicals, and methods. Therefore, the numbers displayed in the following tables do not add up to the total number of papers retrieved.

Of the 432 titles identified during the initial search, approximately 264 were identified for full test retrieval; of those, 232 were able to be obtained and consequentially reviewed.

Table A-1. Summary of Database Count

Total Number of Papers in Database	264
Number of Papers Retrieved	232

The papers in this database that were retrieved were categorized into two different types. The first type included all papers that investigated the basic biological function of a system. This included papers describing alterations in hormone levels and the biological activity of naturally produced hormones and steroids etc. The second type included those papers that investigated the effects of an outside substance on the steroidogenic process or system.

Table A-2. Breakdown of Paper Objectives

Papers Describing Biological Function of a System	200
Papers Describing Chemical Influence on a System	32
Papers Not Yet Retrieved	32

This database contained papers studying multiple species of organisms. As stated previously, many papers included more than one species of organism in their study, so the values given will not add up to the total number of papers in the database.

Table A-3. Breakdown of Species Types Used in Database Research Papers

Species Type	Number of Papers Concerning Species
Rats	92
Humans/Primates	62
Mice	18
Fish	14
Cows	13
Pigs	10
Rabbits	8
Horses	5
Hamster	4
Sheep	4
Birds	4
Reptile	3
Snakes	2
Lizards	2
Frogs	1
Gerbil	1
Guinea Pig	1
Goats	1
Shellfish	1
Crustacean	1
Insects	1

Several papers in the database only reviewed the methods and studies of others. These papers included no new experimental research, therefore not involving any specific species or chemicals.

Table A-4. Listing of Review Papers

Number of Review Papers (no experimental research conducted)	3

Many of the papers in the database were sex-specific studies on certain cells and systems. However, there were several papers that included both sexes. Table A-5 shows the breakdown of studies concerning the male systems, and Table A-6 those concerning female systems.

Table A-5. Papers Investigating the Male Reproductive and Hormonal System

	Total
Tissues/Systems Studied Concerning Male Endocrine System	127
Testes/Testosterone	112
Leydig Cells	47
Male Infertility	1

Table A-6. Papers Investigating the Female Reproductive and Hormonal System

	Total
Tissues/Systems Studied Concerning Female Endocrine System	191
Ovaries/Hormones	170
Granulosa Cells	80
Follicle	88
Placenta	26
Mammary	1
Uterine	3

The database included 59 non-sex-specific studies. These included studies on the adrenal, pituitary, neurological, and urinary systems. Although these papers study the effects of non-sex-specific organs, many of them did study the effects of these organs and their products on sex-specific systems.

Table A-7. Papers Investigating the Adrenal, Pituitary, Neurological, and Urinary Systems

	Total
Adrenal/Pituitary/Neurological/Urinary Papers	59
Adrenal	39
Pituitary	7
Neurological	13
Urinary	3

Many different assay methods were used in the papers included in this database. Radioimmunoassay (RIA) was the most frequently used, but again, many of the papers used more than one method of bioassay.

Table A-8. Methods of Gathering Data

	Total
Radioimmunoassay Procedure (RIA)	138
Scintillation	15
Fluorometry	2
Chromatography	49

Most of the research conducted in the database papers was done *in vitro*, although 44 studies used *in vivo*. Some studies included both methods.

Table A-9. *In Vitro* vs. In Vivo

In vitro studies	249
In vivo studies	53

Multiple hormones, steroids, enzymes, and proteins were studied in these papers. Numbers given in Table A-10 only indicate the presence of a certain chemical in a specific study. It does not indicate that it was the *only* chemical studied, however.

Table A-10. Breakdown of Steroids, Chemicals, and Hormones Examined

Note: Indication of use does not imply that the study only examined one chemical, but rather that it was involved somewhere in the process.

Hormone/Steroid	
Progesterone	140
Testosterone	111
Estradiol	108
Luteinizing Hormone (LH)	93
Human Chorionic Gonadotropin (hCG)	82
Follicle Stimulating Hormone (FSH)	69
Pregnenolone	45
Estrogen	37
Oestradiol	31
Insulin	26
ACTH	24
Aromatase	21
Coritcotropin	15
Prolactin	12
Aldosterone	9
Serotonin	9
Forskolin	8
Growth Hormone (GH)	7
Lutropin	5
Melatonin	3
Oxytocin	3
Gonadotropin Releasing Hormone	2
Moult Inhibiting Hormone	1

Table A-11 lists the specific chemicals studied in the papers describing the effects of an outside chemical on a specific system.

Table A-11. List of Chemicals Examined for Their Possible Role in the Disruption of Steroidogenesis

Chemical	
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)	4
Aspirin	1
Indomethacin	2
Cadmium or Cadmium Salts	4
Colchicine	1
Podophyllotoxin	1
Vinblastine	1
Nocodazole	1
Taxol	1
Dimethyl sulfoxide	1
Danzol	3
Diethylstilboestrol	1
Ethanol	2
Acetaldehyde	1
Etomidate	1
Epostane	1
Trilostane	2
Metyrapone	1
Megestrol acetate	1
Aminoglutethimide	1
Stanozolol	1
Gossypol	1
Ketoconazole	1
Lead	1
Lindane	1
Mercury	1
Methoxychlor	1
MGK repellent-11	1
Mitomycin C	1
Nicotine	1
Anabasine	1
Cotinine	1
Nitric Oxide	1

Chemical	
Nomegestrol	1
Omeprazole	1
Polycyclic Aromatic Hydrocarbons (PAH's)	1

Summary

The following generalities can be made from this database:

- Rat and human steroidogenic systems were studied most frequently when investigating the steroidogenic process.
- The majority of the papers in this database investigate the basic biological function of the system. This includes procedural papers simply describing how to assay and perform experiments that would describe broad functions in the steroidogenic process.
- Radioimmunassay was by far the most commonly used method of ascertaining the presence and amounts of specific hormones.

APPENDIX B:

INTERVIEWS

APPENDIX B: INTERVIEWS

Interviews with Principal Investigators—Published and Unpublished Studies
(See Appendix 1)

Researchers contacted:

Gary R. Klinefelter, Ph.D.
Reproductive Biologist and Toxicologist
Reproductive Toxicology Division, MD#72
National Health and Environmental Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
Phone: (919) 541-5779
Fax: (919) 541-4017
E-mail: klinefelter.gary@epa.gov

Jerome M. Goldman, Ph.D.
Endocrinology Br. MD-72, Reproductive Toxicology Div.
National Health & Environmental Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
Phone: (919) 541-2320
Fax: (919) 541-4017
E-mail: goldman.jerome@epa.gov

Patricia A. Fail
Manager, Laboratory of Reproductive and Endocrine Toxicology
Research Triangle Institute
P.O. Box 12194, 3040 Cornwallis Road
Research Triangle Park, NC 27709-2194
Phone: (919) 541-6079
Fax: (919) 541-5956
E-mail: patf@rti.org
Reproductive and endocrine toxicologist

David T. Armstrong
E-mail: david.armstrong@adelaide.edu.au

John W. Laskey
7017 Branton Drive
Apex, NC 27502
Phone: (919) 362-3945
Fax: (919)362-3946
E-mail: JohnL45198@aol.com

Reproductive and endocrine toxicologist
National Health & Environmental Effects Research Laboratory
U.S. Environmental Protection Agency
Consultant

Clarification of Study Design, Interpretation, and Data Analysis of Studies Through Interviews with These Scientists

The interviewer identified him/herself, identified the contract, and indicated the title and objectives/goals of this particular work assignment. He/she briefly described the study designs under consideration (in vitro culture of ovarian or testicular cells). The questions were designed to be open-ended and to encourage discussion; and follow-up questions were asked, as appropriate. The questions were modified to suit each interviewee's experience, as appropriate.

The written text will become part of the permanent record.

At a minimum, the questions provided in the following template should be answered. Other ideas, opinions, thoughts, suggestions, tangents, or anecdotal type of information will be welcomed and will become part of the permanent record. Other information should be recorded on this form under the "Open Dialogue" heading.

Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up-regulation or down-regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases, it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various *in vitro* methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name of Interviewer: Patricia A. Fail, PhD

Name of Interviewee: Dr ________________________________

Date: __________________________________

Have the questions been responded to in writing by interviewee (circle or omit one)? Yes/No

If so was a follow up phone call made to discuss any of the major points (circle one)? Yes/No

Note to reviewer: Please feel free to add more space or modify question as needed. Please put your modifications in italics.
--Thanks, Pat Fail

QUESTIONS

Q1. Do you know of any references that you would recommend that investigate the status of various *in vitro* methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis?

Specific comment(s) to individual "expert" from me, such as, "Jerry, I have the early ones for ovarian steroidogenesis from John Laskey's work. Are there any of yours we can include or have you published the follicle method yet?"

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or your own, please respond to the following question to the best of your ability.

Each form was customized for the specific scientific "expert."

Ovary

Q2. What are the limitations of this method?

- Whole follicle
- Minced ovary
- Other

Q3. What are the strengths?

- Whole follicle
- Minced ovary
- Other

Q4. What would you recommend to further enhance this method or what changes would you recommend (if any)?

- Whole follicle
- Minced ovary
- Other

Q5. Do you know of any other published literature that corroborates or refutes the findings for these methods?

Q6. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

- Whole follicle
- Minced ovary
- Other

Q7. Quality Control: What are the quality control measures to be included in these assays that you favor?

a. Are there specific or special circumstances when additional quality control measures need to be added?

Q8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

Q9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

a. What specific step of steroidogenesis or organ of steroidogenesis does each access?

Q10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

Q11. Are there variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

Q12. Is there anyone else you can think of that we should contact? Yes/No

a. If so, whom?
b. Can we mention your name when we contact him/her? Yes/No

Q13. Open Dialogue

Testes

Q14. What are the limitations of this method?

Q15. What are the strengths?

Q16. What would you recommend to further enhance this method or what changes would you recommend (if any)?

Q17. Do you know of any other published literature that corroborates or refutes the findings in this paper?

Q18. In running this method/procedure are their any steps that are especially difficult that require special attention (i.e., lessons learned that come after numerous runs that you would like to share) or is there any special set-up strategy you would recommend that would save time or resources that come from experience in running the assay?

Q19. Quality Control: What are the quality control measures to be included in these assays that you favor?

a. Are there specific or special circumstances when additional quality control measures need to be added?

Q20. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an *in vitro* study design?

Q21. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

a. What specific step of steroidogenesis or organ of steroidogenesis does each access?

Q22. Do you have any unpublished data relevant to theses assays that you would be willing to share?

a. If so, are there any restrictions?

Q23. Are their variations of the assay that should be considered that you did not have time to validate or access?

Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

Q24. Is there anyone else you can think of that we should contact? Yes/No

a. If so, whom?
b. Can we mention your name when we contact him/her? Yes/No

Q25. Open Dialogue

Detailed Review Paper on Steroidogenesis
EPA Contract Number 68-W-01-023
Work Assignment # 1-6

Interviewee

Patricia A. Fail, PhD
Manager
Laboratory of Reproductive and Endocrine Toxicology
Research Triangle Institute
P.O. Box 12194 (RTI)
Durham, NC 27709
Phone: (919) 541-6079; 1 800 334-8571 ext. 6079
Fax: 919 541-7208

Instruction for interviewing researchers for input regarding procedures, methods, and lessons learned regarding Steroidogenesis (minced testes, ovary, adrenal leydig cells, or granulosa cells etc). Use a separate template to capture information for each person interviewed. This information can be scribed by hand during the interview or you may ask the person being interviewed if he or she would like to provide his or her own written response to the questions to be followed up with a phone call.

The written text will become part of the permanent record.

At a minimum the questions provided in the template below will be asked. All impromptu questions, thoughts, suggestions, tangents or anecdotal type of information will be captured and become part of the permanent record. This information will be recorded on this form under the "Open Dialogue" heading.

Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name of Interviewer: Carol S. Sloan, MS

Name of Interviewee: Patricia A. Fail, PhD

Date: August 2 and 10, 2001

Have the questions been responded to in writing by interviewee? Yes

If so was a follow-up phone call made to discuss any of the major points? Yes

Q1. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis?

A1. Laskey et al., 1994 and many others. See Literature Search and Methods in Toxicology Vol. 3a and 3b for technical methods. Also see attached list of abstracts and reports from my own CV.

In your papers and reports you describe methods (or assays)—the testicular steroidogenesis and ovarian steroidogenesis assessments *in vitro*—in regards to these papers please respond to the following question to the best of your ability.

Q2. What are the limitations of these methods? [All answers apply to both assays unless otherwise stated]

A2. High variability when assessing individual animal's testis. See Table M-5 of document for a measure of variation. A general test.

Q3. What are the strengths?

A3. Ease, rapidness, fairly sensitive, relatively low cost. For our lab, fewer technician hours dramatically lowers the total cost. This assay can be made more specific by supplying precursors. For example, if a potential toxicant is shown to disrupt testosterone basal release or stimulated release, specific intermediates can be used to test for the "health" of specific enzyme substrates.

This assay can represents one animal's response to *in vivo* or *in vitro* exposure. Requires less time, technical skill or major equipment than cellular isolations such as Leydig cell purification, Granulosa or Theca cell isolation, and/or isolation of whole follicles.

A nice compromise might be a crude Leydig Cell isolation.

Q4. What would you recommend to further enhance this method or what changes would you recommend (if any)?

A4. Use of ED 50 or ED75 (of hCG) but no more than ED100, for the challenge test.

Variability must be addressed by using triplicate determinations (or more) per test point. I also recommend that the variation be summarized for these two specific methods within and between labs and compared with variation in other methods (in literature).

I believe that further reduction in costs are possible by downsizing it—using a smaller volume of media and a decreased amount of testis used per evaluation replicate. Klinefelter et al are now using 50 mg testis/ml media. This can be accomplished by using 1.5 ml vials rather that the 20 ml vials or by using a 24-well plate.

Q5. Do you know of any other published literature that corroborates or refutes the findings in this paper?

A5. All HEERL EPA literature from Laskey et al., Klinefelter et al., and Goldman et al. J. W. Laskey and others taught us to use these assays in a cooperative research program.

Q6. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

A6. I believe that further reduction in costs are possible by using 96 well plate assay and decrease amount of testis used per evaluation replicate.

Q7. <u>Quality Control</u>: What are the quality control measures to be included in these assays that you favor?

A7. Always include negative and positive controls (two compounds) at one or more doses. Doses for the positive control should be on the straight part of the dose response curve. Both the positive and a negative controls should be easily obtainable and reasonable inexpensive. Always include procedural controls (e. g., blank media that receives all treatments).

Q7a. Are there specific or special circumstances when additional quality control measures need to be added?

A7a. For the RIA being used—testosterone, estradiol, and/or progesterone—the test chemicals should be tested for cross reactivity. That is, do these chemicals themselves bind to the antibody used in the RIA?

All labs doing interlab validation should use same RIA kit source or have RIAs done at a central facility. Storage time and temperature should be controlled.

Q8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?

A8. First and foremost, the endproducts of steroidogenesis in the system being tested. For the whole testis—testosterone; for the whole or minced ovary—estradiol. Androstenedione and progesterone are also easily assayed and could be included in the first analyses or as a secondary triggered measure if the primary product is affected.

Q9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

A9. See Table in Goldman interview, incorporated into the text.

Q9a. What specific step of steroidogenesis or organ of steroidogenesis does each access?

A9a. Each targets one or more aspects of gonadal steroidogenesis or the effect of gonadal steroid on the target organ (pituitary and accessory sex organ's receptors). If I understand the major recommendation of EDSTAC, it is to rapidly access the overall synthesis of steroidogenesis. Thus the overall process in ovary (estradiol and or progesterone) or testes (testosterone) should be tested initially. Then if desirable the specific parts or place [of steroidogenesis affected] can be evaluated. The overall processes include the integrity of the LH receptors, P450 enzyme systems, cholesterol uptake (and possible biosynthesis), labile protein synthesis (e.g., STAR), bioactivity of specific steroidogenic enzymes, and precursor uptake. These steps can be tested only if the "pure" mechanism of action(s) are known. I doubt that they do for many toxicants. Do not use cytotoxicants such as EDS.

Q10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

A10. The effective doses of hCG in 1/6 testes slabs. See Table M-2. **<u>At this point, please do not cite, quote or publish</u>**. This information will help us establish the appropriate doses to use for challenge assays. Similar data must be established for ovarian cultures or smaller pieces of testes (i.e., to use in 96 well plates). For all tables, the sponsor will be contacted and the conditions for use established. Also the Methoxychlor data are from an EPA cooperative agreement and have not yet been published in other that abstract form (See reference list).

LIST OF TABLES ATTACHED TO DRP

Table M-1: Effects of daily administration of methoxychlor on testicular testosterone (ng/g testis) In f0 male Long-Evans rats: in vitro incubation

Table M-2: Preliminary studies to define the dose and time response in testicular cultures: in vitro testosterone at 0, 1, 2, and 3 hours after hCG challenge

This is an example of an in vitro study for a dose response to hCG. Any (or several) toxicant(s) would be substituted in appropriate doses

Table M-3: Experimental design: Endocrine toxicity of a toxicant (xxx) on rat testes after in vivo exposure for 2, 7, and 14 days. An example of a definitive study design with *in vitro* testing after in vivo exposure

Table M-4: Preliminary Studies to Define Dose and Time Response in Adult Male Sprague Dawley Rats: Plasma and Testicular Testosterone at 1, 3, or 6 Hours Post-hCG Challenge

Table M-5: Characteristics of Radioimmunoassays Validated for Determination of Testosterone in Adult Male Sprague Dawley Rats

Q11. Are there variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

A11. Differences in aged or prepubertal males; differences in females in different stages of cycle; use of the prepubertal PMSG primed females (also see Laskey's interview); use of ovarian whole follicles (see goldman interview).

Q12. Is there anyone else you can think of that we should contact? Yes

a. **If so, whom?**
b. **Can we mention your name when we contact him/her?**

John Laskey, Jerome Goldman, Gary Klinefelter, David Armstrong.
Also Anita Payne (Leydig cells)

Q13. Open Dialogue

REFERENCES

Publications

Fail, P.A., and R.P. Reynolds (1987). Influence of cortisol on prostaglandin synthesis by fetal membranes, placenta and uterus of pregnant rabbits, Biol. Reprod., 37, 47-54.

Fail, P.A., S.A. Anderson, S.W. Pearce, R.W. Tyl, G. Klinefelter, and L.E. Gray, Jr. (in preparation). Reproductive and endocrine toxicity of vinclozolin administered in corn oil to male Long-Evans hooded rats using the alternative reproductive test protocol, Tox. Sci.

Fail, P.A., S.A. Anderson, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. (in preparation). Endocrine and reproductive toxicities of methoxychlor administered in corn oil to male Long-Evans hooded rats using the alternative reproductive test protocol, Tox. Sci.

Fail, P.A., S.A. Anderson, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. (in preparation). Two-generation study of endocrine and reproductive toxicities of methoxychlor administered in corn oil to female Long-Evans hooded rats using the alternative reproductive test protocol, Tox. Sci.

Abstracts (Poster or Platform Presentations)

Noden, P.A., and J.F. Roux (1981). Prostaglandin-forming cyclooxygenase in rabbit amnion, yolksac splanchnopleure, placenta, decidua and uterus at 20 to 30 days gestation, Society for Gynecological Investigation, St. Louis, MO, March: 89.

Reynolds, R.P., P.F. Noden, and M.E. Greene (1983). Cortisol-induced abortion in rabbits: maternal serum progesterone and cyclooxygenase activity in the gestational tissues, Biol. Reprod., 28: Suppl. 1:112.

Stroud, C., J.M. Whitsett, and P.F. Noden (1984). 2-bromo-a-ergocryptine decreases serum prolactin, flank gland diameter, total accessory sex gland weight, serum testosterone, and testicular weight in prepubertal male golden hamsters, Biol. Reprod., 30: Suppl. 1: 171.

Fail, P.A., S.W. Pearce, S.A. Anderson, and L.E. Gray (1994). Methoxychlor alters testosterone and LH response to human chorionic gonadotropin or gonadotropin-releasing hormone in male Long-Evans hooded rats. Biology of Reproduction, 50, Suppl. 1, p. 102 (Abstract 206).

Anderson, S.A., P.A. Fail, B.T. McTaggart, R.W. Tyl, and L.E. Gray (1994). Reproductive toxicity of methoxychlor in corn oil to male and female Long-Evans hooded rats using the alternative reproduction test protocol (ART). Biology of Reproduction, 50, Suppl. 1, p. 101 (Abstract 186).

Fail, P.A., S.W. Pearce, S.A. Anderson, R.W. Tyl, and L.E. Gray, Jr. (1995). Endocrine and reproductive toxicity of vinclozolin (VIN) in male Long-Evans hooded rats, The

Toxicologist, 15: p. 293 (Abstract 1570).

Anderson, S.A., S.W. Pearce, P.A. Fail, B.T. McTaggart, R.W. Tyl, and L.E. Gray, Jr. (1995). Validation of the alternative reproductive test protocol (ART) to assess toxicity of methoxychlor in rats, The Toxicologist, 15: p. 164 (Abstract 871).

Anderson, S.A., P.A. Fail, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. (1995). Testicular and adrenal response in adult male Long-Evans hooded rats after antiandrogen vinclozolin exposure, J. Andrology, 15 (Suppl. 1), p. P-43 (Abstract 78).

Fail, P.A., and L.E. Gray, Jr. (1996). Endocrine toxicity of vinclozolin in Long Evans hooded male rats: in vivo and in vitro. Presented at 4th Biennial International Symposium on "Alternatives in the Assessment of Toxicity: Issues, Progress, and Opportunities," June 12-14, 1996, Aberdeen Proving Ground, MD, U.S. Army, technical program Abstract 14, p. 26.

Fail, P.A., S.W. Pearce, S.A. Anderson, R.W. Tyl, and L.E. Gray, Jr. (1996). Estrogenicity of methoxychlor in two generations of Long-Evans hooded rats. Presented at the IBC International Environmental Congress of Endocrine Disrupters: Advances in Measuring and Analyzing Their Effects, October 7-8, 1996, Washington, DC.

Fail, P.A., S.W. Pearce, S.A. Anderson, R.W. Tyl, and L.E. Gray, Jr. (1996). Vinclozolin-induced endocrine toxicity in adult male Long-Evans hooded rats via antiandrogenicity. Presented at the IBC International Environmental Congress of Endocrine Disrupters: Advances in Measuring and Analyzing Their Effects, October 7-8, 1996, Washington, DC.

Fail, P.A. (1998). Assessing the estrogenicity and other endocrine activity of chemicals. Presented at 1998 IBC's Third Annual International Congress on Endocrine Disruptors: An Unbiased Examination of the Impact Recent Scientific Developments Will Have on Industry, April 14-15, 1998, Washington, DC.

Study Reports - Commercial Client

Toxicity Testing of a Fungicide, XXXX, in Adult Male CD® Sprague Dawley Rats. P.A. Fail, S.A. Anderson, and S.W. Pearce, Project Number 65C-5703, October 20, 1994, Confidential Client.

Toxicity Testing of a Fungicide, XXXX: Endocrine Toxicology Studies of Testes From Adult Male CD® Sprague Dawley Rats Exposed to XXXX In Vivo. P.A. Fail, S.A. Anderson, and S.W. Pearce. Project No. 65C-6169, September 16, 1996, Confidential Client.

Toxicity Testing of a Fungicide, XXXX: Endocrine Toxicology Studies of Testes From Adult Male CD® Sprague Dawley Rats Exposed to XXXX In Vitro. P.A. Fail, S.A. Anderson, and S.W. Pearce. Project No. 65C-6169, September 16, 1996, Confidential

Client.

Study Reports - Government Client

Two-Generation Study of Methoxychlor Administered in Corn Oil to Long-Evans Hooded Rats Using the Alternative Reproductive Test Protocol. P.A. Fail, S.A. Anderson, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. Project No. 65U-5456, June 3, 1996, U.S. EPA.

Two-Generation Study of Vinclozolin Administered in Corn Oil to Long-Evans Hooded Rats Using the Alternative Reproductive Test Protocol. Volume I: Males, Volume II: Females. P.A. Fail, S.A. Anderson, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. Project No. 65U-5456, February 28, 1997, U.S. EPA.

Synthesis and Testing of New Antiprogestational Agents. Final Report. C.E. Cook, Y.-W. Lee, P.A. Fail, R.D. Bagwell, J.M. O'Reilly, G. Bartley, S.W. Pearce, P.S. Raje, and R.S. Shetty. Project No. 60U-6413, Contract No. N01-HD-3238, September 19, 1998, NICHHD and several others dating back to 1986.

Detailed Review Paper on Steroidogenesis
EPA Contract Number 68-W-01-023
Work Assignment #1-6

INTERVIEWEE: Testicular steroidogenesis

Gary R. Klinefelter, Ph.D.
Reproductive Biologist and Toxicologist
Reproductive Toxicology Division, MD#72
National Health and Environmental Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
Ph: (919) 541-5779
Fax: (919) 541-4017
E-mail: klinefelter.gary@epa.gov

The written text will become part of the permanent record.

At a minimum the questions provided in the following template should be answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information will be welcomed and will become part of the record. Other information should be recorded on this form under the "Open Dialogue" heading.

Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer: Patricia A. Fail, PhD

Name of Interviewee: Dr. Gary Klinefelter

Date of Response: August 1, 2001; follow -up interview on October 11, 2001

Have the questions been responded to in writing by interviewee (circle or omit one) Yes

If so was a follow up phone call made to discuss any of the major points (circle one) Yes/No

NOTE to reviewer: Please feel free to add more space or modify question as needed. Please put your modifications in italics. Thanks, Pat Fail

QUESTIONS

1) Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis?

Salva, A., Klinefelter, G.R., and Hardy, M.P. Purification of rat Leydig cells: increased yields after unit gravity sedimentation of collagenase dispersed interstitial cells, J. Androl., 2001, (In Press).

Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalte. Akingbemi BT, Youker RT, Sottas CM, Ge R, Katz E, Klinefelter GR, Zirkin BR, and Hardy MP. Differential effects of DEHP on Leydig cell function throughout reproductive development, Biol. Reprod., 2001, (In Press).

Klinefelter, G.R., Kelce, W.R., Hardy, M.P.: The isolation and culture of Leydig cells from adult rats. In: Methods in Toxicology, Volume 3, Part A, (Heindel J. and Chapin R., eds.), Academic Press, pp 166-181, 1993.

Klinefelter, G.R., Kelce, W.R. A Comparison of Leydig Cell Responsiveness to Hormonal and Nonhormonal Factors in Vivo and In Vitro. In: The Leydig Cell, (Payne A.H., Russel L. and Hardy M., eds.), pp 535-553, Cache River Press, 1996.

2) In our experiments [for the EPA CA with Earl] we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays, please respond to the following question to the best of your ability.

Testis

a. What are the limitations of this method?

Sensitivity and linearity appear to be issues associated with incubations of minced ovarian tissue

b. What are the strengths?

Incubations of minced testis (or ovarian) tissue permits acquisition of data on individual animals and is relatively easy

c. **What would you recommend to further enhance this method or what changes would you recommend (if any)?**

For testicular assessments, consider going away from minced testis parenchyma incubations and moving forward with modifications of Leydig cell purification that permit a yield of enriched Leydig cells from an individual animals that is sufficient for steroidogenic profile assessment (see Salva et al., J. Androl., In Press).

For ovarian assessments, consider use of preovulatory follicles and in vivo challenges for experimental correlate. Regardless of organ/cell type ensure that linear steroid production is achieved [over time], and that assessments are made within the linear response range.

d. **Do you know of any other published literature that corroborates or refutes the findings in this paper?**

e. **In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.**

f. **Quality control**

1. **what are the quality control measures to be included in these assays that you favor?**

   Tests for linearity of steroid production and cell viability. For testis, include 3B-HSD histochemistry at end of assessment period.

   Incubations with intermediate substrates as well as hCG.

   An *in vivo* experimental correlate. For example challenge in vivo with hCG in control and exposed animals to determine changes in hCG responsivity, examinining steroid in serum as well as in intersititial fluid (testis).

2. **Are there specific or special circumstances when additional quality control measures need to be added?**

3) **Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?**

Degree of LH/hCG responsivity, i.e. to what degree does stimulation with hCG enhance steroid production. If only 2 fold over baseline, cells aren't happy.

Ability to respond to a greater degree to intermediate substrates of steroid production. Does stimulation with Cholesterol increase steroid production over that achieved with hCG stimulation ?

4) Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

Chemicals such as aminoglutithemide, and other suicide substrates for P450 enzyme activity. Chemicals that inhibit membrane signal transduction and cholesterol trafficing (i.e. StAR) should also be evaluated.

Duration [of incubation] should be within the linear range of steroid production.

What specific step of steroidogenesis or organ of steroidogenesis does each access.

5) Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

6)
a. Are there variations of the assay that should be considered that you did not have time to validate or access?
b. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

7) Is there anyone else you can think of that we should contact?

a. If so, whom?
b. Can we mention your name when we contact him/her?

8) Open Dialogue

DETAILED REVIEW PAPER ON STEROIDOGENESIS
EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT #1-6

INTERVIEWEE: Ovarian Steroidogenesis

Jerome M. Goldman, Ph.D.
Endocrinology Br. MD-72, Reproductive Toxicology Div.
National Health & Environmental Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711
(919) 541-2320 FAX: (919) 541-4017
email: goldman.jerome@epa.gov

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At a minimum the questions provided in the following template should be answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information will be welcomed and will become part of the record. Other information should be recorded on this form under the "Open Dialogue" heading.

Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer: Patricia A. Fail, PhD
Name of Interviewee: Dr Jerome Goldman
Date: August 1, 2001

Have the questions been responded to in writing by interviewee (circle or omit one) Yes
If so was a follow up phone call made to discuss any of the major points (circle one) Yes

NOTE to reviewer: Please feel free to add more space or modify question as needed. Please put your modifications in italics. Thanks, Pat Fail

QUESTIONS

1. **Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.**

   Off-hand, I don't know of an article that compares the different ovarian approaches for use in a Tox Study (if that is what you are asking).

   A discussion of ovarian perifusion in Tox studies, along with a presentation of the method, is in: Peluso, JJ, Pappalarda, A. (1993). Ovarian perifusion culture: A tool to assess ovarian toxicity. In: J.J. Heindel, R.E. Chapin (Eds.), Female Reproductive Toxicology (Methods in Toxicology, Vol. 3B). Academic Press: San Diego. pp. 180-193 and in Brannstrom, M. In vitro Perfused rat ovary (same text as above, pp. 160-179). Chapters discussing methods for ovarian cell isolations are in the same text.

   Jodi Flaws and Pat Hoyer have a chapter in Vol. 10 of the Comprehensive Toxicology series (1997) that provides a discussion of follicular isolations as a technique (Flaws, JA, Hoyer, PB. (1997). A new direction in experimental approaches: Follicular isolations. In: P. Hoyer (Ed.), Reproductive and Endocrine Toxicology, Vol. 10, Section II, Female Reproductive Toxicology (Comprehensive Toxicology, I.G. Sipes, C.A. McQueen, A.J. Gandolfi, eds.-in-chief). Pergamon Press: Oxford. pp. 373-377.).

Our lab has used in vitro exposures of pre-ovulatory follicles under hCG-stimulated and non-stimulated conditions (Balchak et al., Repro. Toxicol.14: 533, 2000). A second manuscript has been submitted for publication using our improved approach, but exploring sites of toxic impact along the early portions of the steroidogenic pathway.

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or you own, please respond to the following questions to the best of your ability.

OVARY

2. What are the limitations of this method?

- Whole follicle

This is not a pure cell population. Isolations (of follicles) are more technically demanding than a Laskey-type minced ovary approach.

- Minced ovary

Minced ovaries are much more heterogeneous than either isolated cell preps or follicular preps. Ovarian perifusions, as described by Peluso/Pappalarda and Brannstrom are reasonably time-consuming and more technically demanding, but would generate more reliable data than simply placing minced ovaries in a tube without controlling the oxygen content of the medium.

- Other?

Isolated cell preparations (i.e., granulosa cells or theca cells). Loss of architectural structure and interactions among different cell types that would normally occur.

Likely require longer baselines to characterize "normal" cells.
Viability should be tested.

3. What are the strengths?

- Whole follicle

Maintains follicular structure and intercellular communication (theca-granulosa).

By using immature rats (26d) primed with PMSG for 48h, can obtain first generation pre-ovulatory follicles at a comparable stage of maturation (can select similar-sized follicles).

Better tissue penetration of test compound than with minced ovaries.

Can obtain multiple follicles per rat and randomly assign follicular pairs among treatment conditions to reduce any variability between animals/individual ovaries. Also allows a reduction in the number of animals used per study.

- **Minced ovary:**

  Laskey type incubations- Minimum of preparation time; easy to obtain.
  Ovarian perfusions or perifusions- obtain a picture of the dynamics of steroid secretion under different experimental conditions.

- **Other?**

  Isolated cell preparations (i.e., granulosa cells or theca cells)
  Homogeneous cell types may have advantage in mechanistic studies and improve consistency of hormonal data.
  Better penetration of test compound for in vitro exposures.

4. What would you recommend to further enhance this method or what changes would you recommend (if any)?

- **Whole follicle**

  Besides whole follicles, isolated corpora lutea (from rats made pseudo pregnant) can be used as well. Choice would depend on the nature of the questions asked (pregnancy maintenance/focus on progesterone production, maintenance of cyclicity. Also both can be used in a perifusion system (We've done some unpublished work with CLs. Came out pretty well, but we never took the approach further).

- **Minced ovary**

  This really depends on which approach to ovarian incubations you mean.

- **Other?**

5. Do you know of any other published literature that corroborates or refutes the findings for these methods?

I don't understand. The utility of the approaches?

6. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

For any of the in vitro procedures using steroid secretory endpoints, use of teflon vials/tubes/tubing is the ideal, since the sex steroids (particularly progesterone) adhere quite readily to plastic, less so to glass (see Bruning et al. J. Steroid Biochem. 14:553, 1981; Higuchi and Espey. J. Reprod. Fertil. 87:821, 1989).

Use well-oxygenated media for incubations.

There are always concerns about maintenance of viability over time. Steroid secretion will eventually fall off. For most experiments, 24 hr should be sufficient to determine any toxicant effects on steroidogenesis.

- **Whole follicle:**

  Practice- either for a chemical isolation or a surgical one.

- **Minced ovary:**

  For the perfusion or perifusion approaches, practice and confirmation of a marked increase in steroid secretion under stimulation (e.g., cAMP). This would also be true for a simple minced ovarian prep (a general check on viability). In fact, under experimental conditions, it is always informative to include comparisons of baseline release under control and treatment levels in addition to a response to stimulation (hCG, cAMP, etc.) to evaluate hormonal release when the tissue is "pushed".

- **Other?**

7. Quality Control: What are the quality control measures to be included in these assays that you favor?

pH checks, maintain sterility as much as possible (even though antibiotics are generally included in incubation media).

Discussion with Fail: Likely should characterize how often pH should be measured, or how long it will hold in validation experiments. With oxygenated follicle sealed in flasks, it was stable up to 4 hours.

For most cultures, an atmosphere of 95% Oxygen 5% CO2 works nicely.

Are there specific or special circumstances when additional quality control measures need to be added?

Although it is not generally done, it would be helpful to be able to sample the dissolved oxygen content of the incubation media. However, this can be tricky to do under most circumstances without a more elaborate setup. For some data on the effects of oxygenation on follicular steroid secretion, see Roby KF, Terranova PF. (1990). Effects of tumor necrosis factor-alpha in vitro on steroidogenesis of healthy and atretic follicles of the rat: theca as a target. Endocrinology 126: 2711-2718.

8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/ adrenal steroidogenesis when investigating an in vitro study design?

Depends on the general focus. For toxicant effects on pregnancy maintenance, it would be more appropriate to focus on P4, using corpora lutea. For general effects on ovarian steroidogenesis, the most obvious endpoints for whichever cell/tissue approach employed are E2 and P4 release. Supplementation of the media with known concentrations of hormones such as pregnenolone or testosterone may provide some information about the synthetic capacity of the system under toxic insult and indications of effects on the particular enzymes (3b-HSD, P450arom, or others) involved.

9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

Give me a call to discuss. August 3, 2001 Discussion

Table x.x. Chemical Candidates for Controls

	MOA	hormone
Flutamide	receptor	antiandrogen
Ethynl Estradiol	receptor	estrogenic
Tamoxifen	??receptor	antiestrogen

Ketoconizole	general p450, ? not specific	depress Testosterone
Mthoxychlor	?receptor	estrogenic
Vinclozolin	receptor	antiandrogen
Methyl Testosterone	receptor	ANDROGENIC
Dibutylphthalate	Sertoli cell? General cell toxicity Solubility problems	testosterone
Testosterone Proprionate	receptor	androgenic
DDE	?receptor	antiestrogen
Genestein		
Finasteride	enzyme	% alpha reductase
Dibromo acetate	TBD	Decreased Progesterone in CL fragments

What specific step of steroidogenesis or organ of steroidogenesis does each access?

See above, authors, this aspect needs additional attention. Up to 40 hours could be easily used selecting appropriate controls, but after the assays are selected. PAF August 3, 2001

10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

Maybe. Contact me to discuss circumstances.

11. Are there variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

There are a variety of things to try when issues of sensitivity are considered. Robustness of the assays is always affected by cell/tissue viability.

If the minced ovarian tissue is to be used one should consider using PMSG primed immature female rat to get similar follicles (size and status). The pseudopgegnant females could be used to donate ovaries with coprae lutea dominate status.

12. Is there anyone else you can think of that we should contact?

a. If so, whom?
b. Can we mention your name when we contact him/her?

13. Open Dialogue

The Effective dose issue is an important one. When stimulating tissues, it is important not to overwhelm the system, but the challenge dose must be high enough to evoke a response. In our follicle cultures we add the hCG and the toxicant at the same time and incubate for two hours. That avoids opening the sealed vials to take a sample.

14. Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Specific comment(s) to individual "expert" from me, such as, "Jerry, I have the early ones for ovarian steroidogenesis from John Laskey's work. Are there any of yours we can include or have you published the follicle method yet?"

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or you own, please respond to the following question to the best of your ability.

OVARY

15. What are the limitations of this method?

- **Whole follicle:**
- **Minced ovary**
- **Other?**

16. What are the strengths?
- **Whole follicle:**
- **Minced ovary**
- **Other?**

17. What would you recommend to further enhance this method or what changes would you recommend (if any)?
- **Whole follicle:**
- **Minced ovary**
- **Other?**

18. Do you know of any other published literature that corroborates or refutes the findings for these methods?

19. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?
- **Whole follicle:**
- **Minced ovary**
- **Other?**

20. Quality Control: What are the quality control measures to be included in these assays that you favor?

Are there specific or special circumstances when additional quality control measures need to be added?

21. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/ adrenal steroidogenesis when investigating an in vitro study design?

22. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

What specific step of steroidogenesis or organ of steroidogenesis does

each access?

23. **Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?**

24. **Are their variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?**

25. **Is there anyone else you can think of that we should contact?**

    a. **If so, whom?**
    b. **Can we mention your name when we contact him/her?**

26. **Open Dialogue**

DETAILED REVIEW PAPER ON STEROIDOGENESIS
EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT #1-6

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Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer Patricia A. Fail, Ph.D.
Name of Interviewee : Dr John W. Laskey
Date of Response 08/12/2001

Have the questions been responded to in writing by interviewee (circle or omit one) Yes or NO?
If so was a follow up phone call made to discuss any of the major points (circle one) Yes or NO?

QUESTIONS

1. **Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.**

   Check the References included in my CV – sent separately

   John, I am especially interested in the reference(s) for the testicular assay. I have the ovarian references. Pat - in the listed references there should be several references for the testicular assay G. Klinfelter and I have considered the possible problems with this assay and in consultation with Dr. L. Ewing have concluded that this minced testicular assay is consistent with the isolated Leydig cell assay.

In our experiments (for the EPA CA with Earl Gray) we used both the ovarian steroidogenesis and testicular steroidogenesis methods. With regards to these assays or you own, please respond to the following question to the best of your ability.

OVARY

2. What are the limitations of this method?

i. For all these responses check the attached freelance file Pathway.jpg or Pathway.pre file.

ii. The steroidogenic pathway in humans is somewhat different than in laboratory species probably due to the order of the order of the enzymatic reactions .

iii. The stage of the ovarian cycle (estrus, diestrus, . . . pregnancy) makes a great deal of difference in the response to stimulation and/or inhibition.

iv. Blocking of the study to insure that day to day changes in assay conditions (technical differences, media prep, stimulant prep, inhibitor prep, sample origin, etc.) can be statistically corrected.

3. What are the strengths?

i. Ease of sample preparation.
ii. With good quality/technical control there is excellent reproducibility.

4. What would you recommend to further enhance this method or what changes would you recommend (if any)?

i. With normal laboratory precautions this method doesn't require any changes.

5. Do you know of any other published literature that corroborates or refutes the findings for these methods?

i. I haven't been following the literature for the past five years.

6. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

i. See above

7. Quality Control: What are the quality control measures to be included in these assays that you favor?

i. See above

Are there specific or special circumstances when additional quality control measures need to be added?

i. See above

8. Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/ adrenal steroidogenesis when investigating an in vitro study design?

a. Responses to appropriate hormone/stimulation.

9. Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?

What specific step of steroidogenesis or organ of steroidogenesis does each access?

10. Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?

11. Are there variations of the assay that should be considered that you did not have time to validate or access? Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

12. Is there anyone else you can think of that we should contact?

a. If so, whom?
b. Can we mention your name when we contact him/her?

13. Open Dialogue

REVIEW OF STEROIDOGENESIS ASSAY FOR EVALUATION OF ENDOCRINE DISTRUPTION

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT #1-6

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Template for Steroidogenesis Interviews

Chemicals that interfere with the androgen and estrogen systems can act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors. One prominent mechanism of endocrine disruption is interference with the synthesis of hormones themselves. Such interference is increasingly thought to occur by up regulation or down regulation of the genes responsible for producing the enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction and inhibition of the enzymes themselves. The purpose of this work assignment is to prepare a detailed review paper to survey and investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.

Name Interviewer: Patricia A. Fail, PhD

Name of Interviewee: Dr. D. T. Armstrong

Date: Sept 7, 2001

Have the questions been responded to in writing by interviewee (circle or omit one)

NO Not all of them,

If so was a follow up phone call made to discuss any of the major points (circle one)

NO

QUESTIONS:

1. **Do you know of any references that you would recommend that investigate the status of various in vitro methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) steroidogenesis.**

   There are a lot of papers that use in vitro methods to study regulation of various aspects of steroidogenesis. Many of them could be potentially useful in identification of chemicals that affect the specific steroidogenic outcomes being investigated. I don't know of a specific critical review of the status of such methods for your purposes. It would be a good topic for someone to write; perhaps an honours student with interests along these lines.

   I wrote a review which touched on some of these ideas some years ago. Armstrong DT 1986 Environmental stress and ovarian function. Biology of Reproduction 34: 29-39.

2. **In your experiments did you use both the ovarian steroidogenesis and Testicular steroidogenesis methods?**

With regards to these assays, please respond to the following question to the best of your ability.

OVARY

a. **What are the limitations of this method?**
b. **What are the strengths?**
c. **What would you recommend to further enhance this method or what changes would you recommend (if any)?**

Our research deals primarily with regulation of ovarian steroidogenesis and its relationship to oocyte competence and ovulation. We have used a variety of ovarian cell systems and end points. Studies with isolated systems of single cell types have the limitation that they are able only to identify actions that involve the cell type under study. Since a lot of the more subtle effects of compounds that influence ovarian regulation involve cellular interactions, e.g. between oocytes and granulosa cells, or thecal cells and granulosa cells, their effects may be missed by restricting study of a single cell type. In addition, many agents that alter steroidogenesis do so through extra-ovarian actions such as the hypothalamus or pituitary (or even the liver, as in agents that affect steroid clearance and hence alter feedback regulation of the ovary).

The strength of in vitro methods is their sensitivity, as well as the ability to identify sites and mechanisms of action. In addition to the limitations alluded to above, in vitro methods usually have to make assumptions as to the probable site of action of a given compound. Therefore they may be inappropriate for screening of compounds suspected to affect reproductive functions but for which the site or mechanism of action has not yet been identified. This limitation may be overcome by a sequential strategy that includes a series of tests such as:

whole animal approaches (e.g. ovarian and uterine weights, ovulation, pregnancy} followed by

whole ovary or isolated whole follicle culture in which steroids are measured in culture media;

strategic cell combinations, such as thecal and granulosa cell co-culture; oocyte-granulosa cell co-cultures; macrophage-luteal cell co-culture; isolated single cell types;

subcellular components that enable study of a single component, e.g hormone receptor, signal transduction molecule, specific intracellular reaction (enzymatic, transport mechanisp)

d. **Do you know of any other published literature that corroborates or**

refutes the findings in this paper?

e. **In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.**

f. **Quality control**

1. **what are the quality control measures to be included in these assays that you favor?**
2. **Are there specific or special circumstances when additional quality control measures need to be added?**
3. **Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?**

There are important linkages and interactions between germ cell and somatic cell compartments of the ovary that have not been explored as much as warranted, and that could undoubtedly be exploited for design of specific test systems that could be of interest to your group. Thus, in addition to steroidogenesis end points, oocyte end points could also be used to assess effects of chemicals. Specific oocyte end points that have proven useful in our studies include effects (of regulatory agents) on meiotic maturation in vitro (both spontaneous and gonadotropin-induced), ability to undergo normal fertilization in vitro, and developmental competence after fertilization or artificial activation. All of these end points depend on or are influenced by input from follicular somatic cells, and hence would have potential for development of assay systems that could prove useful for your purposes.

4. **Based on your experience, what chemicals, routes, duration, and doses would you recommend to be used to validate an in vitro steroidogenesis assay/protocol?**

What specific step of steroidogenesis or organ of steroidogenesis does each access?

5. **Do you have any unpublished data relevant to theses assays that you would be willing to share? If so, are there any restrictions?**

6. **Are there variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or**

robustness?

7. **Is there anyone else you can think of that we should contact?**
   a. **If so, whom?**
   b. **Can we mention your name when we contact him/her?**

   Professor Fulvio Gandolfi
   Istituto di Anatomia degli Animali Domestici
   via Trentacoste 2
   I - 20134 Milano. Italy
   Tel: (+39) 02-2154-036
   Fax: (+39) 02-2140-745
   Email: gandolfi@imiucca.csi.unimi.it

8. **Open Dialogue**

Pat, I'm not sure whether I've addressed this in a way that is useful. It will be evident that our research over the years has not been directly aimed at ovarian regulation from the standpoint of chemicals that disrupt/modify ovarian function. Rather it has attempted to better understand normal (physiological) ovarian regulatory processes. New concepts and details are being discovered in this field at an unprecedented rate, as increasingly powerful methodology is focussed on the topic. As our understanding of normal regulatory processes in the ovary increases in both depth and breadth, it should be possible to use this information to design approaches to study specific actions of chemicals such as those that your institute wishes to investigate.

TESTES:

a. **What are the limitations of this method?**
b. **What are the strengths?**
c. **What would you recommend to further enhance this method or what changes would you recommend (if any)?**

d. **Do you know of any other published literature that corroborates or refutes the findings in this paper?**

e. **In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.**

f. **Quality control**

1. **what are the quality control measures to be included in these assays that you favor?**
2. **Are there specific or special circumstances when additional quality control measures need to be added?**

9. **Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on testicular/ovarian/adrenal steroidogenesis when investigating an in vitro study design?**

APPENDIX C:

PARAMETERS FOR COMPARISON SUMMARY OF *IN VITRO* METHODS IN TABLE 4-11

Appendix C: Parameters for Comparison Summary of *in Vitro* Methods in Table 4-11

Cost – The overall cost of doing the screen including labor, cost of animals, and supplies. The relative scale used in the table is defined as: ($) - inexpensive; ($$) - moderately expensive; ($$$) - very expensive. The cost of conducting a steroidogenic assay using sectioned testes was estimated to be $11,600 per chemical (EDSTAC). This was considered an ($) inexpensive assay.

Time - Under this parameter, the time necessary for the initial set-up and time to conduct an assay were evaluated and compared. As for the initial set-up, the evaluation was based on the laboratory equipment and apparatus needed for the assay. Also, the time needed to set-up the apparatus and verify that it was functioning properly. In regard to the time needed to conduct the assay, information from the literature was reported (when ever possible) using the number of preparations that could be used for testing in a day. It does not include the time needed to process samples since that would vary based on the selected endpoints, as well as would not be a distinguishing factor between the assays.

Laboratory - Under this parameter, the level of training and type of equipment were compared for each *in vitro* assay. In regard to the level of training and type of equipment, the *in vitro* methods were designated as either general or specialized. General was used to describe those assays that can be conducted with standard laboratory training and equipment. Specialized describes those *in vitro* methods that require unique equipment, as well as additional and advanced laboratory training in order to be able to perform the assay.

Sensitivity–The ability of the assay to detect an effect. The more sensitive the screening bioassay, the less likely it will be that it fails to detect the positive action of a compound. Failure to detect a positive effect (whether that effect increases or decreases some component of steroidogenesis) is a false negative (a type II error). In the radioimmunoassays (RIAs) or other determination of the hormone concentration, sensitivity is defined as the minimum detectable amount of that hormone for a given set of assay conditions.

Specificity–Specificity refers to the ability to detect very well defined or specific activities. In the case of the steroidogenesis screen, it is more desirable to rank any chemical that interferes with any step of steroidogenesis as having positive activity. It is desirable then for the bioassay to be less specific. For example, any substance that affects the P450 enzymes should cause an altered hormone secretion in the assay. In RIAs, the antibody (detector) used must be characterized to define which if any related substances it is detecting. For example, in a testosterone RIA it is important to know if only testosterone is being detected or if the antibody also recognizes dihydrotestosterone, androstenedione, or other androgens.

Multiple endpoints – The assays were evaluated for their capacity to foster multiple endpoints. The steroidogenic pathway's hormones are the most frequently measured endpoints. The number of different steroid hormones measured are included in the table (in parenthesis) along with the reference. In addition, enzyme activities and architectural changes observed by microscopic examination are also used as endpoints, but less frequently.

Metabolic Activation - An evaluation of whether the assay has an endogenous capacity to metabolize pro-xenobiotics into active toxicants. Also, whether the assay lends itself to exogenous addition of a metabolic fraction, i.e. S9.

Stability - A measure of the time that the preparation has been used by investigators to characterize a steroidogenic response in the presence of a substance being tested. In addition, the times listed in the table were based on papers in the literature that reported some measure of viability. Viability was assessed using data about linear steroid hormone production in the presence of a stimulating agent, e.g. hCG, as well as other viability type tests, e.g. Trypan Blue.

Cytoarchitecture - Describes the degree that the organ remains intact once removed from the animal and is prepared for testing. Preparation of the organ for treatment, e.g. treatment with collagenase in order to isolate cells, then the cytoarchitecture is changed, which may affect how closely the preparation mimics the *in situ* environment.

Repeatability (Inter-assay variability)–Chemicals that test positive (or negative) in the subject bioassay will do so repeatedly and with the same degree of effect. That is, the initial assay is predictive of the same or similar results when repeated in another assay of the same type but set up on different dates. In this case the variation associated with the repeated evaluation is actually characterized (e.g. 10+ 0.5 %; Mean and SEM, n=y dates). For example, Chemical X results in depressed testosterone secretion in a bioassay using adult male rat testes each time it is tested in sequential evaluations and the amount (quantitative) of that suppression is similar. That amount is calculated and reported as a characteristic of the bioassay.

APPENDIX D:

RECOMMENDED PROTOCOL

EPA Contract No.:
Lab Contract No.:
Lab Study Code: **DRAFT: 5/12/02**
Lab Master Protocol No.:

TITLE: Testicular Steroidogenesis Bioassay Screening Protocol

SPONSOR:

TESTING FACILITY:

PROPOSED STUDY IN-LIFE DATES: ________________________

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			
5			

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1.0 OBJECTIVE AND BACKGROUND

Steroid hormones produced by the gonads affect most of the organs in the body including bone, muscle, brain, and reproductive organs. It is for this reason that the EDSTAC recommended the *in vitro* steroidogenesis assay in testicular tissues as a component of the Tier 1 Screening (T1S) battery. The objective of this assay is to detect disruption of the steroidogenic pathway in vitro. It may: (1) be used as one of the protocols recommended by EDSTAC for the Tier 1 screening battery, (2) serve as a follow-up test for certain substances for which additional data are required or desired, and/or (3) predict the likelihood that steroidogenesis and downstream biologically dependent processes would be affected by the same or similar substances *in vivo.* The endpoints were selected for their potential to detect toxicant-induced alterations of steroidogenesis in gonadal tissue.

The Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996 required the EPA to develop and implement a screening program for determining the potential in humans for estrogenic (and anti-estrogenic) effects from pesticides. This program has been expanded on the advice of the EDSTAC to include androgenic (and anti-androgenic) effects and effects from thyroid-hormone (TH)-like (or anti-TH) substances. The EDSTAC, assembled by the EPA in 1996, believed, to the best of its knowledge, that the recommended Tier 1 screening battery, if validated, would have the necessary breadth and depth to detect any currently known disruptors of estrogen, androgen, and thyroid (EAT) hormones.

The suggested T1S protocols are being tested within the Endocrine Disruptor Screening Program (EDSP) "to characterize the nature [and] likelihood of a dose-response relationship of endocrine disruption in humans and wildlife" (EDSTAC, 1998). To this end, the EPA has requested the development of a screening protocol that identifies compounds having the potential to affect steroidogenesis.

The testis steroidogenesis bioassay was selected as a component of the Tier 1 screening (T1S) by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to evaluate the potential toxicity of substances on the endocrine system (EDSTAC, 1998). The T1S tests were selected to obtain minimum yet sufficient estimates of potential endocrine disrupting activity. The Committee stated that these tests should be relatively inexpensive, quick, and technically easy to perform. Furthermore, they should be sensitive and specific, capture multiple endpoints, and be predictive across species, gender, and age. Finally, they should be validated and standardized as soon as possible (EDSTAC, 1998).

The *in vitro* testis steroidogenesis bioassay assesses non-receptor mediated effects on P450 steroidogenic enzymes. This assay has been used with fetal, neonatal, and adult testis, and is not limited to mammalian species, having been used to assess steroidogenesis in fish, reptile, avian, and amphibian systems as well. Thus, the steroidogenesis bioassay as a component in the T1S phase should be broadly understood to screen for any disruption of the overall steroid biosynthetic pathway. Both synthesis and release can be tested in gonads from normal animals.

The goal of the *in vitro* steroidogenesis Tier 1 screen is to evaluate simultaneously all of the processes involved with gonadal synthesis of steroid hormones (receptor binding, signal transduction, transcription, translation, and cellular secretion of the steroids). A number of compounds can inhibit the synthesis of various steroid hormones. These compounds inhibit one or more enzymatic steps in the biosynthetic pathway of steroidogenesis (e.g., aminoglutethimide, cyanoketone, finersteide, ketoconazole). Estrogen biosynthesis can be inhibited by exposure to aromatase inhibitors such as the fungicide fenarimol (Hirsch et al., 1987). In addition, a number of other steps in gonadal steroidogenesis might be disrupted, such as binding of LH to the receptor on the cell membrane, cholesterol synthesis, and cholesterol intracellular transfer.

Antiandrogens and antiestrogens act via a number of direct mechanisms in addition to those that directly involve the steroid hormone receptors (on the target organs). One prominent mechanism of antihormonal activity is inhibition of hormone synthesis by inhibiting the activity of P450 enzymes in the steroid pathway. Such activity will be detected with this *in vitro* procedure using testicular tissue obtained from adult male rats. It will, however, detect only pesticides and other substances that are active without metabolism, that is, the parent material is active. The assay will also detect other mechanisms that alter gonadal steroid synthesis via the LH receptor, cholesterol biosynthesis, and intracellular cholesterol transfer.

Substances that interfere with steroidogenesis primarily by inhibiting cytochrome P450 enzymes in the steroid pathway include two major classes of herbicides, the imidazoles and the triazoles (Taton et al., 1988). They inhibit P450 enzymes in the sterol synthesis pathway for lanosterol, a vital precursor of cholesterol (required for steroidogenesis and a component of fungal membranes). Cytochrome P450 inhibitors tend to be nonspecific, and these fungicides can also inhibit other P450 enzymes such as those required for mammalian steroid hormone synthesis (Murray and Reidy, 1990). Inhibition of mammalian steroid synthesis can potentially result in a broad spectrum of adverse reproductive effects *in vivo*, including abnormal serum hormone levels, pregnancy loss, delayed parturition, demasculinization of male pups, lack of normal male and female mating behavior, altered estrous cyclicity, and altered reproductive organ weights.

Measures of not only testosterone, but also other intermediate hormones of the pathway can be determined from media collected during the incubation period. Aromatase, another P450 enzyme, is also present, albeit at low concentrations, in the testes. Thus, it may also be possible to utilize this assay for determination of aromatase disruption by measuring estradiol concentrations. In support of this notion, Ammonium Perflurooctanoate increased estradiol production is isolated Leydig cell cultures (Biegel et al., 1995). Thus, while testosterone is the ultimate endpoint as it is the end-product hormone, the assay has the advantage of having multiple endpoints that can be used to assess effects at various sites of the pathway.

The purpose of this protocol is to outline a procedure for the quantitation of steroidogenic hormone production from rat testicular tissue. The hormone measured – testosterone – is, if altered, indicative of altered gonadal enzyme activity. This generic protocol is written for 250 mg testicular sections with a focus upon the components that are to be included in a validation

protocol.

2.0 MATERIALS AND METHODS

2.1 Test Substances

2.1.1 Test Substance–A Negative Control

Common Name:
Chemical Name:
Synonyms:
CAS No.:
Molecular Formula:
Molecular Weight:
Appearance:
Odor:
Melting Point:
Density/Specific Gravity:
Solubility:
Vehicle:
Supplier:
Batch/Lot Number:
Purity:
Storage Conditions:

2.1.2 Test Substance–A Positive Control

Common Name:
Chemical Name:
Synonyms:
CAS No.:
Molecular Formula:
Molecular Weight:
Appearance:
Odor:
Melting Point:
Density/Specific Gravity:
Solubility:
Vehicle:
Supplier:
Batch/Lot Number:
Purity:
Storage Conditions:

2.1.3 Test Substance–Each Tested Unknown and Each Stimulant

Unknown test substances will be selected by the client. All information as listed above will be inserted.

2.1.4 Standard Substance–Each Radioimmunoassay Standard

Radioimmunoassay Standards of the highest purity will be obtained and prepared. The following standard curves can be used: Testosterone 0.07 to 500 ng/mL.

2.2 Chemical Safety and Handling

An MSDS for each substance used will be attached.

2.3 Dose Formulation and Analysis

Each test substance, as well as the positive and negative controls, should have known purity, stability in bulk, as well as stability and homogeneity in solution (in the range to be used). The solubility of the test substance in the media to be used must be known. It has been standard procedure to mix the substance in the incubation media. If it is necessary to use a special solvent such as DMSO as a vehicle to enhance solubility in media, then that solvent should be tested alone (as a vehicle control) for its effect in the assay system.

The formulation will be prepared at a frequency determined by stability tests performed prior to the start of the study. Suspensions will be prepared and stored in wide-mouth, amber bottles. The test materials will be suspended in appropriate vehicles or media, with the concentration determined by the experimental design. At least three concentrations of the unknowns should be tested.

An aliquot of each concentration per formulation will be analyzed. The formulation bottles will be identified by a five-digit, random number Rx code and a color code. Personnel, other than the Laboratory Supervisor, Project Toxicologist, and Study Director, will not be informed of the test substances or formulation concentrations until all laboratory work is completed (i.e., the study technicians will be “blind” for substance and dose). Aliquots from the formulations will be collected on the day of treatment of the tissues and will be analyzed.

2.4 Animals

2.4.1 Species and Supplier

The proposed test animals will be the Sprague Dawley Derived Outbred Albino Rat Crl:CD®(SD) IGS BR supplied by Charles River Laboratories, Inc., Raleigh, NC.

2.4.2 Live Animals and Species Justification

The use of tissues from live animals has been requested by the Sponsor. Alternative test systems are not available for the assessment of effects of substances on reproduction and development in intact mammals for determining the potential risk for humans from endocrine-mediated effects of pesticides and other substances. The Charles River CD® rat has been the subject of choice on reproductive and developmental toxicology contracts, and has been used for other reproductive toxicology studies with this test material. Large historical data bases for reproductive performance and prevalence of spontaneous malformations in control rats are available from the supplier (Charles River, 1988). This strain of rat has been proven to have robust fertility and fecundity, and does not present any unusual endocrine patterns. This study does not unnecessarily duplicate any previous study.

2.4.3 Total Number, Age, and Weight

Number of Males: 10
Age on Receipt: ~10-12 weeks

(no less than 8 weeks of age to ensure maturity)

Animal Wt. Range: 250-275 g

NOTE: The number of animals needed will depend upon the size of the bioassay. Ten male rats are sufficient to provide tissue for 80 incubations. Minimum sample size requirements should be verified (based on assay variation). Estimates may be made from existing data. This is essential to providing adequate power for statistical comparison of data among treatment groups and will provide information needed to determine the number of testicular sections assigned to each dose group.

2.4.4 Quality Control (tests of animals to verify antibody free status)

Serological evaluation of animals for fecal or blood viral, bacterial, or protozoan antibodies is not deemed necessary for this protocol as they will not be housed at the laboratory for more than 1 or 2 weeks.

2.4.5 Quarantine

Upon receipt, animals will be quarantined for 7 days. They will be observed daily for general health status and ability to adapt to husbandry conditions. They will be released from quarantine by the attending veterinarian or his/her designate.

2.5 Animal Husbandry

2.5.1 Housing, Feed, and Water

During the quarantine period, animals will be randomly assigned to cages. Animals will be singly housed in solid-bottom polycarbonate cages (8"x19"x10.5") fitted with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ). Sani-Chip® cage bedding (P.J. Murphy, Forest Products, Inc., Montville, NJ) will be used in all solid-bottom cages. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) will be available *ad libitum* for the rats and tap water from the water system will be filtered and available *ad libitum* to all animals via an automatic water delivery system (Edstrom Industries Inc., Waterford, WI). Water is also available in plastic bottles with stainless steel sipper tubes *ad libitum*. The analysis of the rodent chow for chemical composition and possible chemical contamination and analysis of city water will be provided by the suppliers and maintained in the study records if deemed necessary. It is anticipated that contaminant levels will be below certified levels for both feed and water and will not affect the design, conduct, or conclusions of this study. Rat chow will be stored at approximately 60-70°F, and the period of use will not exceed 6 months from the milling date. At all times, animals will be housed, handled, and used according to the NRC Guide (NRC, 1996).

2.5.2 Environmental Conditions

Environmental conditions in the laboratory facility will be continuously monitored, recorded, and controlled during the course of the study by an automated system. Animal rooms used for this study will be maintained on a 12:12 hour light:dark cycle. Target conditions for temperature and relative humidity in the animal rooms will be between 64 and 79°F (18 and 26°C) and 30 and 70%, respectively, with 10 to 15 air changes per hour (NRC, 1996). Temperature and/or relative humidity excursions will be documented in the study records and the final report.

2.5.3 Animal Identification

During quarantine, animals will be individually identified by a cage card. They will not be tattooed or given ear tags. Data generated during the course of this study will not be tracked by these numbers.

2.5.4 Limitation of Discomfort

Discomfort or injury to animals will be limited, in that any animal will be humanely terminated by CO_2 inhalation.

3.0 EXPERIMENTAL DESIGN

3.1 Study Design

The study will consist of 24 treatment combinations (concentration by challenge groups), including at least one vehicle control group. Each group is comprised of at least 3 sections of gonadal tissue (incubated separately) selected at random from a pool of the tissue. Table 1 presents the study design and target doses of the test substance. A graphical representation of the study design is also presented in Figure 1 below.

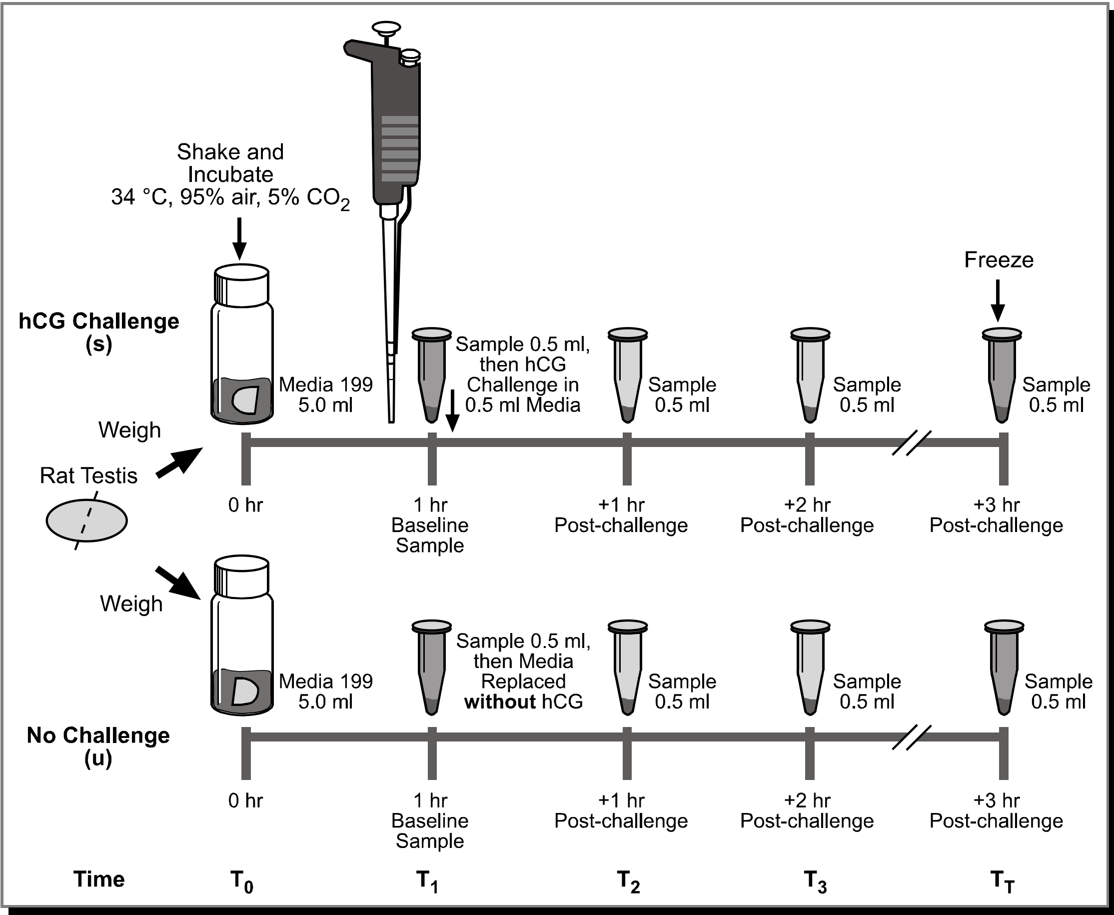

Figure 1. Technical Flow Illustration of the Testicular Steroidogenesis Assay

Table 1. Experimental Design

Treatment	Endocrine Challenge Test	
	hCG Stimulation	Media 199
<u>Vehicle controls</u>		
media alone	3	3
others for solvents (each)	3	3
<u>Negative Control Test Substances</u>		3
one dose used in all assays	3	3
<u>Positive Control</u>		
one dose used in all assays	3	3
<u>Test Substances</u>		
Unknown 1, dose x	3	3
Unknown 1, dose y	3	3
Unknown 1, dose z	3	3
Unknown 2, dose x	3	3
Unknown 2, dose y	3	3
Unknown 2, dose z	3	3
Unknown N, dosex	3	3
Unknown N, dose y	3	3
Unknown N, dose z	3	3

3.1 The Testicular Steroidogenesis Bioassay

Figure 1 represents the technical flow of the testicular steroidogenesis assay. Briefly, the animals are killed and the testes removed, sectioned, weighed, and placed in media in scintillation vials. Vials are kept cold (4° C) until assayed. One testis of each male is separated into four longitudinal sections for an *in vitro* incubation to estimate testosterone synthesis and response to gonadotropin (hCG endocrine challenge test; hCG ECT). Vials contain media (see below) or media plus test substance (1 or more doses). Each condition is represented by three replicates. The vials are placed in the incubator and after the first period of incubation (e.g., 1 or more hours); an aliquot of media (0.5 mL) is collected. The sample is added to a small tube, the tube centrifuged, the sample removed and frozen. (This is the baseline sample or secretion sample.) One half of the replicates then are challenged with appropriate substance (i.e., hCG ECT), at one or more concentrations. Additional samples are removed after various incubation periods (1, 2, and 3 hours), frozen, and later quantified for hormone content. The percentage change in hormone concentration represents the response to hCG ETC. This tests the integrity and function of LH receptors and immediately associated functions (at least through pregnenolone synthesis).

For the testicular *in vitro* incubation, modified medium 199 (Medium 199 [GIBCO BRL, Life Technologies, Inc., Grand Island, NY] with 0.1% bovine serum albumin [BSA], 8.5 mM sodium bicarbonate, 8.8 mM HEPES and 0.0025% soybean trypsin inhibitor, pH 7.4) is used. No phenol red indicator will be used. The vials are incubated at approximately 34°C on a shaker in 5% $C0_2$/95% air. The hCG obtained from Sigma (St Louis, MO) or Calbiochem (San Diego, CA), is added in 0.5 mL media. To the other sections, media without hCG is added. Aliquots (0.5 mL) for testosterone RIA are collected 1, 2, and 3 hours after challenge. Typically, only 10 to 50 microliters are required and duplicate determinations are done. The *in vitro* synthesis and release of testosterone is compared with that released after modified Media 199 alone (media control), both within and between treatment groups.

3.2 Radioimmunoassay of Samples

Media samples from the cultured testicular preparations are assayed for the steroid hormone: testosterone using radioimmunoassays (RIA). Whatever assays are used, the same ones should be used in various laboratories or at the very least the antibody specificity and other assay characteristics should be reported.

Radioimmunoassay standards of the highest purity will be obtained (sigma) and prepared. The following standard curve will be used: Testosterone 0.07 to 500 ng/mL.

4.0 STATISTICAL ANALYSES

The unit of comparison will be hormone concentration (ng/mg testes/hour) for each replicate in the incubation vial (or tubes or wells). (Each replicate determination will be the mean of two replicates in each RIA.) Treatment groups will be compared to the concurrent

control group (within time) using either parametric ANOVA under the standard assumptions or robust regression method (Royall, 1986; Huber, 1967), which does not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960), which is more robust to the underlying distribution of the data than the traditional Bartlett's test. If Levene's test indicates lack of homogeneity of variance ($p<0.05$), then a log 10 conversion of the data will be made. These values will be tested for normalcy and an ANOVA applied to test all treatment effects. If the assumptions of ANOVA are not met, then robust regression methods will be applied. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for overall treatment group differences, followed by individual tests for exposed vs. control group comparisons (via Wald Chi-square tests), if the overall treatment effect is significant. The presence of linear trends (over the time points) will be analyzed by GLM procedures for homogenous data or by robust regression methods for nonhomogenous data (SAS Institute Inc., 1999a,b,c,d,e; 2000). Standard ANOVA methods, as well as Levene's test, are available in the GLM procedure of SAS® Release 8 (SAS Institute Inc., 1999a,b,c,d,e; 2000), and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 7.5.3 (Shah et al., 1997).

If Levene's test does not reject the hypothesis of homogeneous variances, standard ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in SAS® 6.12 will be used to evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group to control via Dunnett's Test (Dunnett, 1955, 1964). A two-tailed test (i.e., Dunnett's test) will be used for all pairwise comparisons to the vehicle control group.

A test for statistical outliers (SAS Institute, Inc., 1990b) will be performed on suspected outliers. If examination of pertinent study data do not provide a plausible technicologically sound reason for inclusion of the data flagged as "outlier," the data will be excluded from summarization and analysis and will be designated as outliers. For all statistical tests, $p \leq 0.05$ (one- or two-tailed) will be used as the criterion for significance.

5.0 RETENTION OF SPECIMENS AND RECORDS

All specimens and records will be retained in archives for two years at the performing laboratory's expense. Beyond two years, continued retention will be at additional cost to the Sponsor.

6.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) prepared for this study.

A list of all laboratory-specific SOPs will be maintained with the study records and available for inspection by the Sponsor's representative.

7.0 REPORTING

An executive summary will be prepared describing the number and strain of rats used in the study, the doses and substances tested, and the effects with levels of statistical significance for all endpoints. Electronic and hard copies of spreadsheets containing the raw data from all animals will be provided for each endpoint. In addition, the spreadsheet should include treatment means, standard deviation, standard error, coefficient of variation, and sample number below each endpoint. Data presented should include sample identification and treatment, and media progesterone, testosterone and estradiol concentrations. A data summary table containing the mean, standard deviation, standard error, coefficient of variation, and sample size for each treatment group should be provided for all endpoints.

Tentative Study Dates[a] (to be added to the protocol by amendment)

Male Rats arrive:
Quarantine period (7 days):
Preparation of chemical solutions (test toxicants):
Sacrifice of animals:
Preparation of tissues and incubation with test toxicants:
Radioimmunoassay of media for hormones:
Statistical analysis:
Submission of nonaudited draft final report:
Submission of audited draft final report:

[a] The end dates are tentative and will depend on the dates of radioimmunoassay completion.

8.0 STUDY RECORDS TO BE MAINTAINED

Protocol and any Amendments
List of any Protocol Deviations
List of Standard Operating Procedures
Animal Requisition and Receipt Records
Quarantine Records
Temperature and Humidity Records for the Animal Room(s)
Animal Research Facility Room Log(s)
Durham City Water Analysis (analyzed monthly, reported annually)
Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results
Dosage Code Records Containing Five-Digit Rx Code, Color Code, and Concentration
Dose Formulation Receipt and Use Records
Statistical Analysis Records
Media Estradiol Analysis (E2)
Media Testosterone (T4)
Media Progesterone Analysis (P4)

Correspondence

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