

DRAFT DETAILED REVIEW PAPER

ON

**STEROIDOGENESIS SCREENING ASSAYS
AND ENDOCRINE DISRUPTORS**

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List of Abbreviations

The following abbreviations are used in this DRP:

ACTH	adrenocorticotrophic 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	17 β -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
3 β -HSD	3 β -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	<i>in vitro</i> 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

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1 **1.0 EXECUTIVE SUMMARY**
2

3 The purpose of this Detailed Review Paper (DRP) on gonadal steroidogenesis is to
4 (1) survey and review the biological mammalian methods that identify substances with direct
5 effects on the steroidogenic pathway; (2) critically evaluate the methods and the individual
6 assays of a given method for their potential use as screening test(s) to identify potential
7 endocrine disruptors, endocrine modulators, or endocrine toxicants; and (3) recommend the next
8 step in developing and evaluating an appropriate screening study protocol using these assays for
9 further standardization and validation. In addition, this document has been written in a manner
10 that can be understood by individuals trained in the biological sciences at the collegiate level but
11 who may have little or no specialty training in reproductive physiology or toxicology, nor any
12 reproductive laboratory experience.
13

14 The United States Environmental Protection Agency (U.S. EPA) is implementing an
15 Endocrine Disruptor Screening Program (EDSP). In 1996, the Food Quality Protection Act
16 (FQPA) amendments were enacted by Congress to authorize the EPA to implement a screening
17 program on pesticides and other substances found in food or water sources for endocrine effects
18 in humans (FQPA, 1996). In this program, comprehensive toxicological and ecotoxicological
19 screens and tests are being developed for identifying and characterizing the endocrine effects of
20 various environmental contaminants, industrial substances, and pesticides. A two-tiered
21 approach will be utilized. Tier 1 employs a combination of *in vivo* and *in vitro* screens, and
22 Tier 2 involves *in vivo* testing methods using two-generation reproductive studies. Validation of
23 the individual screens and tests is required, and the Endocrine Disruptor Methods Validation
24 Subcommittee (EDMVS) will provide advice and counsel on the validation assays.
25

26 A steroidogenesis assay is proposed as one of the Tier 1 Screening Battery assays. This
27 DRP (1) summarizes the state of the science of the *in vivo*, *ex vivo*, and *in vitro* methodologies
28 available for measuring gonadal steroidogenesis; (2) for each methodology, presents a review of
29 the individual assays and representative data generated by investigators that used the assay to
30 evaluate a substance for steroidogenic-altering activity; (3) provides an evaluation of the various
31 methodologies and the assays as tools for screening substances with suspected steroidogenic
32 activity; (4) recommends a particular screening method and assay as a screening tool; and
33 (5) describes the strengths, weaknesses, and implications for further research associated with the
34 recommended screening assay.
35

36 In addition to the Executive Summary (Section 1), this DRP is organized into five other
37 sections:
38

- 39 2. Introduction to the Endocrine Disruptor Screening Program
- 40 3. General background on reproductive endocrinology and steroidogenesis
- 41 4. Bioanalytical methodologies for measuring gonadal steroidogenesis
- 42 5. Candidate protocol
- 43 6. Developmental status of the assay.
44

45 References and appendices provide additional, more detailed information.
46

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

10
11 The steroidogenic pathway of interest in this DRP was limited to the biochemical
12 pathway located in the gonads of male and female animals. In the male, steroidogenesis occurs
13 in the Leydig cells of the testis and, in the female, this pathway is found in the follicle of the
14 ovary. At the cellular level, a series of biochemical reactions are initiated upon stimulation of
15 receptors located in the membranes of these cells. Activation of the LH/FSH receptors initiates a
16 series of enzymatic reactions that culminate in the biosynthesis of end-product hormones, i.e.,
17 testosterone (male) and estradiol/estrone (female). This DRP is intended to address the pathway
18 after the receptor, beginning with the second messenger and ending with the end-product
19 hormone. In particular, this DRP seeks to survey and review methodologies that can be used to
20 identify substances that alter steroidogenesis by a direct interaction with one or more of the
21 substrates, enzymes, or other cellular components that constitute this pathway. Only assays that
22 evaluate these components of the pathway were reviewed and assessed as a possible
23 steroidogenic screening tool.

24
25 The most promising assay for use as a screening tool was based on advantages and
26 disadvantages of the various methodologies and criteria established for the optimal screen.
27 These attributes were reported by the Endocrine Disruptor Screening and Testing Advisory
28 Committee (EDSTAC) in their final report, as well as other literature references
29 (Gray et al., 1997; EDSTAC, 1998). Based on this information, the methodologies and their
30 respective assays were compared to the optimal screen criteria in order to select the most
31 promising assay for use as a screening tool. For a given method, the assays evaluated were the
32 Endocrine Challenge Test (ECT), which is an *in vivo* method; the whole testis or ovary used in
33 simple incubation, perfusion, or perfusion assays, as well as the sectioned testis, minced ovary,
34 isolated and cultured crude or purified Leydig cells, isolated and cultured granulosa cells, and
35 cell line assays, which are all *in vitro* assays; or a combination of the *in vivo* and *in vitro*
36 methods, which constitute the *ex vivo* assays.

37
38 The ***in vitro* sectioned testis assay** was selected as the most promising screening tool for
39 identifying substances with steroidogenic-altering activity. Based on the advantages and
40 disadvantages of the *in vivo* and *in vitro* methods, the *in vitro* methodology was in better
41 agreement with the characteristics of an optimal screen. In addition, it was determined that this
42 single assay would suffice as a screen for females as well, because the steroidogenic pathways of
43 the two genders are very similar, the testes provide more organ for testing, and organ isolation
44 and preparation are technically easier to accomplish using the male organs. Finally, based on the
45 criteria used, the sectioned testis assay is recommended, because it can be conducted at a
46 minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory
47 training; the preparation is stable; the organ remains viable (over several hours); the assay is

1 relatively sensitive and specific; it maintains the cytoarchitecture of the organ; the assay uses a
2 reduced number of animals (quartered sections); the assay will be relatively easy to standardize
3 (by optimization or consensus); and the assay has well-defined and multiple endpoints.
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1 **2.0 INTRODUCTION**

2
3 **2.1 Developing and Implementing the Endocrine Disruptor Screening Program (EDSP)**

4
5 In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and
6 Amendments to the Safe Drinking Water Act (SDWA) mandated the United States
7 Environmental Protection Agency (U.S. EPA) to screen substances found in drinking water
8 sources of food to determine whether they possess estrogenic or other endocrine activity
9 (Federal Register, 1998a, 1998b). Pursuant to this goal, the U.S. EPA is required to “develop a
10 screening program, using appropriate validated test systems and other scientifically relevant
11 information, to determine whether certain substances may have an effect in humans that is
12 similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect...”
13 (FQPA, 1996). The U.S. EPA established the Endocrine Disruptor Screening and Testing
14 Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for
15 developing a testing paradigm for compounds that may have activities similar to
16 naturally-occurring hormones. Following the recommendations made by EDSTAC in its final
17 report (EDSTAC, 1998), the U.S. EPA established the Endocrine Disruptor Screening Program
18 (EDSP). The program’s aim is to develop a two-tiered approach, e.g. a combination of *in vitro*
19 and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2)
20 for identifying and characterizing endocrine effects of pesticides, industrial substances, and
21 environmental contaminants. To date, the U.S. EPA has implemented the program on two
22 fronts: (1) the development of the Endocrine Disruptor Priority Setting Database, and the
23 approach that will be used to establish priorities for screening compounds, and (2) prevalidation
24 and validation studies of some of the Tier 1 and Tier 2 assays that are likely to be included in the
25 testing battery. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) has been
26 set up to advise and review new and ongoing work in the validation of these assays.

27
28 **2.2 The Validation Process**

29
30 The U.S. EPA (and EDMVS) chose to follow the validation process established by the
31 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), of
32 which the U.S. EPA was a charter member, for validation of the EDSP screening and testing
33 methods. ICCVAM was established by the National Institute of Environmental Health Sciences
34 (NIEHS) as a standing interagency committee to aid in the validation, acceptance, and
35 harmonization of test methods designed to reduce animal use, refine procedures involving the
36 use of animals so that they would experience less stress, and to replace animal tests whenever
37 appropriate (ICCVAM, 2000). To this end, ICCVAM defined a flexible, adaptable framework
38 for test method validation that was applicable to conventional and alternate methods, and could
39 be applied to the needs of different agencies and regulatory processes.

40
41 The purpose of the validation is to establish the reliability and relevance of a test method
42 with respect to a specific use. The process is science-driven, and addresses the scientific
43 principles of objectivity and experimental design (NIEHS, 1997). In addition, as stated in the
44 ICCVAM report, “A test is considered validated when its performance characteristics,
45 advantages, and limitations have been adequately determined for a specific purpose.”
46 (NIEHS, 1997).

1 The validation process consists of four discrete phases: (1) initial protocol development,
2 (2) prevalidation studies, (3) validation studies, and (4) external scientific peer review. The
3 initial protocol, developed from existing information and experience (past and current research),
4 serves as the starting point for initiating the validation process. Prevalidation studies consist of
5 further development and optimization of specific initial protocols through targeted
6 investigations. Either before or during prevalidation, a detailed review paper addressing all
7 critical areas outlined in *Validation and Regulatory Acceptance of Toxicological Test Methods*
8 (NIEHS, 1997) is prepared for each method to summarize, explain, and document decisions
9 regarding the relevant principles, methods, and techniques recommended for the initial protocol.
10 Targeted prevalidation investigations are designed to address questions necessary for completing
11 an optimized, transferrable protocol suitable for interlaboratory validation studies. Validation
12 studies consist of comparative interlaboratory studies to establish the reliability and relevance of
13 the protocols developed in the prevalidation stage. Validation requires the development of a
14 detailed review paper to document what is known about the assay system proposed for
15 validation.

16
17 A test is considered validated when its performance characteristics, advantages, and
18 limitations have been adequately determined for a specific purpose. The measurement of a test's
19 reliability and relevance are independent stages in the validation of a test method, and both are
20 required. Reliability is an objective measure of a method's intra- and interlaboratory
21 reproducibility. If the test is not sufficiently reliable, it cannot be used for its intended purpose.
22 Alternatively, if the test is not relevant, of questionable relevance to the biological effect of
23 interest, or if it is not an appropriate measure of the effect, its reliability is academic. The
24 relevance of a test may be linked to the mechanism of the toxic effect it measures and to its
25 proposed uses (NIEHS, 1997). The studies conducted will be used to develop, standardize, and
26 validate methods, prepare appropriate documents for peer review of the methods, and develop
27 technical guidance and test guidelines in support of the EDSP.

28
29 Following the validation studies, results of an external scientific peer review of the study
30 and the optimized protocols will be used to develop the U.S. EPA test guidelines.

31 32 **2.3 Purpose of the Review on Steroidogenesis**

33
34 The purpose of Work Assignment 2-6 is to prepare a Detailed Review Paper (DRP) to
35 survey and investigate the status of steroidogenic assay methodologies. The steroidogenic
36 pathway includes several potential target sites for pesticides, industrial substances,
37 environmental contaminants, and other such substances. For this reason, an *in vitro*
38 steroidogenic assay is proposed to be used in the Tier 1 Screening Battery. It is also the purpose
39 of this DRP to identify the most promising assay that could be used as a screen from among the
40 various steroidogenic assays, as well as the steps that are necessary to develop and evaluate the
41 assay's protocol for further standardization and validation.

42 43 **2.4 Objective of the Steroidogenic Screen Assay**

44
45 The objective of the steroidogenic screen assay is to detect any substance that would
46 disrupt estrogen and/or androgen gonadal steroid hormone production. In this way, the assay
47 will complement the other Tier 1 assays so as to provide the necessary breadth and depth to

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

15 16 **2.5 Methodology Used in the Analysis**

17
18 Appendix A describes the methods employed for the literature search (i.e., key words,
19 databases used, results, etc.). Briefly, after key papers were identified, retrieved, and read for
20 content, pertinent information was extracted and synthesized to generate this DRP. In addition
21 to the literature review, interviews with experts were conducted to obtain current views and
22 opinions regarding assays, methods, procedures, and measurement endpoints that hold promise
23 for identifying and developing the most promising screening assay to identify substances that
24 affect (i.e., inhibit or enhance) steroidogenesis. The results of the interviews are found in
25 Appendix B. Finally, accompanying this report is a CD ROM that has the Reference Manager
26 Database of all documents reviewed. This database includes the reference citation and abstract,
27 in addition to summary information from each article.

28 29 **2.6 Definitions**

30
31 The definitions of terms that were utilized throughout the DRP are presented below:

32
33 *ex vivo* - treated *in vivo* but observed *in vitro*

34
35 hypophysectomized - removal of the pituitary gland

36
37 *in vitro* - outside the body, in an artificial environment

38
39 *in vivo* - within the body

40
41 perfusion - media is pumped into the organ via cannulated blood vessels

42
43 perfusion - media is pumped through a chamber containing the organ (the media
44 surrounds the organ)

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3.0 GENERAL BACKGROUND ON REPRODUCTIVE ENDOCRINOLOGY

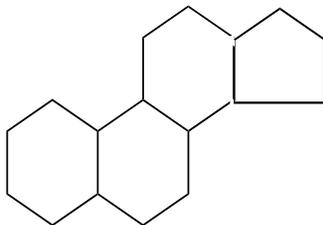
The following material provides background information about the mammalian endocrine system, especially as it pertains to relevant aspects of the reproductive system. The hormones of the reproductive system are synthesized in a steroid-producing sequence of reactions termed steroidogenesis, which is 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.)

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

10 **3.1.2 Hormone Release and Transport**

11
12
13 Release of hormones varies by the type of hormone and the way in which the hormone is
14 stored. Hormones stored in storage granules, i.e., amines and polypeptides, are released by
15 exocytosis. As for the steroid hormones, their release is dependent on a stimulus for production
16 and, once synthesized, their release can occur as a result of passive diffusion. The steroid
17 hormone flows down a concentration gradient created by the site of production relative to the
18 dynamic flow of the blood circulation. Thus, for steroid hormones, the limiting factor in their
19 release is the rate of production.

20
21 The rate of release of hormones varies, at times, in a rhythmic fashion. For example, the
22 hypothalamus releases gonadotropin releasing hormone (GnRH) in a pulsatile pattern. GnRH, in
23 a receptor-mediated process, stimulates cells of the anterior lobe of the pituitary to secrete the
24 gonadotrophins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are
25 also released in a similar pulsatile pattern. Pulsatile patterns of GnRH releases continue
26 throughout adulthood. The specific patterns of GnRH release define the effect on release of FSH
27 and/or LH in synchronous or asynchronous patterns.

28
29 Transport of hormones occurs via the circulatory system. While the amine and peptide
30 hormones are soluble enough to be carried in the plasma, the steroid hormones are not. A
31 transport protein is required for the steroid hormones. The steroid hormones are transported
32 bound to a plasma protein, such as albumin. In addition, there are specific binding transport
33 proteins. For example, testosterone-binding globulin transports testosterone in the circulatory
34 system.

35 **3.1.3 Hormone Action at the Cellular Level**

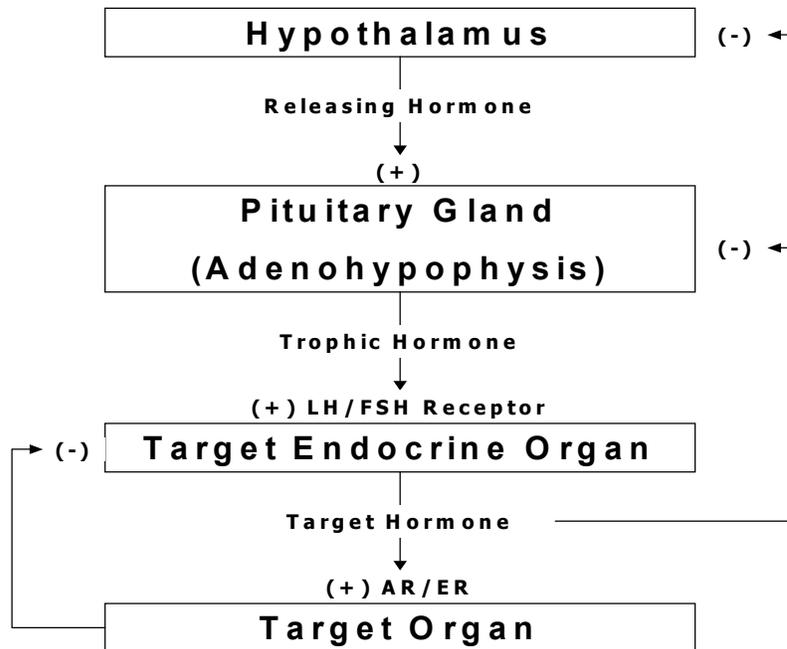
36
37
38 Hormone effects are dependent upon the hormone binding with a specific receptor.
39 Receptors for hormones are found on the cell surface or inside the cell. The receptor site for the
40 steroid hormones is intracellular. Briefly, steroid hormones, once released from a transport
41 binding protein, diffuse into the cell and bind to an intracellular receptor, which can be located in
42 the cytoplasm or nucleus. This hormone-receptor complex initiates a series of reactions in the
43 cytoplasm or regulates transcription. If the complex affects gene transcription, then a mRNA
44 molecule is formed, which is transported to the cytoplasm, where proteins are synthesized for
45 mediating the effect of the steroid hormone.
46

1 **3.1.4 Hormone Metabolism and Excretion**

2
3 The pathway for inactivation of a hormone depends on the type of hormone. Peptide
4 hormones are metabolized by proteases. Steroid hormones are metabolized by enzymes that
5 reduce, oxidize, hydroxylate, etc., the molecule for subsequent addition of a glucuronide or
6 sulfate conjugate that can be more readily excreted. Excretion occurs in the urine, bile or feces.
7 Steroid hormones that are excreted in the bile may be hydrolyzed in the gastrointestinal tract and
8 then reabsorbed back into the body through the portal system circulation.
9

10 **3.1.5 Control of Hormonal Secretion**

11
12 Feedback systems control the secretion and synthesis of hormones from endocrine glands
13 (Figure 3-1). In general, when affected by a stimulus, a given endocrine gland will increase
14 secretion of its hormone. However, once the desired physiological effect occurs, information is
15 fed back to the endocrine gland responsible for producing the hormone, and further secretions
16 are reduced or stopped. In contrast, under-secretion of a given hormone will stimulate cells in
17 the endocrine gland to increase production and secretion. This negative feedback mechanism is
18 specific for each producing gland and its hormone.
19



20
21 **Figure 3-1. Endocrine System Regulation – A Feedback Mechanism**
22

1 The hormonal feedback system comprises four primary organs: the hypothalamus, the
2 pituitary gland, the hormone secreting target gland, and the target organ. These four sites are in
3 communication with each other via the bloodstream, and they interact to regulate hormone
4 synthesis and secretion. In males, this feedback system is referred to as the
5 Hypothalamic-Pituitary-Testicular Axis; likewise, in females, it is referred to as the
6 Hypothalamic-Pituitary-Ovarian Axis.
7

8 The following sections summarize the function and interrelated operation of the
9 hypothalamus, the pituitary, and the hormone secreting target glands. In addition, descriptions
10 using examples from the reproductive system are included to provide additional information
11 about steroidogenesis.
12

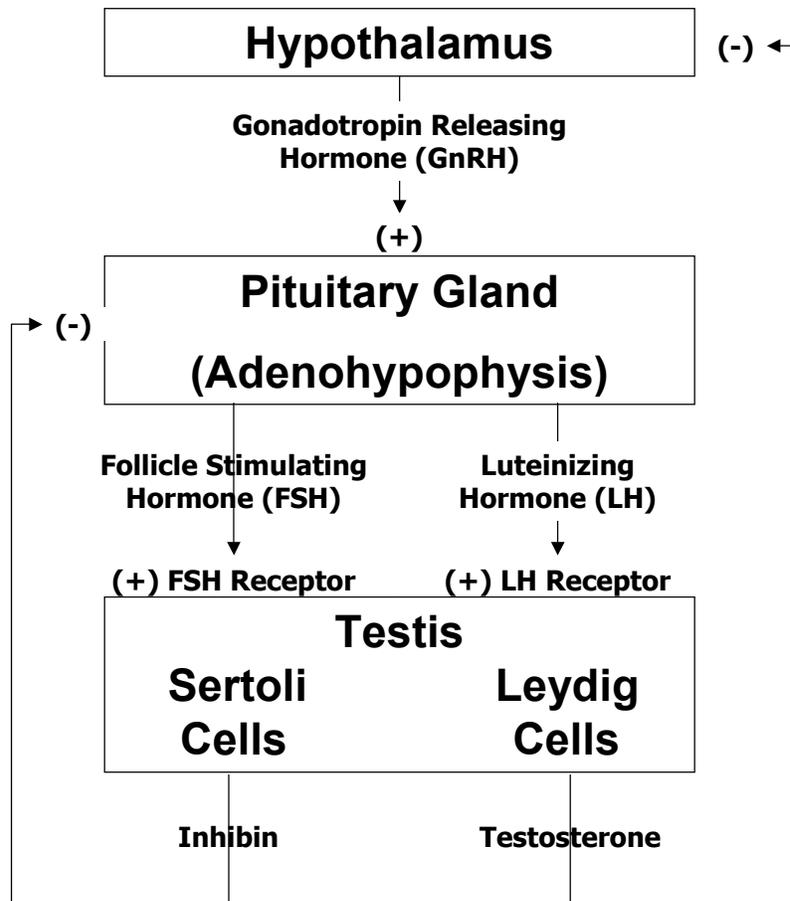
13 **3.1.5.1 Hypothalamus.** The hypothalamus, located in the diencephalic region of the
14 brain, receives signals from other components of the central nervous system. These signals are
15 used to control the secretion of substances from the pituitary gland, which is attached by a stalk
16 to the hypothalamus. Hypothalamic-directed control of pituitary gland hormones occurs via a
17 neuro-secretory neuron network (neurohypophysis) or a hypothalamo-hypophyseal portal system
18 blood vessel network (adenohypophysis). The hypothalamus exerts control over the
19 reproductive system by secreting releasing or inhibitory factors into the hypothalamic-pituitary
20 portal system, which then act on the anterior pituitary gland. The hypothalamus releasing factors
21 pertinent to controlling reproduction are the follicle-stimulating releasing factor (FRF),
22 luteinizing hormone releasing factor (LRF), and prolactin-inhibiting factor (PIF). The releasing
23 factors FRF and LRF are also referred to as the gonadotropin-hormone releasing factors (GnRH).
24

25 It is generally believed that the central nervous system (CNS) is the trigger point for
26 initiation of sexual maturation in the male and female rat (Goldman et al., 2000; Stoker et al.,
27 2000). GnRH levels can be viewed as an indicator of initiation of sexual maturation. GnRH is
28 present in the fetal brain and slowly increases until the second postnatal week in females, and the
29 third postnatal week in males. At that point, GnRH increases steeply and remains elevated until
30 puberty. At puberty, the GnRH neurons undergo a morphological change, developing spiny-like
31 processes that may be related to an increase in synapses on the cells. It has been shown that, at
32 puberty, the GnRH neurons become more responsive to neurotransmitter (norepinephrine and
33 dopamine) stimulation.
34

35 **3.1.5.2 Pituitary Gland.** The pituitary gland is found at the base of the brain and is
36 composed of two main lobes: the adenohypophysis and neurohypophysis. The adenohypophysis
37 is composed of different cell types that are richly innervated by a capillary system. The different
38 types of cells can be differentiated by specific histological stains, and each is generally
39 associated with the production of a specific hormone. The cells, when stimulated by the
40 hypothalamic releasing hormones, secrete hormones into the blood vessels. The
41 adenohypophysis secretes numerous hormones such as the gonadotropins FSH and LH, and the
42 lactating promoting hormone prolactin (Prl). LH and FSH travel via the blood supply to the
43 target endocrine organs, e.g., the testes (male) and ovaries (female). These hormones are
44 essential for sexual maturation and reproductive activity. The neurohypophysis has no role in
45 the reproductive system or its development.
46
47

1 **3.1.5.3 Hormone-Secreting Target Gland.** The reproductive system's target endocrine
2 organs are the testes and ovaries. These organs release hormones that regulate the target organs,
3 e.g., the mammary glands, uterus, or other structures involved in reproductive activity.
4

5 In the testis, regulation of hormone releases involve coordinated communication among
6 different cells and tissues (Figure 3-2). LH binds to receptors on the interstitial cells of Leydig
7 to stimulate the synthesis of testosterone. FSH binds to receptors on Sertoli cells that release and
8 metabolize factors required for spermatogenesis. FSH also increases the number of LH receptors
9 in the testis, which in turn increases testosterone production and testis growth. Sertoli cells have
10 receptors for both FSH and testosterone for additional coordination between the Sertoli and
11 Leydig cell populations within the testis (Russell et al., 1990). The Sertoli cells also produce a
12 glycopeptide, inhibin, that provides negative feedback on the release of FSH from the anterior
13 pituitary gland.
14



15
16 **Figure 3-2. Feedback System of the Hypothalamic-pituitary-testicular Axis**
17

1 In humans, a 28-day cycle is used to describe the changes in the ovary. This cycle has
2 three phases: follicular phase, luteal phase, and menstrual phase (hormonal withdrawal). At the
3 end of one cycle and the beginning of the next, the concentrations of estrogen and progesterone
4 begin to decrease, which stimulates the anterior pituitary gland to secrete FSH. As FSH
5 increases, numerous follicles in the ovary begins to develop. The developing follicle secretes
6 increasing amounts of estradiol from the granulosa cells. A positive feedback mechanism causes
7 the estradiol level to begin to increase.
8

9 It is during the follicular phase that LH stimulates thecal cells to increase steroid
10 hormone biosynthesis. The product is androstenedione, which crosses into the granulosa, where
11 it is enzymatically converted by aromatase to estrone (see the section on steroidogenesis). FSH
12 stimulates this conversion. Also during the follicular phase, progesterone, secreted from the
13 follicle, begins to rise. Just before ovulation, estradiol secretion peaks, inducing an LH surge to
14 occur, in turn inducing the follicle to rupture, thereby resulting in ovulation. As the luteal phase
15 begins, FSH and LH decrease and progesterone levels increase. Also, estrogens begin to rise
16 again, which further decreases FSH and LH levels. As the luteal phase nears the end, the
17 estrogen and progesterone levels fall, inducing the menstrual period. Low levels of these
18 hormones result in stimulating the release of FSH, and the cycle starts again.
19

20 **3.1.6 Sexual Development**

21
22 Androgens and estrogens are essential in the development of the reproductive system. In
23 addition, these hormones are needed for feedback regulation of the hypothalamic-pituitary axis,
24 sex accessory organ development and maintenance, spermatogenesis in males, and oogenesis in
25 females (Goldman et al., 2000; Stoker et al., 2000). The differentiation of tissue into a male or
26 female gonad is chromosome dependent. The presence of the Y chromosome results in testicular
27 development, and, if the Y chromosome is absent, then ovaries develop. Gonadal development
28 dictates the types of hormones produced, which determine whether the embryo exhibits a male or
29 female phenotype. The wolffian and müllerian ducts, found in the early embryo, give rise to
30 either male or female sex characteristics. The presence of testes results in the production of two
31 hormones: müllerian-inhibiting substance (antimüllerian hormone-AmH) and testosterone. AmH
32 causes the müllerian ducts to disappear and suppresses uterine and fallopian tube development.
33 Testosterone causes the wolffian ducts to develop into the epididymides, vasa deferentia, and
34 seminal vesicles. For the female, the müllerian ducts develop into the fallopian tubes, uterus,
35 and upper vagina, but the wolffian ducts disappear. Thus, differentiation of these two tissues
36 into male or female is dependent on the production of androgens from the testes. If present, then
37 male; if absent, then female. The ovary is not necessary for female development.
38

39 Testosterone and dihydrotestosterone (DHT) are the two most active androgens. In the
40 male, testicular descent and development; maturation of the epididymides, vas deferens, seminal
41 vesicles, levator ani/bulbocavernosus; and other aspects of the male reproductive tract are
42 dependent upon testosterone. DHT is responsible for development of the male urethra and
43 prostate and the formation of the penis and scrotum, and male secondary sexual characteristics
44 such as scrotum and penis development.
45

46 Target organs in males and females also includes the primary sexual organs (penis and
47 clitoris), the secondary sex targets (e.g., muscle, bone, skin, hair follicles, and sweat glands), and

1 sex accessory glands (e.g., seminal vesicles and prostate gland in males, and the uterus and
2 breasts in females). These male and female target organs respond to testosterone in males and
3 estradiol or progesterone in females. These target organs require steroids for functional integrity
4 and growth (in juveniles). Changes in hormones in a cyclic manner (over days) are responsible
5 for maintenance of the menstrual cycle in human females and the estrus cycle in domestic
6 animals.

7 8 **3.2 Steroidogenesis**

9
10 The endocrine system synthesizes three types of hormones—polypeptides, amines, and
11 steroids. Synthesis of the latter hormone type is referred to as steroidogenesis. In short,
12 steroidogenesis is the biosynthetic pathway that produces steroid hormones. Although
13 steroidogenesis is a general term that refers to the biosynthesis of any chemical substance with a
14 steroid nucleus, in the context of this review paper it will be used to describe the production of
15 gonadal steroid hormones. Steroid hormones participate in the control and regulation of the
16 reproductive system.

17
18 The following subsections focus on the sites and pathway of the steroidogenic
19 biosynthetic processes for the reproductive system. It is important to point out that although a
20 lot of detail about the steroidogenic pathway is presented, an assay used as a screen will not
21 provide specific information about the site or mechanism of action of a test substance. However,
22 a thorough description of the steroidogenic pathway is believed necessary in order for one to
23 appreciate the possible number of sites in which an endocrine disruptor compound can act.

24 25 **3.2.1 Site of Steroidogenesis**

26
27 The reproductive system steroid hormones are produced primarily in the gonads,
28 although some steroidogenic chemical reactions are also found at peripheral tissue sites. For the
29 male, the steroidogenic pathway is found in the testes and, to a much lesser extent, the adrenal
30 glands. Within the testis, steroidogenesis occurs in the Leydig cell. The Leydig cells are
31 interstitial cells that lie interdispersed among the seminiferous tubules. Inside the Leydig cell,
32 the steroidogenic pathway begins in the cytoplasm and includes chemical reactions that occur in
33 the mitochondria and smooth endoplasmic reticulum, where the final end-product hormone, i.e.,
34 testosterone, is produced (Chen et al., 1996). Other active androgenic hormones are produced in
35 the testis and at peripheral tissue sites. Several peripheral tissues are involved in testosterone's
36 role as a prohormone (Figure 3-3). For example, testosterone is converted to estradiol in the
37 liver and brain (hypothalamus) and converted to dihydrotestosterone in the liver, brain, prostate,
38 and external genitalia.

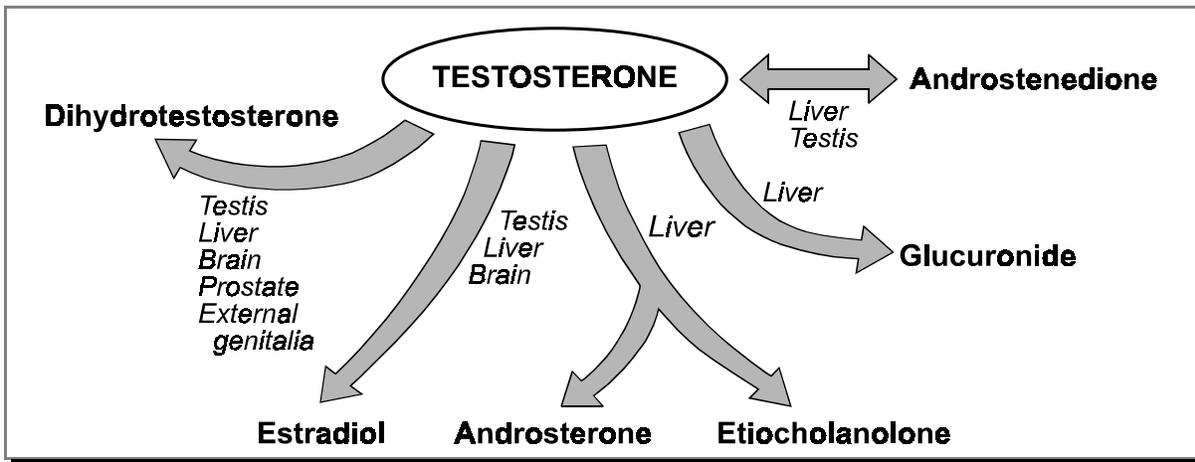


Figure 3-3. Testosterone Conversion in Peripheral Tissues

2 Source: Federman (1981)

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32

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 mitochondrial 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.

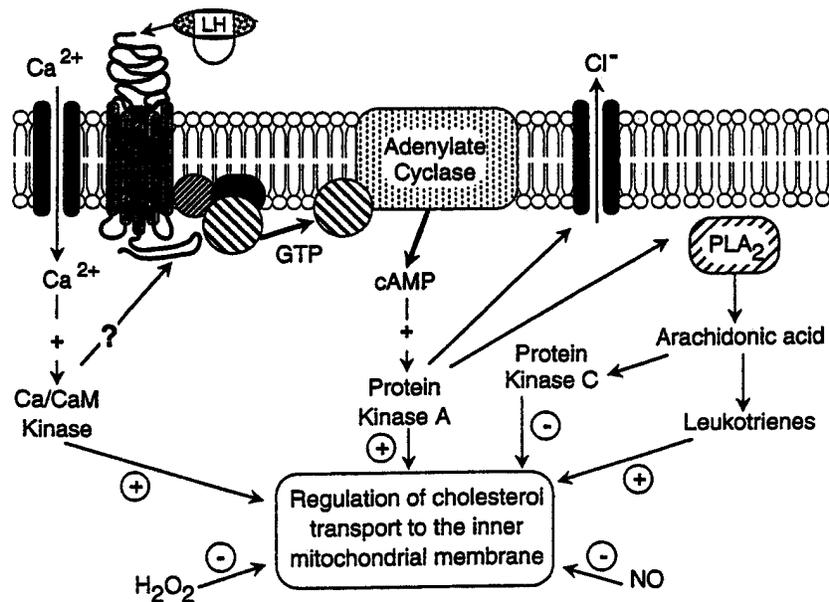


Figure 3-4. Signal Transduction in the Leydig Cell

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 (Ca²⁺) 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.

1 LH stimulation increases the release of arachidonic acid in the Leydig cell (Naor, 1991;
2 Cooke, 1996). Arachidonic acid appears to act as an intracellular mediator and also appears to
3 produce a direct inhibitory effect and an indirect stimulatory effect on steroidogenesis. Steroid
4 hormone production is inhibited when arachidonic acid activates protein kinase C. However,
5 metabolism of arachidonic acid to its metabolites, e.g., leukotrienes, stimulates cholesterol
6 transport into the mitochondria, thereby enhancing steroid hormone production.
7

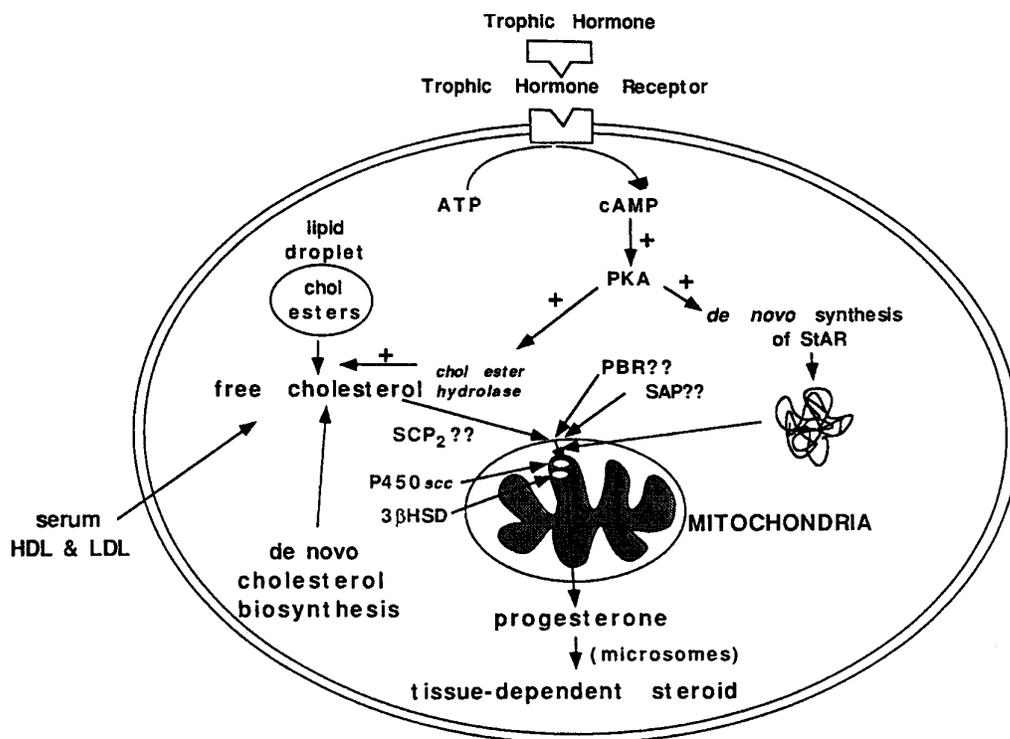
8 Other intracellular substances shown to affect steroidogenesis include free radicals,
9 i.e., superoxide anion and hydroxyl free radical, as well as hydrogen peroxide and nitric oxide.
10 Molecular oxygen is needed for proper function of the cytochrome P₄₅₀ enzymes, which are used
11 in the synthesis of intermediary and end-product hormones. However, molecular oxygen leads
12 to the formation of free radicals, which damage Leydig cells (Kukucka and Misra, 1993).
13 Hydrogen peroxide (H₂O₂) inhibits steroidogenesis by affecting cholesterol transport and
14 inhibiting 3β-hydroxysteroid dehydrogenase, the enzyme that converts pregnenolone to
15 progesterone (Clark et al., 1994). As for nitrous oxide (NO), this molecule diffuses through cell
16 membranes, is formed in the testis, and affects steroidogenesis (Davidoff et al., 1995). Although
17 its site of action is not clear, nitrous oxide has a negative effect on steroid hormone production.
18

19 In summary, stimulation of the LH membrane-bound receptors initiates intracellular
20 events, and this post-receptor signal transduction constitutes the beginning stage of
21 steroidogenesis. Steroidogenic signal transduction involves the second messenger, cyclic AMP,
22 and stimulation of protein kinase A, which leads to increased cholesterol transport and utilization
23 for the production of steroid hormones. The pathways and molecules that comprise
24 steroidogenic signal transduction can be altered by several substances, and this alteration can
25 have a stimulatory or inhibitory effect on steroid hormone production. Thus, it is important to
26 understand the role signal transduction plays in steroidogenesis to better identify substances that
27 could potentially alter steroid hormone production through interactions at intracellular sites.
28

29 **3.2.2.2 Cholesterol Synthesis and Transport.** Steroidogenic signal transduction
30 initiates events that result in the next stage of steroidogenesis: intracellular biosynthesis of
31 cholesterol, mobilization of intracellular storage depots of cholesterol, and cholesterol transport
32 from the cytoplasm to the mitochondria. These events were elegantly determined and are
33 reviewed in detail by Stocco (1999). The intracellular events involving cholesterol in the
34 biosynthesis of steroid hormones are illustrated in Figure 3-5.
35

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

1 of cholesterol across this membranous, aqueous, mitochondrial gulf is the rate-limiting step in
 2 steroidogenesis.
 3



4
 5 **Figure 3-5. Intracellular Biochemical Pathway Following Trophic Hormone Stimulation**

6
 7 Source: Stocco (1999)

8
 9 The transport of cholesterol from the outer to the inner mitochondrial membrane requires
 10 a transport protein. LH stimulation of steroidogenic cells activates *de novo* production of the
 11 cholesterol transport protein. This protein is essential for steroidogenesis and, since it mediates
 12 the rate-limiting step of steroid hormone production, it is referred to as the steroid acute
 13 regulatory (StAR) protein. Investigations that led to the identification of the StAR protein
 14 reported that it was rapidly synthesized, cycloheximide-sensitive (dependent on *de novo* protein
 15 synthesis), and highly labile (short half-life). The StAR protein is synthesized in the cytoplasm
 16 as a precursor molecule. It is transported to the mitochondria, where it is cleaved to its active
 17 form. In the mitochondria, StAR protein transports cholesterol to the inner mitochondrial
 18 membrane, where the first cytochrome P450 enzymatic conversion takes place. At this site, the
 19 side-chain cleavage enzyme (P450_{scc}) is found. This enzyme catalyzes cholesterol into
 20 pregnenolone.
 21

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

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

5
6 In summary, cholesterol is the common precursor molecule for production of all steroid
7 hormones. Cholesterol is synthesized, mobilized from storage depots, and transported to the
8 mitochondria following LH-stimulated signal transduction. Transport of cholesterol from the
9 outer to the inner mitochondrial membrane is the rate-limiting step of steroidogenesis.
10 Cholesterol is carried between the membranes by the StAR protein. At the inner membrane, the
11 first of a series of enzymatic reactions occurs, whereby cholesterol is converted to pregnenolone
12 by P450_{scc}.

13
14 **3.2.2.3 Enzymatic Conversions.** Enzymatic conversion of cholesterol to pregnenolone
15 constitutes the initial step in a series of biochemical reactions that culminate in end-product
16 hormone production. Figure 3-6 summarizes the final stage of the steroidogenic biosynthetic
17 pathway, as well as the cell types for males and females and the intracellular location of various
18 enzymatic steps of the steroidogenic pathway. The remainder of this section describes the
19 enzymatic reactions in detail.

20
21 The first enzyme reaction is the conversion of cholesterol to pregnenolone by the
22 cytochrome P450 cholesterol side-chain cleavage enzyme (P450_{scc}). P450_{scc} activity is also
23 considered to be a rate-limiting step in the production of gonadal steroid hormones. This
24 reaction occurs on the inner membrane of the mitochondria and involves three sequential
25 oxidation reactions, each requiring molecular oxygen and NADPH. The reactions add two
26 hydroxyl groups to cholesterol (at C₂₂ and C₂₀) followed by cleavage between the added
27 hydroxyl groups. As a result of these reactions, cholesterol, a 27-carbon sterol, is cleaved of its
28 6-carbon group termed the “side chain,” thereby resulting in production of pregnenolone, a 21-
29 carbon steroid (Kagawa & Waterman, 1995).

30
31 The second enzymatic reaction results in the conversion of pregnenolone to progesterone
32 by the enzyme 3 β -hydroxysteroid dehydrogenase/^{a5} - ^{a4} isomerase (3 β -HSD). This reaction is
33 also believed to occur on the inner membrane of the mitochondria. It is hypothesized that an
34 interaction of the StAR protein with the inner mitochondrial membrane could cause the
35 formation of P450_{scc} and 3 β -HSD, thereby allowing cholesterol to be converted to pregnenolone
36 and then to progesterone rather quickly (Stocco, 1999). 3 β -HSD catalyzes dehydrogenation and
37 isomeration of pregnenolone to progesterone, which converts a ^{a5}-3 β -hydroxysteroid to a
38 ^{a4}-3-ketosteroid, the active form of steroid hormones. It is also possible that pregnenolone is
39 converted to progesterone in the cytosol by 3 β -HSD, as well. Thus, the steroidogenic pathway
40 bifurcates into a ^{a5}- hydroxysteroid pathway (starting with pregnenolone) and a ^{a4}-ketosteroid
41 pathway (starting with progesterone) and, even though the same enzymes use different substrates
42 along the parallel pathways, both pathways converge. The result is the production of
43 androstenedione. 3 β -HSD converts the ^{a5}- hydroxysteroid pathway substrates,
44 17 α -hydroxypregnenolone and dehydroepiandrosterone (DHEA), into their respective
45 ^{a4}-ketosteroids, 17 α -hydroxyprogesterone and androstenedione, respectively.

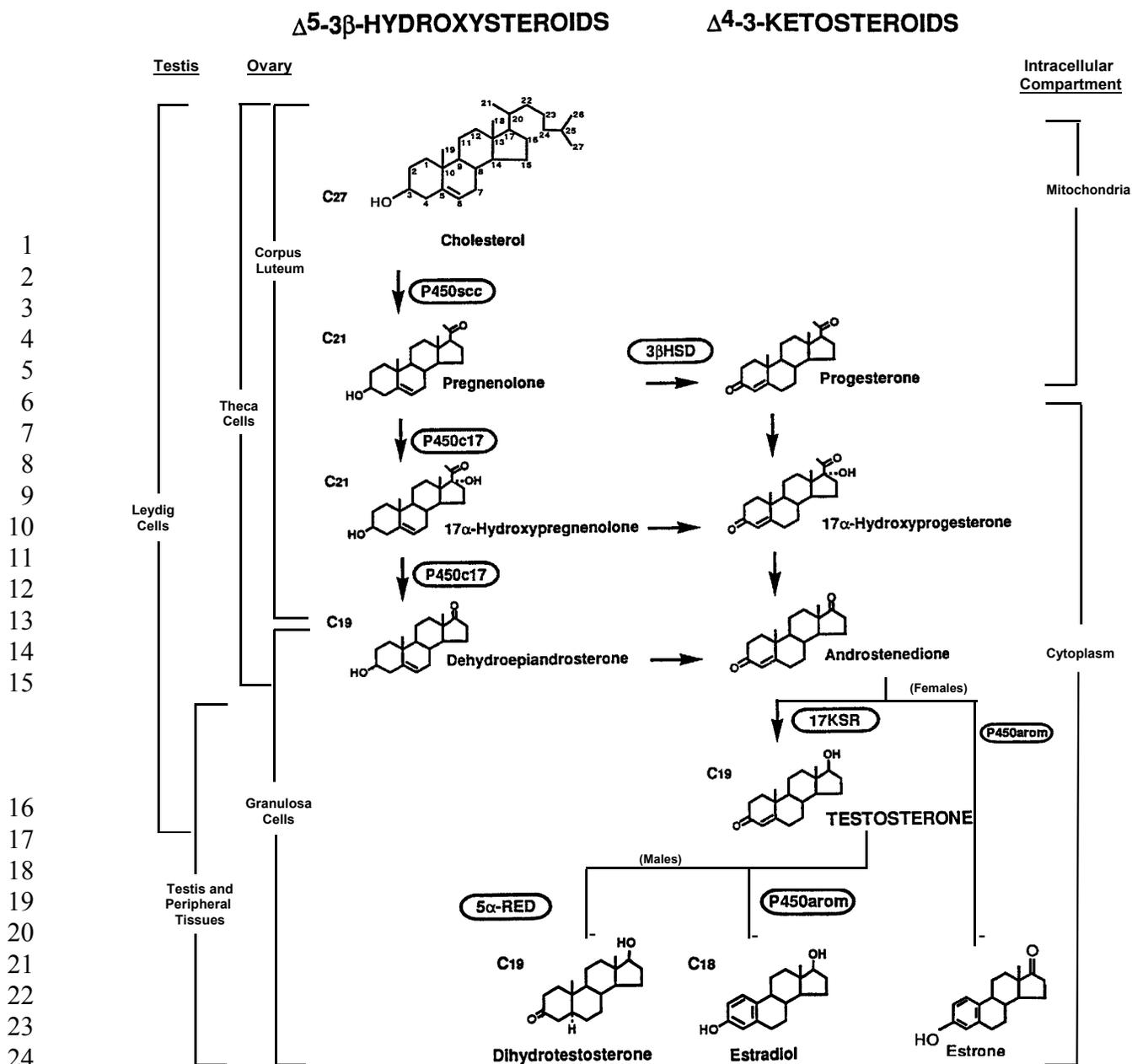


Figure 3-6. Enzymatic Conversions of Cholesterol and Intermediate/End-Product Hormones

The third enzymatic reaction involves cytochrome P450 17 α -hydroxylase/C₁₇₋₂₀ 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

1 17 α -hydroxypregnenolone, which is then converted to DHEA. As mentioned above, DHEA is
2 converted to androstenedione by 3 β -HSD. Likewise for the ^{a4}- ketosteroids, P450c17 converts
3 progesterone to 17 α -hydroxyprogesterone, which is then converted to androstenedione. The
4 lyase activity of P450c17 differs for the intermediate substrates among species. For example, in
5 the human, P450c17 converts 17 α -hydroxypregnenolone to DHEA (^{a5}- hydroxysteroid pathway)
6 but not 17 α -hydroxyprogesterone to androstenedione (^{a4}- ketosteroid pathway). In comparison,
7 in the rat, P450c17 converts the intermediates of both the ^{a5}- hydroxysteroid and ^{a4}- ketosteroid
8 pathways equally. Such differences in the P450c17 lyase activity may explain species-dependent
9 differences in response to substances that alter steroidogenesis.

10
11 The next enzymatic reaction involves the conversion of androstenedione to testosterone
12 by 17-ketosteroid reductase (17KSR), which is also referred to as 17 β -hydroxysteroid
13 dehydrogenase (17 β -HSD). The production of testosterone is considered an end-hormone
14 product. A second possible reaction involving androstenedione occurs in the female, whereby
15 androstenedione is converted to estrone by aromatase. (Aromatase is described in further detail
16 below). The conversion of androstenedione to testosterone is reversible and dependent on
17 product concentrations. 17KSR is able to catalyze either the reduction (forward) or the oxidation
18 (reverse) reactions. The cofactors NADH/NAD⁺ are used in this interconversion.

19
20 In the male, testosterone is converted to dihydrotestosterone (DHT) by 5 α -reductase,
21 which is found in the cellular membranes, nuclear envelope, and endoplasmic reticulum. DHT is
22 significantly more potent as an androgen than testosterone and is also considered an end-product
23 hormone. DHT is produced primarily in peripheral tissues, although it is also found in the testis.
24 The activity of 5 α -reductase in the Leydig cells and testes varies with age; the highest activity
25 occurs around puberty.

26
27 The last enzyme in the steroidogenic pathway is aromatase. Aromatase converts
28 testosterone into estradiol and, in the female, androstenedione into estrone. In short, aromatase
29 converts androgenic substances into estrogenic substances. As mentioned above for testosterone
30 and DHT, estradiol and estrone are considered end-product hormones of the steroidogenic
31 pathway. Aromatase is found in many different peripheral tissues, as well as male and female
32 gonadal tissue. The activity of this enzyme varies with species and age. Aromatase is an
33 enzyme complex (two cytochrome P450 enzymes: a reductase and an aromatase) bound to the
34 endoplasmic reticulum. The complex catalyzes two hydroxylation steps and the aromatization
35 (hence the name aromatase) of Ring A in the steroid nucleus, which results in the loss of the
36 C-19 carbon atom, thereby producing a C-18 molecule characteristic of estrogens. The reaction
37 requires molecular oxygen and NADPH.

38
39 Production and activity of these enzymes are under hormonal control. The P450_{SCC} and
40 P450c17 enzymes are regulated by LH. In the male, FSH stimulates release of a Sertoli cell
41 factor that increases the effect of LH on 3 β -HSD activity. For females, FSH increases the
42 activity of aromatase, thereby enhancing the conversion of androstenedione to estrone. In
43 addition to regulatory effects of gonadotropins, gonadal hormones regulate steroidogenic
44 enzymes. For example, testosterone inhibits P450c17 activity, which occurs via effects on the
45 second messenger cAMP pathway. Testosterone also suppresses 3 β -HSD through inhibitory
46 effects on cAMP-mediated 3 β -HSD mRNA.

1 In summary, cholesterol is the common precursor for production of steroid hormones. A
2 series of biochemical reactions involving different enzymes results in conversion of cholesterol
3 to end-hormone products: testosterone, DHT, estradiol, and estrone. The steroidogenic pathway
4 is regulated by gonadotropins and end-product hormones. An alteration of the regulatory
5 mechanisms, as well as direct effects on the substrates and enzymes of the steroidogenic
6 pathway, can affect end-hormone product formation, thereby possibly resulting in reproductive
7 system toxicity.

8 9 **3.3 Steroidogenic Pathway Defects and their Effects on Sexual Development**

10
11 Alteration of the hormonal steroidogenic pathway can lead to abnormal sexual
12 development. If sex chromosomes are altered at fertilization due to mutations, cross-over
13 abnormalities, chromosomal aberrations, etc., then there will be abnormal gonad development,
14 which, in turn, may lead to defects in hormonal steroidogenesis. A deficiency in an enzyme at
15 any one step of the steroidogenic pathway will change the pattern of production and eventually
16 secretion of steroid hormones. This section summarizes many of the disorders in mammalian
17 male and female sexual development that occur as a result of defects in the steroidogenic
18 pathway. In general, any defect in steroidogenesis at the embryonic stage can lead to
19 pseudohermaphroditism or defective masculinization in mammals. (Kelce and Wilson, 1997;
20 Gray et al., 1999). Although pseudohermaphroditism is caused by many different defects, the
21 following information will focus on gonadal steroidogenic pathway deficiencies and the defects
22 that develop (Wilson and Griffin, 1994).

23 24 **3.3.1 StAR Gene Suppression**

25
26 Negative regulation of the StAR gene has been associated with adrenal hypoplasia
27 congenita (AHC) and hypogonadotropic hypogonadism (HHG) (Stocco, 1999). These diseases
28 are attributed to mutations in the DAX-1 gene, which expresses the dosage-sensitive sex reversal
29 transcription factor (DAX-1). Also, duplication of that part of the X chromosome where the
30 DAX-1 gene is found results in a male-to-female sex reversal. Excessive expression of the
31 DAX-1 gene leads to excessive levels of DAX-1, which inhibits expression of the StAR gene
32 (Zazopoulos et al., 1997). Complete blockage of the active StAR protein is the cause of
33 congenital lipid adrenal hyperplasia (CAH). This disease occurs because the StAR protein is
34 truncated by 28 or 93 amino acids. In addition to blocking gonadal steroid hormones,
35 mineralocorticoid and glucocorticoid production is inhibited, thereby causing death shortly after
36 birth if left undetected.

37 38 **3.3.2 Cholesterol Side Chain Cleavage (P450_{sc}) Enzyme Deficiency**

39
40 This defect is also called lipid adrenal hyperplasia. It occurs as a result of the absence
41 of the P450_{sc} enzyme. Apparently, an error on chromosome 15, where this enzyme is encoded,
42 results in no conversion of cholesterol to pregnenolone. Most individuals die at infancy from
43 this defect due largely to adrenal gland insufficiency. A necropsy of these infants shows that
44 males are incompletely masculinized, whereas females have normal genital development. Also,
45 laboratory tests find no detectable steroids in the urine.

1 **3.3.3 3 β -Hydroxysteroid Dehydrogenase/Isomerase Deficiency**

2
3 This defect is attributed to an error on chromosome 1, where this enzyme is encoded. As
4 a result, pregnenolone is not converted into progesterone in the male and female. In this
5 disorder, males develop a vagina and show varying degrees of feminization, including breast
6 development at puberty. Plasma testosterone levels are low and Delta 5 pathway precursors are
7 elevated. Females exhibit some varying degrees of masculinization. The urine contains no
8 Delta 4 steroids for those individuals with the complete deficiency.

9
10 **3.3.4 17 α -Hydroxylase/17, 20-Lyase Deficiency**

11
12 These two defects are attributed to an error on chromosome 10, where the DNA code for
13 P450_{17 α} resides. Both of these enzymes are mediated by the 17 α P450 enzyme. A deficiency in
14 the activity of these two enzymes alters the conversion of progesterone to androstenedione in the
15 male, and this alters the conversion of both pregnenolone (Delta 5 pathway) and progesterone
16 (Delta 4 pathway) to androstenedione in the female.

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

22
23 In the 17, 20-lyase deficiency, the male exhibits varying degrees of
24 pseudohermaphroditism but can show some masculinization at puberty. No information
25 regarding the physical appearance of the female was found.

26
27 **3.3.5 17 β -Hydroxysteroid Dehydrogenase Deficiency**

28
29 This defect is the most common of the alterations to the hormone steroidogenic pathway.
30 This enzyme converts androstenedione into testosterone in both the male and female. The
31 46,XY individual has a female appearance, which includes a vagina, as well as abdominal testes.
32 At puberty, masculinization occurs. Penile and breast enlargement and facial/body hair
33 development occurs. Plasma testosterone levels are low to normal.

34
35 **3.3.6 5 α -Reductase Deficiency**

36
37 This enzyme converts testosterone to dihydrotestosterone in peripheral tissues. In this
38 defect, abnormal masculinization is localized during embryogenesis to the urogenital sinus and
39 the external genitals, an effect mediated by dihydrotestosterone but not testosterone.
40 Testosterone and estradiol levels are normal, because aromatase catalyzes the conversion of the
41 former to the latter. This deficiency is characterized in the male by a blind vaginal pouch, testis,
42 no enlarged breasts, no internal female genitals, and masculinization at puberty.

1 **3.3.7 Aromatase Deficiency**

2
3 Aromatase catalyzes the conversion of testosterone to estradiol in the peripheral tissues.
4 This deficiency causes disorders of bone maturation in the male and sexual development in the
5 female.

6
7 **3.3.8 21-Hydroxylase or 11 β -Hydroxylase Deficiency**

8
9 The disorder that results from the deficiencies of these enzymes is referred to as
10 congenital adrenal hyperplasia. The enzyme 21-hydroxylase uses a steroidogenic hormone,
11 17-hydroxyprogesterone, as a substrate for the production of an intermediate in the
12 glucocorticoid pathway, 11-deoxycortisol. Obviously, this defect is *not* due to a direct disorder
13 of the steroidogenic pathway; rather this defect is a secondary disorder due to the decreased
14 formation of cortisol, which results in a compensatory increase in ACTH. The effect of
15 increased ACTH not only increases the secretion of cortisol, but it also increases the production
16 of androgenic hormones. In the male, this results in premature masculinization, i.e., early
17 maturation of the penis and secondary sex characteristics. The male may or may not be able to
18 exhibit spermatogenesis, depending on a negative or positive effect of adrenal androgens on the
19 release of GnRH. In the female, masculinization is apparent at birth. The female genitals are
20 abnormal in appearance. At puberty, the female does not exhibit normal female sexual
21 development or menstrual period. Plasma progesterone and 17-hydroxyprogesterone are
22 increased.

23
24 The enzyme 11 β -hydroxylase is also part of the glucocorticoid pathway. It converts
25 11-deoxycortisol to cortisol. A defect in this enzyme results in phenotypic patterns similar to
26 those described above for 21-hydroxylase.

27
28 **3.4 Steroidogenesis: Toxic Effects of Substances**

29
30 Exposure to substances that are not endogenous to the body can lead to chemical
31 reactions that alter the outcome of biochemical pathways. Altering any one step in the
32 steroidogenic pathway for the production of reproductive hormones has the potential to cause
33 toxicity. In general, the disruption of gonadal steroidogenesis could result in an increased
34 concentration of one or more steroids, a decreased concentration of one or more steroids, and/or
35 new steroid synthesis products. Toxic responses to the reproductive system can result in such
36 adverse effects as abnormal sexual and physical development, diminished fertility or sterility,
37 and cancer, to name just a few (Kelce and Wilson, 1997; Gray et al., 1999). The focus of this
38 section is to provide examples of substances that alter specific steps in the steroidogenic pathway
39 as well as to provide a summary table of substances known to produce direct effects on the
40 steroidogenic pathway (Table 3-1).

1 **Table 3-1. Substances and Conditions That Directly Alter Steroidogenesis ^a**
 2

Site of Chemical Action	Reference
<i>cyclic-AMP Second Messenger System</i>	
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
<i>StAR Protein</i>	
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
<i>P450_{scc}</i>	
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
<i>3β-HSD</i>	
Lead	Huang et al., 2002
Daidzein/genistein/biochanin A	Ohno et al., 2002
Lithium chloride	Ghosh et al., 1991
Mibolerone	Fanjul et al., 1989

Table 3-1. Continued

Site of Chemical Action	Reference
Danazol (ethinyltestosterone)	Barbieri et al., 1977
Cyproterone acetate	Lambert et al., 1987
Ethionine	Goldberg et al., 1969
Cyanoketone (WIN-19578)	Goldman et al., 1965
Mitomycin C	Deb et al., 1980
Aflatoxin	Verma and Nair, 2002
<u>P450c17 (17α-hydroxylase/C₁₇₋₂₀ lyase)</u>	
Ethanol (17 α -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
<u>17KSR</u>	
Cotinine	Yeh et al., 1989
Danazol	Barbieri et al., 1977
Cyclosporin A	Kasson and Hsueh, 1985
Lithium chloride	Ghosh et al., 1991
<u>5α-Reductase</u>	
Finasteride	Morris, 1996
<u>Aromatase</u>	
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

Table 3-1. Continued

Site of Chemical Action	Reference
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.

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

The first step in the conversion of cholesterol to steroid hormones involves the enzyme P450_{sc}. 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 11 β -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_{sc}. 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-10 β -oxiranyl-), or C-4 substituted (OH-, formestone), or C-7 substituted

1 (7 α -SC₆H₄-p-NH₂-), or 2 β , 19 bridged (2 β , 19-methylene-), fadrazole, letrozole, and arimidex, to
2 name just a few.

3
4 The reproductive effects of inhibiting aromatase activity are dramatic. At birth, females
5 exhibit pseudohermaphroditism and as adults are amenorrheic, have small breasts, and can
6 develop cystic ovaries. In males, the epiphyseal plates do not calcify, which results in elongated
7 bones and tall stature, as well as osteopenia and sterility (Simpson et al., 2002).

8 9 **3.4.3 Multiple-Site Chemical Inhibitors of Steroidogenic Pathway Enzymes**

10
11 Several substances are recognized for inhibiting more than one of the enzymes of
12 the steroidogenic pathway. The chemical danazol inhibits 3 β -hydroxysteroid dehydrogenase and
13 17 α -hydroxylase, thereby having direct inhibitory effects on the production of steroidogenic
14 hormones. In addition, this chemical inhibits the glucocorticoid enzymes 11 β - and
15 21-hydroxylase, which indirectly affects the synthesis of steroidogenic hormones as previously
16 described. Another chemical that affects multiple sites is spironolactone. This chemical inhibits
17 17 α -hydroxylase of the steroidogenic pathway and 21-hydroxylase of the glucocorticoid
18 pathway. Ketoconazole, an imidazole used as an antifungal agent, inhibits P450_{scc} and C17,
19 20-lyase (Morris, 1996).

20 21 **3.5 Conclusion**

22
23 The hormones of the reproductive system are synthesized in the steroidogenic pathway.
24 The operation of this pathway is critical to the endocrine system, as well as sexual function and
25 development. Any chemical interference to steroidogenesis, e.g., altering enzymatic activity or
26 hormone production, altering precursor availability, interfering with control mechanisms, etc.,
27 can cause adverse effects to the reproductive system. It is for these reasons that an assay is
28 needed to identify substances that could produce toxicity by altering the steroidogenic pathway –
29 an assay that can identify substances that inhibit or stimulate steroid hormone production. Such
30 an assay should identify alterations at the biochemical level – not the physiological level.

31
32 Thus, since the goal of this review paper is to lay the foundation for selecting an assay for
33 screening substances that are disruptors of the endocrine system, the next section provides
34 background information about methods currently used to measure steroidogenesis in mammals.
35
36

1
2
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4

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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:

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

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

1 **4.1 Whole Animal Methods (*In Vivo*)**

2
3 **4.1.1 General Assays**

4
5 Intact animals can be used to measure androgenic and estrogenic activity. *In vivo*
6 methods were the earliest papers cited in the literature; dating back more than 70 years
7 (Allen and Doisy, 1924; Hershberger et al., 1953). Yet, *in vivo* studies still represent a useful
8 method for measuring estrogenicity and androgenicity as demonstrated by more recent
9 investigators (Gray and Ostby, 1998; Kelce et al., 1997). *In vivo* methods take advantage of the
10 animal's innate hormone-directed tissue development and maintenance responses and uses these
11 responses to identify whether administered substances alter the normal response. For this
12 method to work, the animal's inherent capacity to produce the hormones that stimulate the tissue
13 response must be removed. Since this inherent capacity resides in the gonads, *in vivo* studies
14 often involve an ovariectomy or castration. Otherwise, the hormones from the gonadal organs
15 would interfere with the response being measured by the substance being tested. Alternatively,
16 immature or juvenile animals have been used. In this way *in vivo* methods are useful for
17 identifying substances that disrupt the endocrine system.

18
19 *In vivo* studies used for evaluating substances for estrogenicity or androgenicity are not
20 necessarily useful tools for evaluating a direct effect on steroidogenesis. As mentioned above,
21 two widely used bioassays, i.e., uterotropic and Hershberger, generally use gonadectomized
22 animals, thereby removing the organs where steroidogenesis occurs. There is, however, an *in*
23 *vivo* method that uses the fully intact animal and measures a direct effect of a substance on
24 steroidogenesis. The endocrine challenge test (ECT) is a bioassay that can be used to evaluate
25 the gonadal response to a substance by measuring the steroid hormone production and release
26 following trophic hormone stimulation.

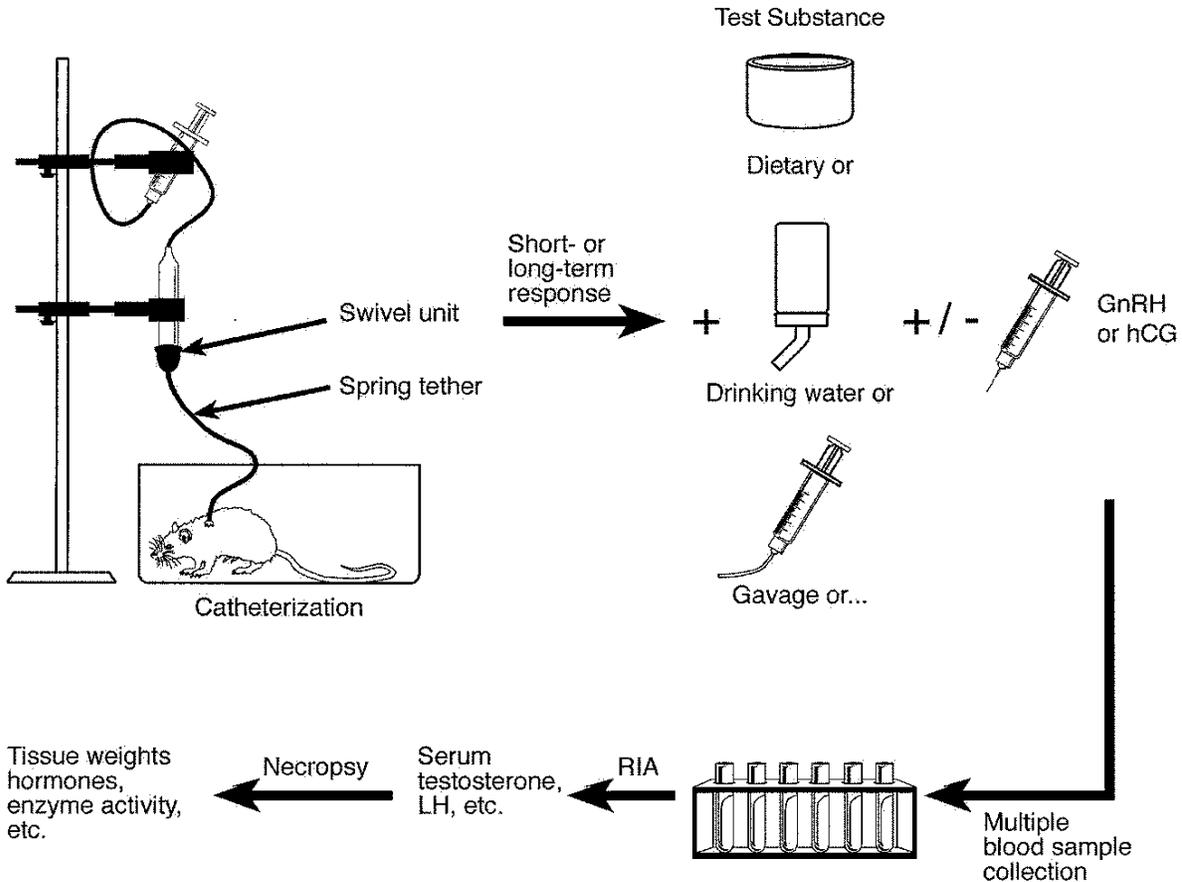
27
28 **4.1.2 Endocrine Challenge Test (ECT)**

29
30 ECT is a recently developed *in vivo* method for measuring changes in steroid hormone
31 production. Although the ECT is an accepted diagnostic method in human and veterinary
32 medicine, only in the past decade, has it been incorporated into endocrine toxicology
33 experiments (Fail et al., 1994; 1995; 1996a, b, c; Anderson et al., 1992).

34
35 **4.1.2.1 Scope of the Test.** In the ECT, the intact mature animal is challenged with
36 GnRH or an LH- or FSH-like substance that stimulates a hormonal response. Serial blood
37 samples are then collected and measured to evaluate whether the substance being tested has
38 androgenic, e.g., increased testosterone, or anti-androgenic e.g., decreased testosterone, activity.
39 The ECT is viewed by some investigators as the best way to detect altered steroidogenesis *in*
40 *vivo* (EDSTAC, 1998). During the past 10 years, ECT has been used to evaluate the functional
41 capacity of the gonads or pituitary (Fail et al., 1992); determine the effects of a substance on
42 GnRH or hCG challenged steroid hormone production (Fail et al., 1994; 1995); assess
43 reproductive toxicity (Fail et al., 1996b; 1996c); and measure thyroid hormone production
44 (Fail et al., 1999).

1 **4.1.2.2 Description of the Test.**

2 A flow diagram for the *in vivo* ECT is illustrated in Figure 4-1.



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

6

7 The ECT can be used to test the functionality of hypothalamic, pituitary, or gonadal

8 responses. To test the functionality of the pituitary for LH or FSH release, the animal is given an

9 injection of GnRH. At designated time periods, samples of blood are taken for measurement of

10 FSH and/or LH concentrations, thereby assessing the release of these adenohipophyseal

11 hormones. If the concentrations of FSH and/or LH are altered, then the functionality of the

12 pituitary is compromised, as would be the target organs and glands that are affected by FSH and

13 LH. In a similar manner and of more significance for this DRP, gonadal steroidogenesis can be

14 tested by injecting an animal with FSH or LH. Alternative stimulants to FSH and LH are

15 pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The

16 primary stimulant used is hCG. Steroidogenesis is stimulated in the gonads by hCG, thereby

17 allowing one to measure the effect of a substance on steroid hormone production.

1 **4.1.2.3 Experimental Design Information.** The ECT has unique aspects in regard to
 2 the approach used to identify steroidogenic inhibitory substances. Other assays have single point
 3 endpoints at necropsy, whereas the ECT collects samples over time. In this way subtle effects
 4 can be detected (Gray et al., 1997; Fail and Anderson 2002). However, the collection of multiple
 5 specimens, i.e., blood samples, can be difficult and require expertise. Techniques used include
 6 tail vein sampling, tail vein treatments and/or jugular catheterization (Fail et al., 1995; Fail and
 7 Anderson, 2002).
 8

9 A number of different endpoints have been used with the ETC. The endpoints with the
 10 most application for evaluating a direct effect on steroidogenic hormone production are plasma
 11 testosterone (males) or estradiol (females), before (basal) and after LH stimulation
 12 (Fail et al., 1995; 1996b). Other endpoints include epididymis weight, caudal sperm count,
 13 testicular sperm heads, and sperm motility (Fail et al., 1995; 1996b).
 14

15 **4.1.2.4 Representative Studies from the Literature.** The hCG-stimulated plasma
 16 testosterone response has been characterized. Fail et al. (1996a) treated adult male
 17 Sprague-Dawley rats with hCG by tail vein injection at dosages of 0, 0.2, 0.4, 0.8, or 8 IU/100 g
 18 BW. Blood and testicular samples were collected at specified time intervals after dosing, and
 19 testosterone concentrations were measured by RIA. Significant changes in testosterone were
 20 observed for 1 to 6 hours after dosing in both testicular and blood samples. Whole testicular
 21 concentrations of testosterone were four times higher than plasma testosterone (Table 4-1).
 22
 23

24 **Table 4-1. Expanded Data Summary for *In Vivo* Preliminary Studies: Defining Dose and**
 25 **Time Responses in Adult Male S-D Rat Plasma and Testicular Testosterone**
 26 **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

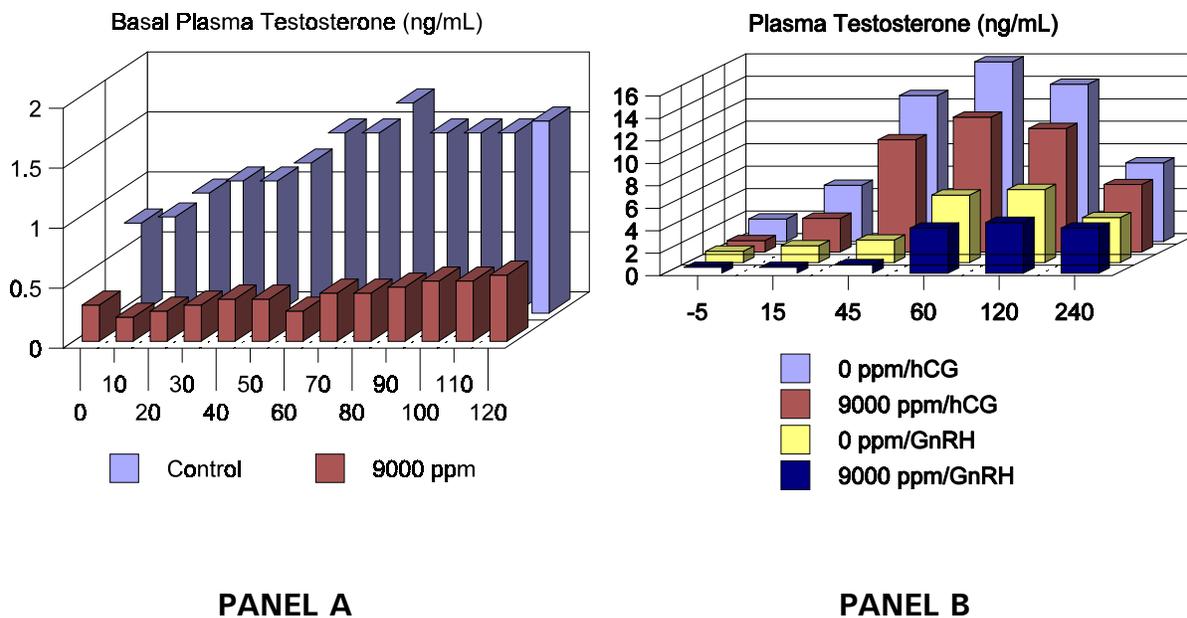
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 50 ^a Values are means ± standard error of the means; n=6.

51 * Significantly different from control (0 IU; within sacrifice time); Dunnett's; p<0.05. (rows)

52 H Significantly different from 1 hour time point (within hCG dose); Dunnett's; p<0.05. (columns)

53
 54 Source: Fail et al. (1996a)
 55
 56

1 A study using ECT for determining whether boric acid had androgenic or anti-androgenic
 2 activity was conducted by Fail and coworkers (Fail et al., 1992; 1998; Anderson et al., 1992).
 3 Data from this study are shown in Figure 4-2.
 4



5
 6
 7
 8 **Figure 4-2. Example Data from an ECT Assay**
 9

10 *Notes: Sprague-Dawley rats, cannulated to collect serial blood samples (every 10 minutes for*
 11 *2 hours) for serum hormone analysis, were treated with boric acid in the feed at a concentration*
 12 *of 0 or 9000 ppm for 2, 9, or 14 days. Gonadal response to trophic stimulation was tested by*
 13 *administering GnRH, 25 ng, iv or hCG, 2.5 IU, iv. Basal serum testosterone was decreased 3- to*
 14 *5-fold at all collection time points (Panel A). Even after challenge with GnRH or hCG, serum*
 15 *testosterone remained lower at most time points (Panel B). Serum FSH was increased about*
 16 *2-fold for the boric acid group on Day 14 but not at Days 2 or 9. Serum FSH concentrations*
 17 *increased at all time points in response to GnRH stimulation (data not shown). The*
 18 *investigators concluded that boric acid compromised steroidogenesis and the ability of the testis*
 19 *to respond to gonadotropin stimulation.*

20
 21 Source: Fail et al., 1998
 22

23 Other studies in the literature that use the ECT for evaluating the androgenicity of a
 24 substance are summarized in Table 4-2.
 25
 26

1 **Table 4-2. Representative Studies Using the ECT Assay**
 2

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	9 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	9 basal plasma testosterone and LH 9GnRH or hCG-stimulated plasma testosterone 8 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	8 basal plasma testosterone and LH but no effect on basal plasma ACTH, or corticosterone 9 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	9 basal plasma testosterone Response to hCG was blocked and/or delayed 8 plasma LH response to GnRH	Fail et al., 1996a

15
16
17 **4.1.2.5 Distinguishing Features of the Method.**
18

19 **Stability**

20 Primarily dependent on catheter patency or viability of tail veins to allow
 21 for serial collection of blood samples. (Animals can be euthanized at each
 22 time point – a cross sectional design – but this decreases the power of
 23 variation). Sample number and volume are critical when repeated samples
 24 are collected in short time periods. Animal growth can affect catheter
 25 placement. Heparanization of animal can occur as a result of leakage from
 26 catheter. Clot formation can occur, which occludes the catheter. Assay is
 27 easily used over a period of a few days and has been used as long as
 28 2 months.

29 **Standardization**

30 Assay has not been standardized. Standardization would involve a
 31 procedure for purity, challenge dosage, method of sample collection,
 32 times for collection, types of samples to be collected, and analysis
 33 methods. Procedures require skill and experience, thereby making
 standardization between laboratories difficult. Other factors for

1 consideration include circadian rhythms and effects of acute stress
2 (Gray et al., 1997).

3
4 **Sensitivity** Increased when collecting basal and challenge response. Increased over
5 other assays in that repeated measurements of hormone levels are possible
6 within an animal rather than one measurement at necropsy or use of
7 multiple animals. False negatives could occur if the substance being
8 tested induces negative metabolism of the hormone being measured in the
9 plasma. If metabolic induction occurs, then an increase in the plasma
10 hormone concentration (caused by the test substances) would not be
11 observed.

12
13 **Specificity** Stress may reduce testosterone levels and increase prolactin and
14 corticosterone, which may interfere with results. Hormone circadian
15 rhythms can also affect assay results (EDSTAC, 1998). Use of cannulated
16 animals and a tether apparatus allows samples to be collected without
17 handling the animal, thereby reducing stress and precluding the need for
18 anesthesia (Fail et al., 1992).

19
20 **Metabolic Activity** Yes. Test substances that require metabolic activation to produce an
21 effect would be identified in this assay. Metabolic induction of the
22 hormone could also occur giving the false negative results described
23 above (see sensitivity).

24
25 **Equipment** Animal facilities. Standard and specialized laboratory equipment. Use of
26 permanent ports provide use (up to 2 months).

27
28 **Training** Animal husbandry, dosing, and necropsy skills. Canula and port insertion
29 surgery, cannula sampling, and tail vein sampling are all specialized skills.
30 Cannulation requires surgical training and sterile technique.

31
32 **Sample Scheduling** Multiple sampling, e.g., every 10 minutes for 2 hours, causes method to be
33 labor intensive. Time to perform study using this method is measured in
34 several days to a couple of weeks, primarily the time it takes to conduct
35 the in-life phase and collect and analyze the samples.

36
37 **Animal Usage** Whole animal assays use relatively more animals than some *in vitro*
38 assays, e.g., sectioned testis, or cell line assays. However, of the whole
39 animal assays, ECT allows data to be collected over time from the same
40 animal, thereby reducing the number of animals used relative to other
41 types of whole animal assays.

42
43 **4.1.2.6 Conclusion.** The ECT is a relatively new test for toxicity evaluation and/or
44 evaluating steroidogenesis, as well as the androgenic/anti-androgenic or estrogenic/
45 anti-estrogenic effects of a substance after the animal is challenged with a substance that
46 stimulates hormone-producing organs. ECT requires a high degree of specialized training and
47 laboratory skills. While it may be useful for identifying the anti-pituitary or anti-gonadal effects

1 of a substance, the technical difficulty of performing the test may preclude its use as a screening
2 tool.

3 4 **4.2 Combination of Whole Animal and Isolated Organs Method (*ex vivo*)**

5 6 **4.2.1 Scope of the Method**

7
8 The *ex vivo* method is a combination of *in vivo* and *in vitro* procedures. More
9 specifically, study designs using the *ex vivo* method include administration of a substance to the
10 whole animal based on a certain dosing regimen and, at the conclusion of dosing, removal of one
11 or both testes or ovaries and culturing them for further treatment and testing. The *ex vivo*
12 method not only allows the effect of a toxicant on the hypothalamic-pituitary-gonadal axis to be
13 assessed, but it also permits evaluation of any change in the capability of the gonad to produce
14 steroid hormones.

15
16 The *ex vivo* method is readily accepted and widely used. Investigators find it a robust
17 method for evaluating substances for their capacity to alter steroid hormone production and
18 secretion as well as characterize the mechanism of action of a substance. In short, it has been
19 used to evaluate reproductive toxicity (Berman and Laskey, 1993) and physiological processes
20 (Bambino and Hsueh, 1981) in cycling, pregnant, or hypophysectomized rats (Laskey and
21 Berman, 1993; Bambino et al., 1980; Piasek and Laskey, 1994). The wide appeal of the *ex vivo*
22 method seems to be that it combines the various *in vivo* dosing regimens with all the *in vitro*
23 organ function assays and procedures. Further, it allows for metabolism of substances, thereby
24 detecting possible active metabolites derived from active or inactive parent compounds,
25 e.g., vinclozolin.

26 27 **4.2.2 Description of the Assay**

28
29 A flow diagram of an *ex vivo* method is illustrated in Figure 4-3. The *ex vivo* method
30 first involves treating immature or adult animals according to a selected dosing regimen. Route
31 and duration of exposure are not constrained. In addition to the acute exposures, as described for
32 the *in vivo* methods, exposures for *ex vivo* studies can actually begin as early as gestation and
33 continue for as long as the life expectancy of the animal being tested. During the in-life
34 exposure period, blood samples can be collected at specified times for serum hormone analysis.

35
36 After the exposure period is completed, the animal is killed, at which time final necropsy
37 specimens can be collected, and the testis or ovary isolated. The gonads are then processed
38 according to the type of *in vitro* preparation that was selected to assess the toxicant's effect.
39 [The different types of *in vitro* preparations, e.g., whole organ, sections, isolated/cultured cells,
40 etc., are described in sections 4.3 and 4.4.] The *in vitro* preparation is treated with or without a
41 stimulant, e.g., hCG or LH, in order to evaluate whether the toxicant perturbs gonadal function.
42 In addition, the activity of steroidogenic enzymes can be assessed by adding different substrates.
43 This assessment indicates which enzyme(s) is/are affected based on whether intermediate
44 and/or end-hormone production occurs (Gray et al., 1997).

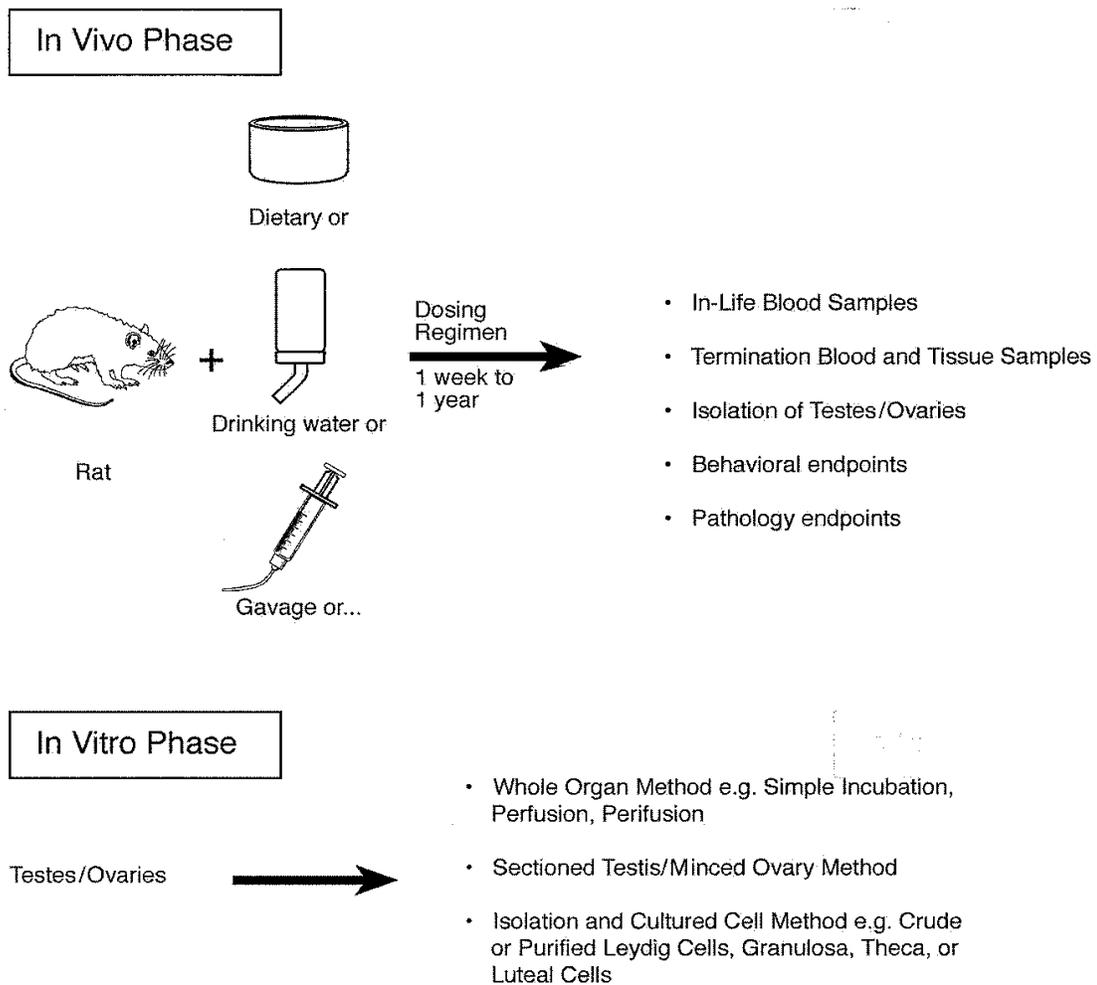


Figure 4-3. Schematic Diagram of *ex vivo* Method

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

1 keyed to hormone changes. Laskey and Berman (1993) used a vaginal lavage technique and
2 observed three classes or stages, based on the kinds of cells present:

- 3
- 4 ■ Estrus—Only cornified epithelial cells
- 5
- 6 ■ Diestrus—Only leukocytes and cornified and a few nucleated epithelial cells
- 7
- 8 ■ Proestrus—cornified and nucleated epithelial cells.
- 9

10 Thus, by sampling the rats during the pre-study phase, animals with regular cycles that are
11 clearly classified can be identified to reduce the variability in study results. Also, necropsy
12 would be performed at a specific stage, e.g., diestrus (or proestrus).
13

14 The developmental stage of the male is an important experimental design consideration.
15 The Leydig cells of the testis are where the enzymes concerning the steroidogenic pathway are
16 located, and the responsiveness of these cells to endogenous endocrine and paracrine factors, as
17 well as hormone production rates, change during an animal's life time. Stoker, et al. (2000),
18 have provided a thorough review about pubertal development in the male rat, including a list of
19 substances known to alter male reproductive development (endocrine disruptors).
20

21 Payne et al. (1996) has also reviewed the changes that occur in mammalian Leydig cells
22 during the fetal, prepubescent, adolescent, and adult stages. Briefly, during the fetal stage, the
23 testis contains progenitor Leydig cells (maximal production by gestation day, or GD, 21). These
24 cells produce testosterone (maximum production GD18 to GD20) and androsterone (produced up
25 through GD 25). From then until GD 35 to 40, immature Leydig cells predominate (maximum
26 production by GD 35). These cells produce testosterone and 3 α -androstenediol. From GD35 to
27 GD90, Insulin Growth Factor (IGF-1) is believed to stimulate the immature Leydig cells into
28 mature Leydig cells, a process that is completed by GD 90. This development of the Leydig cell
29 is also regulated by LH, which has been reported to occur on GDs 25, 33, 35 (surge), 40, and
30 60 (surge). It is from these elegant studies and reviews that seemingly disparate results are better
31 explained and understood. For example, Molenaar et al. (1985) showed that EDS was toxic to
32 Leydig cells from adult rats, but Risbridger et al. (1989) observed no degeneration by EDS when
33 testes from 10- or 20-day-old rats were tested. Kelce et al. (1991) exposed adult and immature
34 rat Leydig cells (purified) to EDS using both *in vivo* administration and *in vitro* testicular
35 perfusion and reported that the reduced sensitivity could be attributed to an intrinsic factor
36 within the immature Leydig cell. This intrinsic capacity of the fetal Leydig cells has yet to be
37 fully characterized. Nevertheless, these studies demonstrate the importance of giving careful
38 consideration to the age and developmental stage of the testis and Leydig cells when designing a
39 study to identify substances that can alter the hormonal products (qualitative and quantitative) of
40 steroidogenesis.
41

42 Experimental design flexibility varies widely for the *ex vivo* method; first for the *in vivo*
43 phase and, second, for the *in vitro* phase. Variations of the *in vivo* phase include different dosing
44 regimens and routes of administration chosen to evaluate a given kind of exposure. Various
45 endpoints can be utilized such as behavioral parameters, cytology (vaginal smears), blood and
46 tissue collection, as well as different types of test groups, e.g., exposed and unexposed F₁. The
47 *in vitro* phase can be varied to involve any procedure based upon the best endpoint for evaluating

1 a particular aspect of steroid production and secretion. The different types of *in vitro* procedures
 2 are discussed in subsections 4.3 and 4.4.

3
 4 **4.2.4 Representative Studies from the Literature**

5
 6 The *ex vivo* method has been employed to evaluate and, ultimately, identify a test
 7 substance that can alter steroidogenesis (e.g., Fail et al., 1996c). In this study, adult female
 8 Long-Evans rats were treated with methoxychlor by gavage at dosages of 50 or 200 mg/kg/day
 9 for 18 weeks. After the specified exposure period, the ovaries were isolated and baseline values
 10 for progesterone, testosterone, and estradiol were obtained. Next, the ovaries were stimulated by
 11 treatment with 100 IU of hCG (0.5 mL of media). Ovarian weight was significantly increased
 12 by methoxychlor treatment. The production of ovarian progesterone and estradiol were
 13 significantly increased at all time points, as was testosterone production at the 2-hour time point
 14 for the high methoxychlor dosage (Table 4-3).

15
 16 Other studies that have employed the *ex vivo* method for evaluating the effect of a
 17 substance on steroidogenesis are summarized in Table 4-4. There are numerous examples in the
 18 literature that describe investigations that used the *ex vivo* method to assess substances for
 19 altering steroid hormone production and secretion—those shown in the table represent an
 20 attempt to illustrate a fair sample of all these studies.

21
 22 **Table 4-3. Example of *Ex Vivo* Study Results: The Effects of Daily Administration of**
 23 **Methoxychlor on Ovarian Hormone Production in Long-Evans Rats**

24

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)**

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 41 ^a Values are mean ± SEM. Units are indicated for each parameter.

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

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

Animal (<i>in vivo</i>)	Substance Tested	Preparation (<i>in vitro</i>)	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	8testosterone 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	9 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	9 basal testosterone @ 19 and 25 mg/kg/day 9 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	8basal and hCG-stimulated testosterone and estradiol (diestrus) 9Basal 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	9 ovarian estradiol & testosterone secretion @ 250 or 500 mg/kg/day; no effect on progesterone 9 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 Fg/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	9 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	9testosterone production after 24 hours @ 75 or 100 mg/kg with hCG stimulation	Klinefelter et al., 1991
Adult male SD rats	TCDD @ 100 Fg/kg, gavage, single administration 7 days before necropsy	Isolated testicular perfusion; stimulated by 50 to 4050 mIU hCG	9basal and hCG-stimulated testosterone	Kleeman et al., 1990

Table 4-4. Continued

Animal (<i>in vivo</i>)	Substance Tested	Preparation (<i>in vitro</i>)	Measured Response	Reference
1 2 3 4 Immature hypophysectomized male SD rats	dexamethasone @ 10 Fg/day, injection, for 5 days; FSH, 4 Fg/day R5020 (synthetic progestin) @ 500 Fg/day, injection, for 5 days	Testicular cell suspension; stimulated 500 ng hCG	9basal and hCG-stimulated androstenediol no effect on basal or hCG-stimulated androstenediol	Bambino and Hsueh, 1981
5 6 Adult male OFA rats	20-438 (anti-spermatogenic) @ 50 mg/kg, gavage, single administration	Sectioned testis; incubated with ¹⁴ C-pregnenolone	9testosterone and androstenedione 8progesterone, 17" - hydroxyprogesterone, and estradiol	Gurtler and Donatsch, 1979

7
8
9 **4.2.5 Distinguishing Features of *Ex Vivo* Methods**

10
11 **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.

12
13
14
15
16
17
18 **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.

19
20
21
22
23
24
25 **Sensitivity**

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

26
27
28
29 **Specificity**

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

30
31 **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.

1 **Equipment,**
2 **Training, and**
3 **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 CO₂ 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.

10
11 **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.

17
18 **4.2.6 Conclusion**

19
20 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.

27
28 **4.3 Isolated Organ Methods (*in vitro*)**

29
30 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.

1 Several terms may be used to classify the *in vitro* studies included in this section. In the
2 literature, the terms “whole,” “sectioned,” and “minced” are used to indicate the way in which
3 the organ is processed. If the method uses **the whole organ**, then after the organ is removed and
4 placed in media, no further processing occurs. In contrast, the whole organ can be further
5 processed into sections, or even more finely processed, i.e., minced.
6

7 Testes are often sectioned, but ovaries are rarely, if ever, sectioned. The consistency of
8 the ovary does not readily permit sectioning. A **sectioned organ** is also commonly referred to in
9 the literature as a slice or slab. For the testes, an organ section is generally understood to refer to
10 an organ that has been cut such that each section constitutes 1/8 to 1/2 the size of the whole organ,
11 i.e., ~ 50 to 250 mg (rat testis). **Minced organ** generally refers an organ that has been cut with a
12 scissors, knife, or razor blade into very small sizes, i.e., 1 to 50 mg (rat testis). The literature
13 also uses the generic term “piece” to describe any organ that has been processed such that it is
14 less than the whole. The terms “whole,” “sectioned,” and “minced” appear to be the most
15 descriptive, and will be used for purposes of this review.
16

17 **4.3.1 Whole Testis/Whole Ovary Methods**

18
19 **4.3.1.1 Scope of the Method.** A whole organ *in vitro* method uses the whole ovary or
20 whole testis after removal from an untreated animal. The organ is placed into an artificial
21 environment that as closely as possible simulates *in situ* conditions, in order to test substances
22 for estrogenicity or androgenicity. These whole organ *in vitro* methods allow toxicant exposure
23 of the steroidogenic cells while maintained in their normal cytoarchitectural environment. In
24 addition, *in vitro* methods are not affected by neuronal influence. These assays allow for study
25 of hormonal and/or toxicant influence on gonadal steroid hormone production over several
26 hours.
27

28 Whole organ methods have been used to investigate a variety of endocrine functions.
29 The simple whole organ incubation procedure has been used to investigate steroidogenic
30 responses to treatment with LH or cAMP (Leung and Armstrong, 1979). The perfused ovary
31 has been used to study follicular growth (Peluso, 1988), steroidogenesis (Soendoro et al., 1992a,
32 1993), ovulation (Peluso, 1990), and function of the corpus luteum (Nulsen et al., 1991). The
33 isolated perfused ovary has been used to study ovulation and the mechanism of ovulation (Koos
34 et al., 1984), oocyte maturation (Brannstrom et al., 1987), and ovarian physiology (Brannstrom
35 and Flaherty, 1995). The perfused testis provides a method to simulate biosynthesis and
36 secretion of testicular steroid hormones (Chubb and Ewing, 1979a; 1979c) and to detect
37 inhibitors of testosterone biosynthesis (Chubb and Ewing, 1979b).
38

39 **4.3.1.2 Description of the Method.** Three techniques can be used to describe
40 variations of the *in vitro* whole organ method: simple incubation, perfusion, and perfusion. The
41 procedure used to combine the organ and the medium is what distinguishes these techniques. In
42 incubation, the organ is placed in and remains in a medium; in perfusion, the medium passes
43 over and around the organ; and in perfusion, the medium passes through the vasculature of the
44 organ, as described in more detail below.
45

46 The media surrounding the tissue can be static or dynamic. In the **static approach**, used
47 for simple incubations, steroids accumulate in a “tissue culture system” over a specified period

1 of time. This approach may (1) use periodic media replacement as samples are collected for
2 analysis or (2) leave the media undisturbed. If the media are left undisturbed, then the analyte
3 being measured will accumulate. In both situations, the analyte being measured in the media
4 increases over time. However, the rate of production can change over time due to negative
5 feedback effects of accumulating steroid hormones, decreased precursor concentrations, changes
6 in cAMP, etc. By contrast, a **dynamic approach**, used for perfusion and perfusion, allows the
7 tissue to be in a “stream” of media. The medium is continually pumped past or through the
8 tissue. In this approach, the feedback effects are minimized and, therefore, the tissue and steroid
9 production more closely represent the tissue’s environment in a biological organism.

10
11 The *in vitro* system used to bathe the removed organ can be closed, half-open, or open.
12 In a **closed system**, no medium is replaced and one sample is collected. In this kind of system,
13 product accumulation occurs. A **half-open system** has medium replaced at certain time
14 intervals; samples are collected when the medium is replaced; product accumulation occurs; and
15 the organ is altered each time the medium is replaced. In an **open system** (also called a
16 superfusion system), the medium is continually replaced and samples can be collected at any
17 desired frequency without affecting the organ. The use of one system over another is based on
18 choosing between finding a technically simple assay that minimizes the amount of external
19 interference with the incubation (closed system is best) and being able to get samples at various
20 time points, realizing that the preparation will be altered by doing so (half-open system). The
21 open system can be used to circumvent interference with the organ while, at the same time,
22 allowing for multiple samples to be collected; however, this system is technically more difficult
23 to set up and operate, which requires more labor over time and decreases the number of organs
24 that can be evaluated.

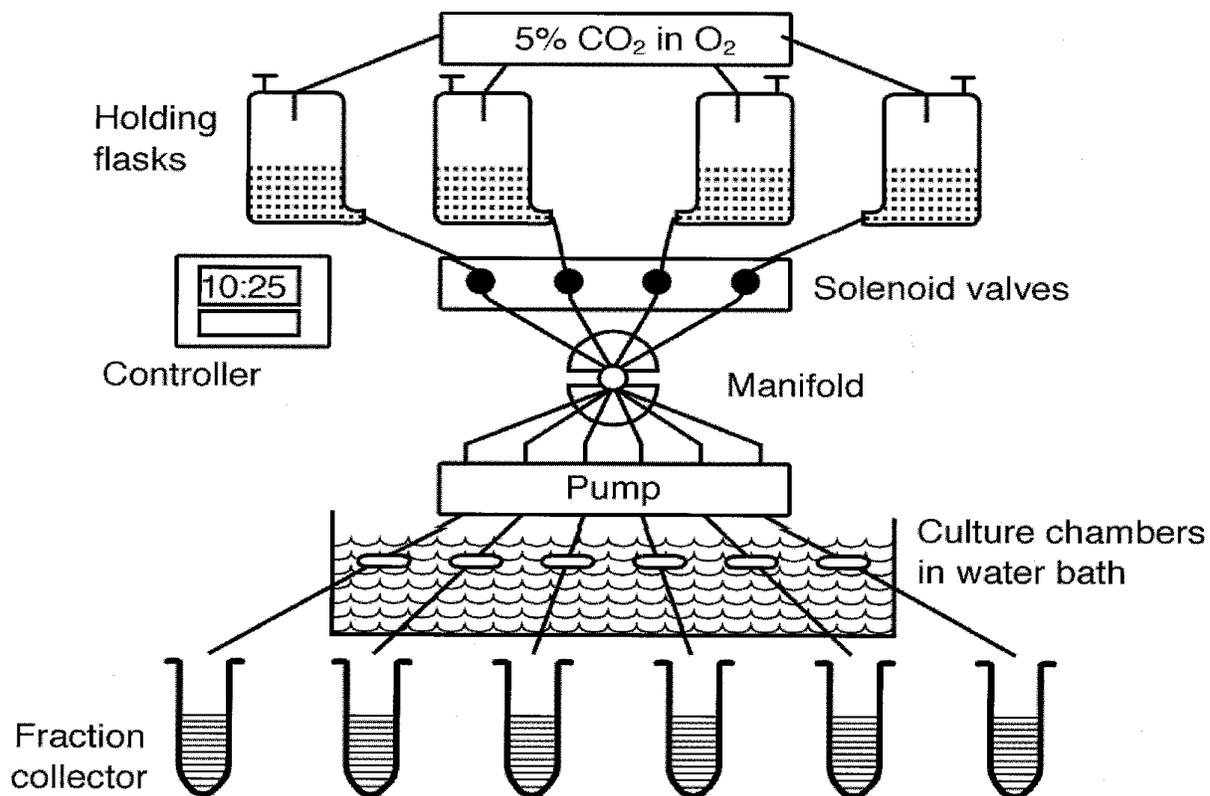
25 26 **Simple Whole Organ Incubation**

27
28 The simple incubation technique involves removal of the testis or ovary and incubation
29 of the organ in medium for testing. For the whole testis (Deb et al., 1980), the animal is
30 anesthetized or euthanized and the testes removed. The testes are incubated in
31 Krebs-Ringer-bicarbonate solution (pH 7.6) at 37EC and an atmosphere of 95 percent O₂ and
32 5 percent CO₂. The testes are incubated with or without the substance being tested, as well as
33 with or without stimulant, e.g., LH. For the whole ovary, a procedure has been used to assess the
34 effect of a toxicant on ovarian steroidogenic function with and without stimulation (Berman and
35 Laskey, 1993; Laskey and Berman, 1993; Laskey et al., 1995; Piasek and Laskey, 1999). As
36 with the testis, the ovarian method is relatively simple. The whole ovary culture for evaluating
37 steroid production (non-stimulated) involves removing the ovary from an anesthetized or
38 euthanized animal and incubating the ovary at 34EC and 5 percent CO₂ for 1 hour with slow
39 shaking (the vials are slowly agitated and the duration can range from 1 to 24 hours). Finally,
40 the vials are centrifuged and the supernatant decanted and stored frozen for later analysis.

41 42 **Perifusion**

43
44 The perifusion system (medium passing over and around the tissue in a chamber) is the
45 most technically difficult of the three whole organ methods, but mimicks *in vivo* conditions and
46 allows ovarian function to take place over several days. Perifusion keeps the organ viable long
47 enough to characterize more complicated developmental processes, e.g., corpus luteum

1 formation. The perfusion system for the ovary is described in detail by Peluso and Pappalarda
2 (1993). The perfusion apparatus is shown schematically in Figure 4-4. Perfusion systems may
3 involve the use of a computer-controlled apparatus to regulate the release of the test substance in
4 a pulsatile pattern (Soendoro et al., 1992a).
5



6
7 **Figure 4-4. Schematic Diagram of the Perfusion Apparatus**
8

9 Source: Peluso and Pappalarda (1993)
10
11

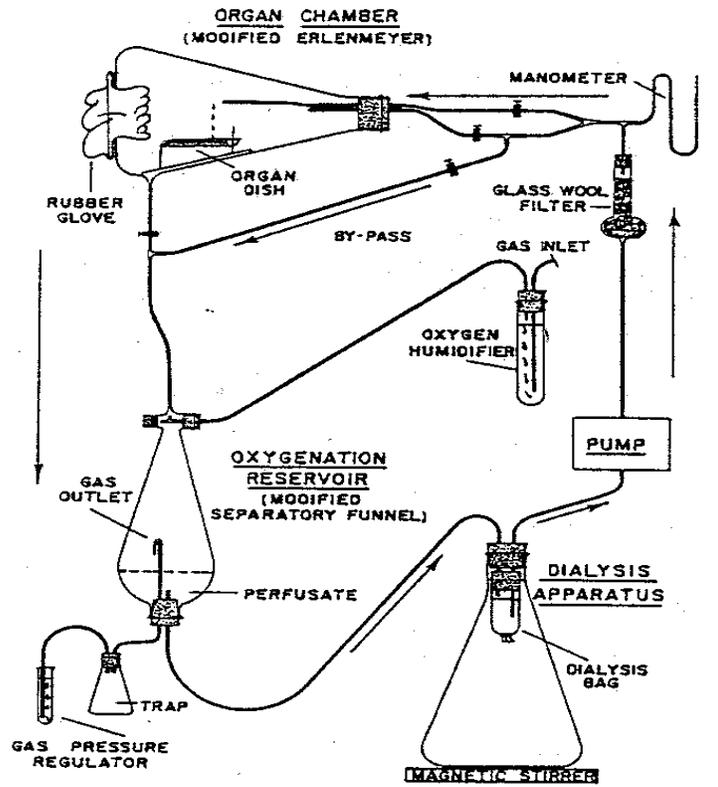
12 The ovarian perfusion procedure requires a pre-study set-up check. Prior to conducting
13 the study the perfusion system is sterilized and tested for optimal operation, e.g., rates of
14 perfusate media flow need to be equal for all chambers. Verification of the assembly operation
15 is extensive, e.g., autoclaved components, elaborate tubing set-up, medium preparation (laminar
16 flow hood), and aseptic technique required for set-up. Once the perfusion system is set up,
17 immature rat ovaries are collected from anesthetized rats and placed in heparinized medium.
18 The ovaries are incubated at room temperature for a few minutes (allowing microvasculature to
19 dilate and reducing blood content and blood clot formation). The ovaries are placed into the
20 perfusion chamber, the chamber is connected to the perfusion system, and the system started.
21 The perfusate gas tension (95% O₂ and 5% CO₂), temperature (37EC), pH (7.4), and flow rate
22 (6 mL/hr) are carefully maintained. The preparation is viable for a few days. A test for

1 detecting toxic effects on ovarian function involves adding a toxicant to the perfusion system.
2 The response of the ovary is determined by removing samples of perfusate for analysis.

3
4 **Perfusion**

5
6 The perfusion system (medium passing through the vasculature) generally involves a
7 laparotomy to cannulate the aorta and vena cava, as well as to isolate and remove the gonad. A
8 perfusion apparatus is connected to the vasculature, and the medium is perfused through the
9 organ in a chamber. The medium is oxygenated, the pH adjusted, the temperature maintained,
10 the perfusion pressure controlled, and the flow rate maintained. Perfusion of the isolated testis
11 or ovary is also a technically difficult procedure, but one that closely mirrors physiological
12 conditions.

13
14 The perfused rat testis procedure was developed for the rabbit by Vandemark and
15 Ewing (1963) and modified for the rat by Chubb and Ewing (1979a). An illustration of the
16 perfusion apparatus is shown in Figure 4-5.
17



18
19 **Figure 4-5. Schematic Diagram of a Testicular Perfusion Apparatus**

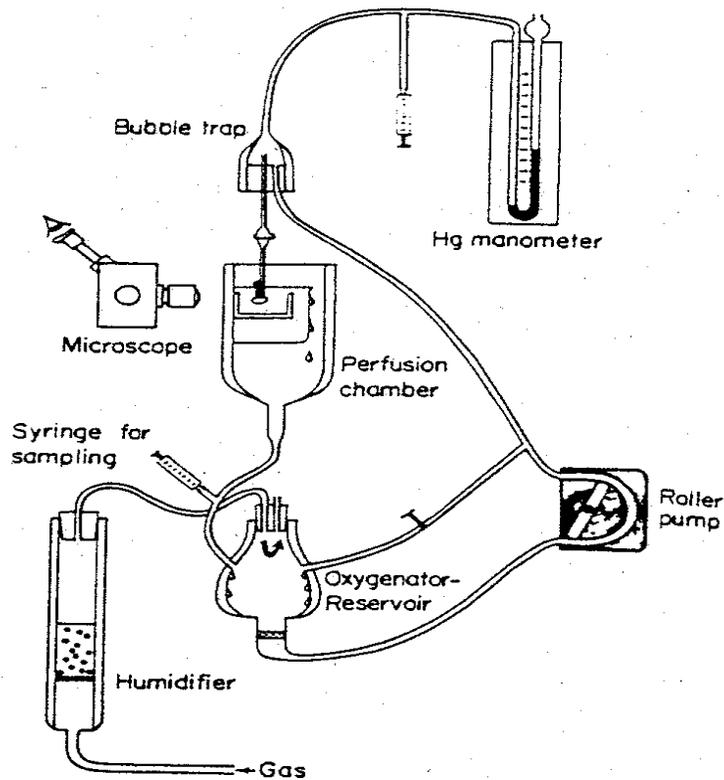
20
21 Source: Vandemark and Ewing (1963)

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

9
10 The perfused rat ovary procedure was described by Koos et al. (1984) and
11 Brannstrom et al. (1987). An illustration of the ovarian perfusion apparatus is shown in
12 Figure 4-6. Briefly, the procedure depicted in Figure 4-6 involved anesthetizing the rat and
13 removing the ovary with its arterial supply and venous drainage vessels intact. The ovarian
14 vasculature is cannulated and the assembly placed in a 37EC sodium chloride solution. The
15 ovary is pre-perfused manually with a syringe to remove blood from the preparation. During this
16 time, the ovary is carefully examined to ensure that there is no leakage and the perfusate flows
17 out only from the venous vasculature. Next, the ovary assembly is connected to the perfusion
18 apparatus (Figure 4-6). This recirculating perfusion system pumps medium from the
19 oxygenator-reservoir to the bubble trap and directly back to the reservoir through the shunt line.
20 From the bubble trap, the medium is pumped through the arterial catheter to the ovary, which
21 rests immersed in media in the perfusion chamber. The medium is oxygenated (95 percent O₂
22 and 5 percent CO₂), the temperature (37EC) and pH (7.4) maintained, perfusion pressure
23 monitored (55 and 80 mmHg), and the flow rate carefully adjusted (0.6 to 0.9 mL/min). The
24 preparation can be maintained for approximately 20 hours. Samples are removed from the
25 perfusate for analysis.

26
27 **4.3.1.3 Experimental Design Information.** The simple incubation assay is technically
28 simple, easy to set up, and can be rapidly used to assess several substances and concentrations
29 simultaneously. The experimental design issues associated with this assay have been described,
30 e.g., age of animal, stage of development, medium, incubation times, etc. In addition, since most
31 investigators are interested in the mechanism and site of action of a toxicant, the simple
32 incubation assay has not been frequently used (to avoid feedback of accumulating steroids),
33 thereby limiting the number of citations available for inclusion in this review paper. This,
34 however, does not preclude it from further consideration as a screen tool for steroidogenesis.

35
36 Ovarian maturation, to the extent that normal steroidogenesis occurs, requires a precise
37 sequence of hormonal stimuli. The *in vivo* pattern that brings this about can be mimicked using
38 **perifusion** (Soendoro et al., 1992a). The design of this procedure requires mathematical
39 calculations between the following parameters: the hormone concentration in the holding tank
40 (C_i) and the perifusion chamber (C_f), the rate (R) of delivery, the initial volume (V) of medium,
41 and time. The formula that integrates these relationships with the hormone concentration (C) at
42 any given time (t) is: $C(t) = C_f + (C_i - C_f) e^{-Rt/V}$. In practice, these variables are controlled with a
43 microprocessor and solenoid valves. The numerous calculations needed to simulate a complex
44 hormonal gonadotropin release pattern is facilitated with a computer software program or
45 spreadsheet (Peluso and Pappalardo, 1993). In addition to controlling the gonadotropin release,
46 selection of a release pattern is important. The gonadotropin release pattern can cause



2
3 **Figure 4-6. Schematic Diagram of an Ovarian Perfusion Apparatus**

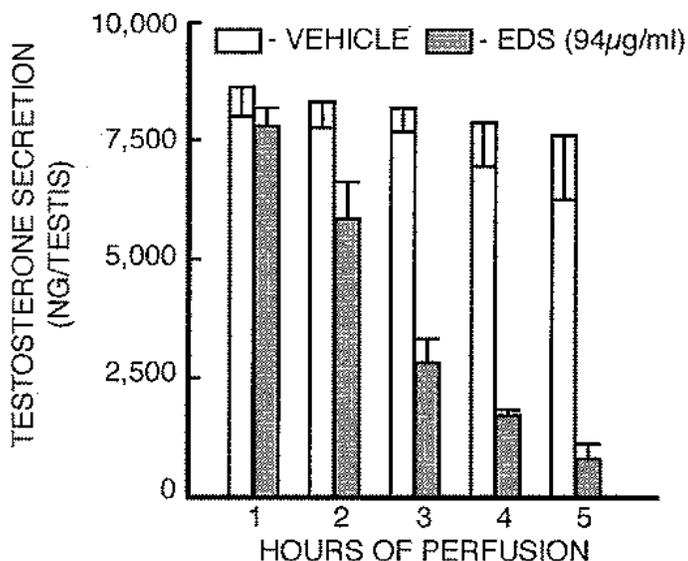
4
5 Source: Brannstrom et al. (1987)

6
7
8 differences in production and secretion of the steroid hormones (Soendoro et al., 1992a; 1993).
9 Furthermore, the developmental status of the ovary affects the response. An ovary from a mature
10 rat (PMSG-treated) responds with a slow and sustained increase in estradiol output, whereas the
11 ovary from an immature rat responds with an acute, transient increase in estradiol.
12 Developmental differences were also noted to affect testosterone and progesterone secretion
13 patterns (Soendoro et al., 1992a). Finally, the size, shape, and/or surface area of the ovary
14 preparation that is exposed results in different estradiol, testosterone, and progesterone profiles
15 under gonadotropin stimulation (Soendoro et al., 1992b).

16
17 The **perfused rat ovary** assay requires special consideration of a number of issues, some
18 of which are unique to this assay (Koos et al., 1984; Brannstrom et al., 1987; Brannstrom and
19 Flaherty, 1985; Dharmarajan et al., 1993; Zimmerman et al., 1985). First, regarding the test
20 system, immature rats rather than mature rats are often used because the latter require monitoring
21 the estrous cycle. By using immature rats, the results do not need to be interpreted based on
22 consideration of the endogenous steroidogenic hormone pattern. Second, the perfused rat ovary
23 assay produces a response that is supported by *in vivo* results for such parameters as ovulation

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

8
9 **4.3.1.4 Representative Studies from the Literature.** Perfusion of the whole testis has
10 been shown to identify substances that alter steroidogenesis. Kelce et al. (1991) used the
11 perfused testis technique to measure the effect of ethane dimethanesulfonate (EDS) on
12 testosterone production. Figure 4-7 summarizes some of the results of this study.
13



14
15 **Figure 4-7. Example Data from Study Using a Perfused Testis Method**
16

17 *Notes:* Testes were obtained from adult SD rats and perfused as described above. The testis were
18 perfused with artificial medium containing ovine luteinizing hormone (100 ng/mL) and either vehicle
19 (DMSO, 0.2 percent) or 94 Fg of EDS (the ED₅₀ concentration for purified Leydig cells and the
20 approximate concentration following an *in vivo* exposure of 85 mg/kg). Samples of the testicular venous
21 effluent were measured for testosterone concentration at selected time points during the 5 hour perfusion.
22 Testes perfused with the media and DMSO maintained a high rate of LH-stimulated testosterone
23 production. EDS treatment reduced testosterone production and secretion by approximately 10 percent
24 of control by the fifth hour of testing.
25

26 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 Fg/Kg, gavage, single administration and terminated after 7 days (<i>ex vivo</i> - see text) / hCG @ 50 to 4050 mIU/mL	\uparrow basal and hCG-stimulated testosterone \uparrow secretion of pregnenolone, 17 α -hydroxypregnenolone, progesterone, 17 α -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 Fg/mL/LH @ 100 ng/mL hydroxyflutamide @ 1 Fg/mL/LH @ 100 ng/mL	\uparrow basal and LH-stimulated testosterone	Gangnerau & Picon, 1987
Adult male rats/Whole testis	mitomycin C @ 50, 100 or 200 Fgm/mL	\uparrow $^{5-3\beta}$ -HSD and glucose- 6-phosphate dehydrogenase activity	Deb et al., 1980
Adult male SD rat/Perfused testis	aminoglutethimide @ 30 to 150 FM SU-10603 @ 1 to 40 FM Medrogestone @ 1 to 40 FM	\uparrow testosterone; inhibition of pregnenolone biosynthesis \uparrow testosterone; \uparrow pregnenolone and progesterone; inhibition of 17 α -hydroxylase and C-17, C-20-lyase \uparrow testosterone and inhibition of $^{5-4}$ isomerase	Chubb and Ewing, 1979b

1 **4.3.1.5 Distinguishing Features of Whole Organ Methods.**

2
3 **Stability**

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

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

9
10 The isolated perfused ovary is viable for approximately 20 hours based on
11 capacity for the ovary to ovulate when stimulated with LH or PMSG,
12 histology, and steroidogenic hormone production and secretion
13 (Koos et al., 1984).

14
15 **Standardization**

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

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

28
29 The isolated perfused ovary assay is not standardized. This assay would
30 also require standardization of several critical design considerations.
31 Many of the factors are similar to the ones listed above for perfusion.

32
33 **Sensitivity**

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

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

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

43
44
45 Testosterone production was decreased 1 to 95 percent when 15 different
46 inhibitors of steroidogenesis were tested at 30 FM using the perfused
47 testis assay (Chubb and Ewing, 1979b).

1 **Specificity**

2 Perfusion and perfusion of the ovary respond to LH and PMSG by
3 producing and secreting steroidogenic hormones in a manner similar to *in*
4 *vivo* responses. Using perfusion, stimulation of steroid release from the
5 ovary occurred above a threshold of 3 to 8 mIU/mL of LH and 4 to
6 10 mIU/mL of FSH and the slope of the increase was important
7 (Soendoro et al., 1992a).

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

11
12 **Metabolic Activity**

13 None.

14 **Equipment**

15 Perfusion and perfusion procedures require standard and specialized
16 equipment. Examples of the specialized laboratory set-up are shown in
17 Figures 4-7, 4-8, and 4-9. Perfusion in particular requires a
18 microprocessor, solenoid valve set-up, and a computer in order to achieve
19 accurate and precise control of the perfusion patterns of the
20 gonadotropins.

21 **Training**

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

28 **Sample Scheduling**

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

34 **Animal Usage**

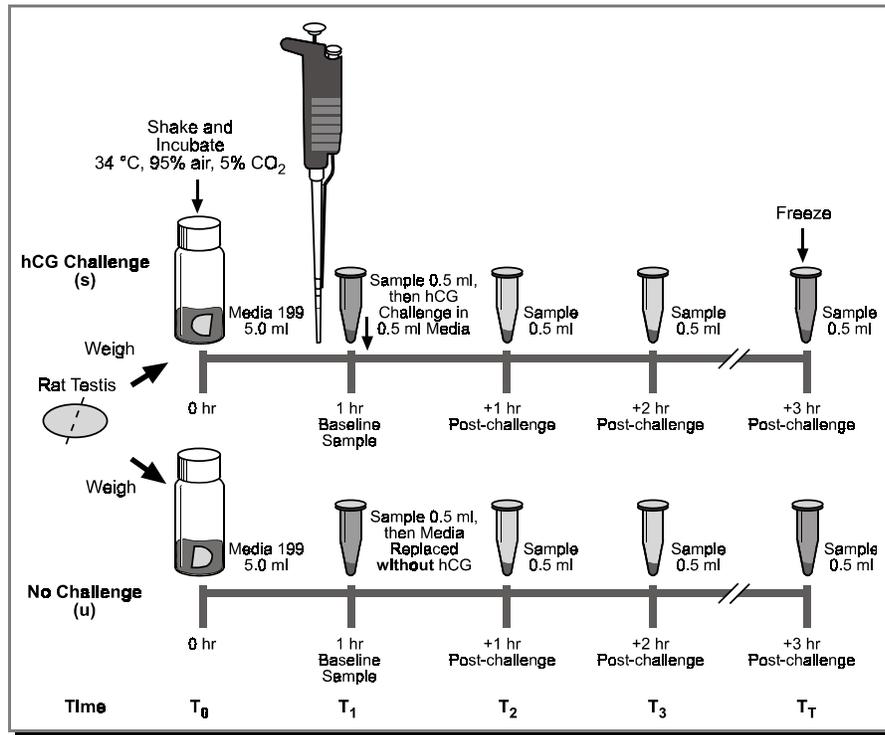
35 Whole organ assays are similar to whole animal assays, which result in an
36 assay that uses relatively more animals than other assays. Since each
37 animal contributes two gonads and each gonad generates one set of data, a
38 reduction in the number of animals used is limited.

39 **4.3.1.6 Conclusion.** Isolated whole organ methods are useful for identifying
40 substances that alter steroidogenesis. The different procedures that use isolated whole organs
41 vary greatly in their degree of technical difficulty. The simplest procedure is isolation of the
42 testis or ovary followed by incubation with a stimulant and/or substance for testing. However,
43 the literature infrequently cites this as a method used to identify substances that alter
44 steroidogenesis. The other procedures, perfusion and perfusion, are very labor-intensive and
45 seem to have better application for characterizing physiological and/or biochemical processes
46 that take long periods of time to investigate.

1 **4.3.2 Testicular Sections or Minced Ovary Methods**

2
3 **4.3.2.1 Scope of the Method.** The testicular section and minced ovary methods use
4 untreated animals as a source for the organs used. After removal of the whole organ from an
5 anesthetized or euthanized animal, the testes or ovaries are further processed into smaller sizes
6 for use as sections (testis) or for mincing (ovary). A method to prepare the organs for measuring
7 steroidogenesis was worked out in detail for the ovaries by Laskey et al. (1991, 1994) and in the
8 testes by Sikka et al. (1985). The testicular section and minced ovary methods are reliable
9 assays in that they are relatively easy to conduct, the cellular architecture is preserved, different
10 species can be used, and the assay can be conducted relatively rapidly (Gray et al., 1997). The
11 primary limitation of these methods is the absence of metabolism for those substances that
12 require metabolic activation.
13

14 **4.3.2.2 Description of the Method.** A flow diagram for the isolated testicular section
15 method is illustrated in Figure 4-8.
16



17 **Figure 4-8. Technical Flow Illustration of the Testicular**
18 **Steroidogenesis Assay**
19

1
2 The **testis section procedure** involves euthanizing the animal and removing the testes.
3 The testes are decapsulated, weighed, and sectioned. One testis is separated into 2 to
4 8 longitudinal sections. Each section is placed in a vial containing media alone or media plus
5 toxicant. The medium that has been used in such studies is modified medium 199 with
6 0.1 percent bovine serum albumin (BSA), 8.5 mM sodium bicarbonate, 8.8 mM HEPES and
7 0.0025 percent soybean trypsin inhibitor at pH 7.4. The vials containing the testicular sections
8 and media are incubated at 34EC on a shaker in 5 percent CO₂/95 percent air. After the first
9 period of incubation, e.g., 1 hour, an aliquot of media is collected. This sample is the baseline or
10 secretion sample. Next, one half of the replicates are challenged with a stimulant, e.g., hCG, and
11 the other half are not, which provides information regarding the toxicant's effect when the organ
12 sample is stimulated to initiate steroidogenesis and when it is functioning at a basal level.
13 Additional samples are collected from the vials after various incubation periods, e.g., 1, 2, and
14 3 hours. The media samples are then frozen for later analysis or analyzed immediately for
15 selected hormones using an appropriate method, which is usually RIA.

16
17 The **minced ovary procedure** involves euthanization of the animal and removal of the
18 ovaries (Laskey et al., 1995). Euthanization must be carefully controlled to ensure that animals
19 are in the same stage of their estrus cycle. The ovaries are trimmed of fat and other extraneous
20 tissue, weighed, and placed in media (as previously described). Each ovary is minced with
21 scissors while in the media. The vial containing the minced ovary and media (with and without
22 toxicant) are incubated for one hour at 34EC in a 5 percent CO₂ environment. The vials are
23 centrifuged and the supernatant decanted and stored frozen for later analysis (baseline). Media
24 containing hCG is added to half the minced ovary samples (stimulated and non-stimulated). The
25 minced ovary samples are resuspended in media, incubated for an additional hour, centrifuged,
26 and the supernatant decanted and stored frozen for later analysis. This process is repeated for
27 sample collection at 2 and 3 hours after stimulation. The media samples are analyzed for
28 selected hormones using an appropriate analytical method.

29
30 **4.3.2.3 Experimental Design Information.** Various incubation conditions are used for
31 *in vitro* methods. Gurtler and Donatsch (1979) incubated ¹⁴C-pregnenolone with sectioned testis
32 in order to measure the conversion of this steroidogenic pathway substrate into testosterone,
33 androstenedione, and other hormones. They incubated the testis sections for 2 hours in
34 Krebs-Ringer bicarbonate solution (pH 7.4; 95 percent O₂/5 percent CO₂, at 30EC) with 3 F moles
35 of NADPH and NADP, 20 F moles glucose, 40 F moles glucose-6-phosphate and 5 IU
36 glucose-6-phosphate dehydrogenase. Incubation conditions that have been used for minced
37 ovaries (Piasek and Laskey, 1999) include incubation (34EC, 5 percent CO₂) for up to four hours
38 in M-199 culture medium supplemented with sodium bicarbonate, HEPES, BSA, and soybean
39 trypsin inhibitor.

40
41 Careful consideration must be given to the developmental stage of the animal when
42 designing a study for organ collection. The use of immature versus mature animals was
43 described previously (Section 4.3.1.3 - Experimental Design Information for Whole Organ).
44 With regard to immature rats from 2 to 5 weeks of age, an evaluation of ovarian steroid
45 production using the minced ovary procedure indicated that 3- and 4-week-old animals were
46 favorable (Laskey and Berman, 1993). Ovaries from the 2-week old animals produce low
47 estradiol, progesterone, and testosterone levels, whereas 5-week-old animals could begin

1 cycling, which would produce more steroids. Similar consideration must be exercised for
 2 studying ovarian steroidogenesis in cycling versus pregnant animals. For example, Piasek and
 3 Laskey (1999) used proestrus rats and pregnant rats at gestation days 6 and 16. Proestrus was
 4 selected because it is when maximal steroid production and secretion occurs. Gestation day 6,
 5 the time following implantation, is the onset for increased progesterone production and gestation
 6 day 16, the time following organogenesis, is when the second peak of progesterone production
 7 occurs.

8
 9 **4.3.2.4 Representative Studies from the Literature.** The minced ovary method
 10 described above was used by Laskey et al. (1995) to assess toxicant effects on the steroidogenic
 11 pathway in the rat. The results of their investigation are illustrated in Figure 4-9.
 12

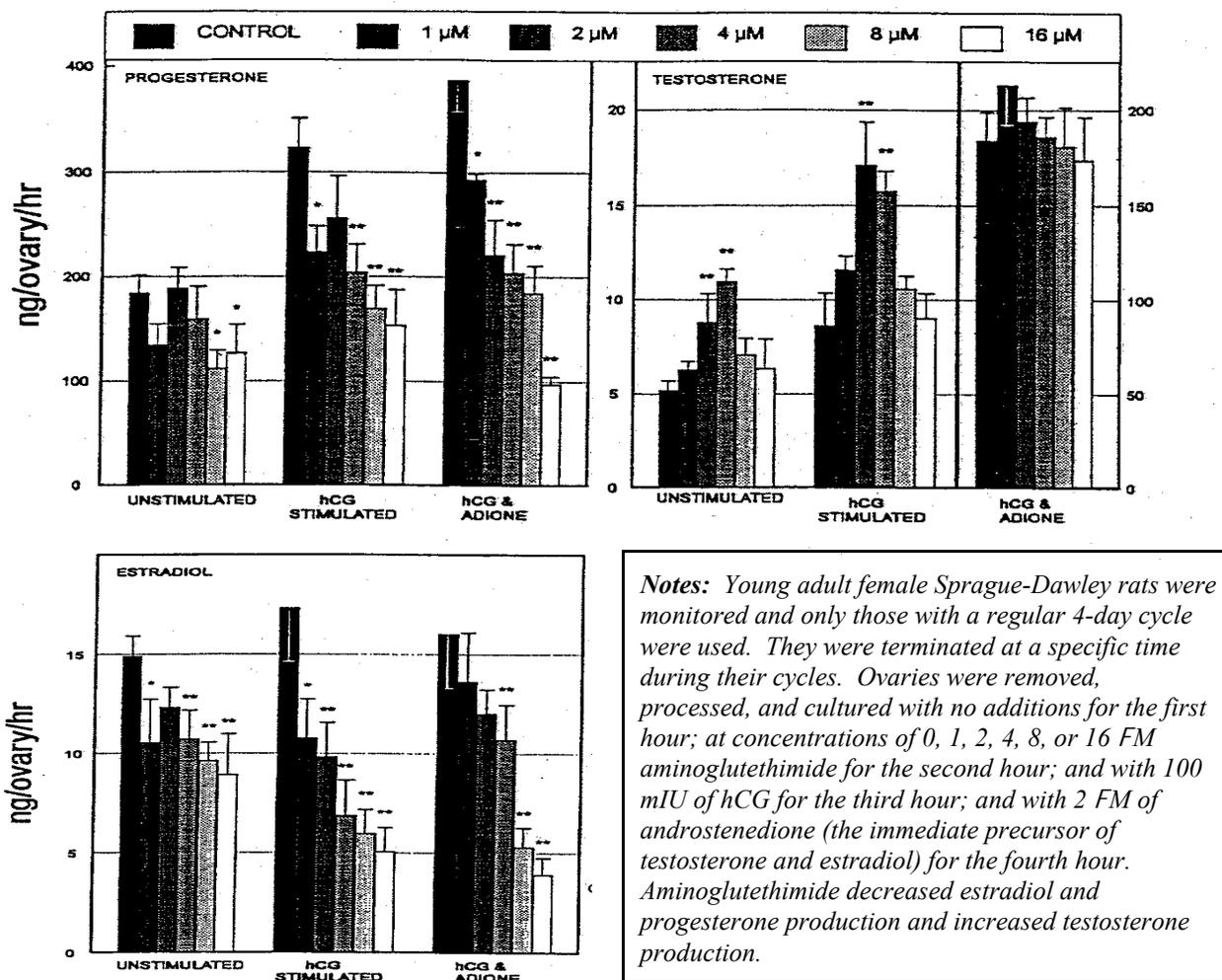


Fig. 6. Steroid production and T/E ratio from ovary cultures

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

14 Source: Laskey et al., Figure 6; copyright (1995), with permission from Elsevier Science.
 15
 16

1
2 Examples of experimental studies from the literature that used isolated testes sections or
3 minced ovary for measuring steroidogenesis are summarized in Table 4-6. The studies in this
4 table used untreated animals prior to collecting the testis sections or minced ovary. It is
5 important to note that the testis section and minced ovary preparations are also used in
6 conjunction with treated animals as described above in the discussion of *ex vivo* methods.
7

8 **Table 4-6. Representative Studies Using the Isolated Testis Sections or Minced Ovary**
9 **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 FM/ 100 mIU hCG	<p>♀ progesterone in proestrus and pregnant rats</p> <p>♀ testosterone in proestrus and pregnant rats</p> <p>♀ no effect on estradiol in proestrus and pregnant rats</p>	Piasek and Laskey, 1999
Adult male Long-Evans rats/Testes Sections (1/4)	Ethane dimethanesulfonate @ 0, 3, 10, 32, 100, 320, 1000, or 3200 Fg/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	<p>♀ basal testosterone production</p> <p>no effect on HCG-stimulated testosterone production</p>	Fail et al., 1994
Adult male SD rat/Testes sections(1/4)	Ethane dimethanesulfonate @ 0, 500, or 3000 FM/ 100 mIU/mL hCG	♀ testosterone production	Laskey et al., 1994
Adult female SD rat/Minced Ovary	Phenolsulfonthalein @ 20 mg/L	<p>♀ progesterone production</p> <p>some alteration to estradiol and testosterone production</p>	Berman and Laskey, 1993
Female rat/ Minced ovary	Methoxychlor & hCG	Progesterone, estradiol, & testosterone	Cummings and Laskey, 1993

Table 4-6. Continued

Animal/Type of Preparation	Treatment & Stimulant	Measured Response	Reference
Adult female Golden Hamsters/ Quartered Ovary	Aromatase Inhibitors: testolactone @ 10, 100, or 1000 FM /LH @ 100 ng/mL CGS 16949A @ 0.004 to 400 FM/ LH @ 100 ng/mL 4-hydroxy-androstenedione @ 0.33 to 330 FM/ LH @ 100 ng/mL aminoglutethimide @ 3, 30, or 300 FM/ LH @ 100 ng/mL	9estradiol and estrone; no effect on progesterone or testosterone 9estradiol and estrone; 8testosterone; no effect on progesterone 9estradiol, estrone, and progesterone; no effect on testosterone 9estradiol, estrone, testosterone, and progesterone	Hausler et al., 1989
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 Testes 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.

1 **Sensitivity** These methods provide a technique for evaluating a wide range of toxicant
2 concentrations. Testable low-level concentrations are generally in the
3 FM range and high concentration levels are limited by the solubility of the
4 substance in the carrier and media.
5

6 **Specificity** Preparations of the parenchyma (whole or sectioned) have
7 maximal-stimulating testosterone production that is within approximately
8 10-fold of the physiological levels (Klinefelter and Kelce, 1996). The
9 selectivity of the *in vitro* method was evaluated by Hausler, et al. (1989),
10 using aromatase inhibitors and evaluating the production and secretion of
11 estradiol, estrone, progesterone and testosterone (see table for results).
12

13 **Metabolic Activity** Little to no metabolism.
14

15 **Equipment** Animal facilities. Standard and endocrine laboratory equipment,
16 e.g., balance. Necropsy equipment. Specialty equipment needed is an
17 incubator and -70EC freezer.
18

19 **Training** A simple assay that requires some practice but can be readily learned by a
20 general laboratory technician. RIA method requires the most training.
21

22 **Sample Scheduling** Methods can be set up and conducted, with RIA samples analyzed, within
23 1 to 2 days.
24

25 **Animal Usage** The process of sectioning or mincing the organ reduces the number of
26 animals used relative to whole animal or whole organ assays but not to the
27 extent made possible by isolated cell or cell line assays.
28

29 **4.3.4 Conclusion**

30

31 The organ section/minced method is not technically difficult and takes relatively little
32 time to conduct. In addition, the method maintains the cytoarchitecture of the organ. The organ
33 preparations remain stable for a sufficient period of time to identify changes in steroid hormone
34 production and appear to be sensitive and specific enough based on the information collected to
35 date. However, one limitation of these preparations is the absence of metabolism pathways for
36 activation of pro-xenobiotics.
37

38 **4.4 Isolated and Cultured Cell Method (*in vitro*)**

39

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

1 androgenicity. The isolated and cultured cell method can be used to evaluate the potential of a
2 direct effect of a substance on the steroidogenic pathway and for identifying the site of action in
3 the pathway.
4

5 The testicular and ovarian cell isolates are enriched with the cells that synthesize the
6 steroid hormones. The cells are isolated to different levels of separation. Generally, the cells are
7 described as being in either a crude or a purified preparation. A **crude** preparation includes
8 much of the cellular debris and tissue remnants that remain as a result of processing the organ
9 into cells but the cells are not subjected to additional clean-up steps. A crude preparation of
10 Leydig cells, also called an interstitial cell preparation, generally has approximately 15 percent
11 Leydig cells. In contrast, a purified preparation involves additional clean-up steps. A **purified**
12 preparation is obtained through procedures such as column separation, centrifugal elutriation,
13 histological separation, or biochemical centrifugation to separate the desired cells from other
14 debris. A purified preparation is commonly understood to be approximately 95 percent isolated
15 cells.
16

17 Once the cells are isolated, they must also be cultured. Culturing the cells involves
18 placing the cells in an environment that will maintain their viability and extend their longevity.
19 The optimization and maintenance of the cells in culture media requires attention to specific
20 procedures (Klinefelter et al., 1993; Treinen, 1993), which are described in further detail below.
21

22 A wide variety of cell preparations have been used for studying the steroidogenic
23 capacity of isolated testicular and ovarian cells. Testicular preparations include fragments of
24 testicular parenchyma, dispersed testicular interstitial cells, purified Leydig cells, and tumor
25 Leydig cells (Klinefelter and Kelce, 1996). It is important to remember that these cell
26 preparations will not take into account the interaction among surrounding cells found within the
27 organ. For example, the isolated purified Leydig cell preparation will not include Sertoli cells,
28 and the granulosa cell preparation will not include many theca cells. Nor will the paracrine
29 factors of the organs be of influence in the responses being measured in these cell preparations.
30 This isolation is both an advantage and a disadvantage. In the following subsections, the crude
31 (interstitial cell preparation) and purified Leydig cell preparations and granulosa cell preparation
32 will be used as examples of the isolated and cultured cell *in vitro* method.
33

34 **4.4.1 Isolated Leydig Cell Culture Method**

35
36 **4.4.1.1 Scope of the Method.** Isolated and cultured Leydig cells provide an *in vitro*
37 method for assessing the androgenic or anti-androgenic effects of a substance. While the
38 isolation and culturing of these cells is a multi-step process, isolated Leydig cells are used to
39 minimize factors, e.g., interstitial or germ cells, that can compromise the response of the
40 substance being tested on steroid production; as a model for understanding mechanistic effects of
41 a toxicant; and evaluating the reversibility of toxicant effects. Extensive reviews about Leydig
42 cells include those by Klinefelter et al. (1993) and Payne et al. (1996). From these reviews,
43 information was evaluated and extracted based on its usefulness for evaluating this procedure as
44 a possible screen for substances to be tested for steroidogenic-altering effects.
45

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

1 summary table below), as well as to investigate the mechanism of Leydig cell tumorigenesis
2 (Biegel et al., 1995); steroidogenic cytochrome enzyme activity using immunohistochemistry
3 techniques and ultrastructural alterations using electron microscopy (Thoreux-Manlay et al.,
4 1995); comparison of species responses (Laskey et al., 1994); and the mechanism of action of
5 toxicants at the cellular, biochemical, and physiological levels (Klinefelter et al., 1994;
6 Bambino and Hsueh, 1981).

7
8 **4.4.1.2 Description of the Method.** The interstitial cell preparation (crude Leydig cell
9 preparation) is simpler and faster than the purified preparations. The procedure reported by
10 Laskey and Phelps (1991) involves anesthetizing or euthanizing the animal and then removing
11 and decapsulating the testes. The testes are incubated in media containing collagenase for
12 10 minutes at 37EC with gentle shaking. The cells are then washed with fresh media, filtered
13 (50 FM nylon mesh), centrifuged, and resuspended in fresh media to the desired number of cells
14 per unit volume.

15
16 For the purified Leydig cell preparation, the procedure is substantially more laborious. A
17 flow diagram for the isolation of a purified Leydig cell preparation is shown in Figure 4-10a,
18 with expanded details shown in Figures 4-10b and 4-10c.

19
20 Isolation and maintenance of Leydig cells involves a multi-step procedure:

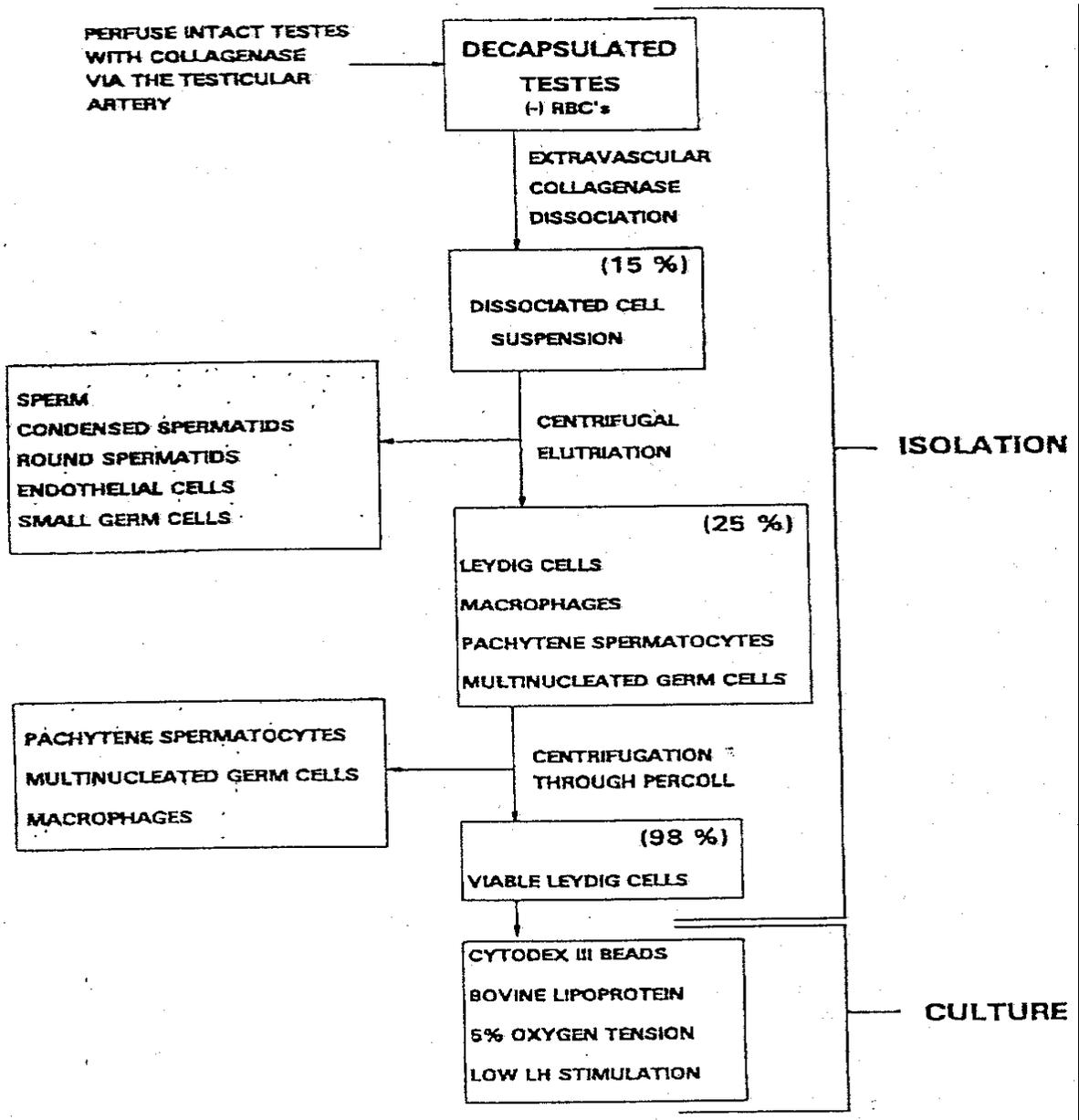
- 21 ■ Collagenase dispersion of testicular interstitial cells
- 22 ■ Separation of smaller cells by centrifugal elutriation
- 23 ■ Purification of cells by density gradient centrifugation
- 24 ■ Culturing of purified cells (Klinefelter et al., 1993).

25
26
27 Each of these steps is described briefly in the following paragraphs.

28
29 **Collagenase Dispersion.** This step provides enzymatic separation of the Leydig cells
30 within the testis. The testes from untreated, adult rats are quickly removed and placed in
31 ice-cold medium, e.g., M199D. The testicular artery is catheterized and the testis is perfused
32 with collagenase. The perfusion both removes red blood cells from the organ and initiates the
33 cellular dispersion process. After perfusion, the testis is decapsulated and the parenchyma
34 collected into centrifuge tubes containing media. The tubes are incubated at 37EC for several
35 minutes to initiate intravascular enzymatic digestion. Collagenase dispersion is terminated by
36 adding fresh media. The dispersed cells are filtered through doubled 100 Fm nylon mesh,
37 centrifuged, and the pellet resuspended in fresh media. The purity of the cell preparation is
38 approximately 15 percent after this step.

39
40 **Centrifugal Elutriation.** This step separates Leydig cells from other cells and debris
41 based on cell size. The elutriation system (peristaltic pump, loading tube, bubble trap/loading
42 chamber, and centrifuge with elutriator rotor) is filled with media and residual air removed to
43 prevent back pressure and altered buffer flow velocity in the chamber. The media flow rate is
44 optimized for collection of dissociated Leydig cells. The harvested cell suspensions are pooled,
45 placed in a loading tube, set in the elutriator, passed through the system, and collected in a
46 separation chamber. The purity of the cell preparation is approximately 25 percent after this

1 step. The other cell types included with the Leydig cells at this stage include macrophages,
 2 pachytene spermatocytes and multinucleated germ cells.
 3



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

6
 7 Source: Klinefelter et al. 1993
 8

1

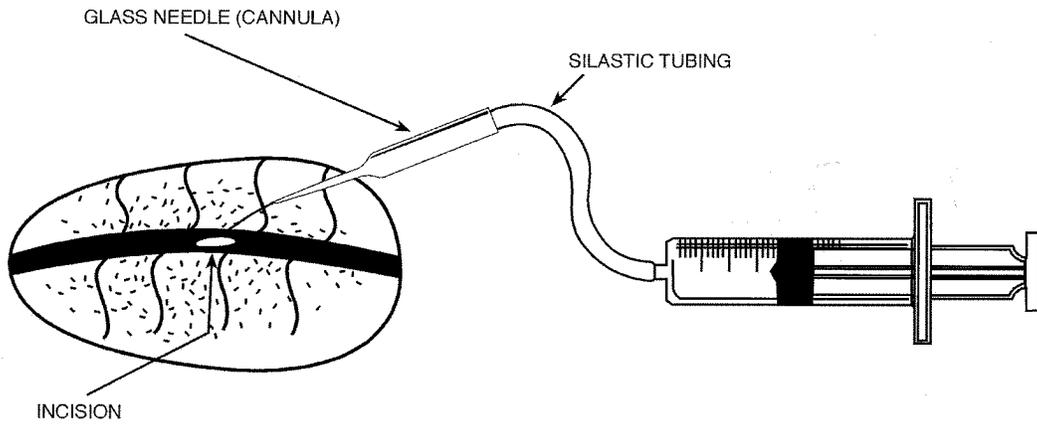


Figure 4-10b. Testicular Perfusion Detail

Source: Klinefelter et al. (1993)

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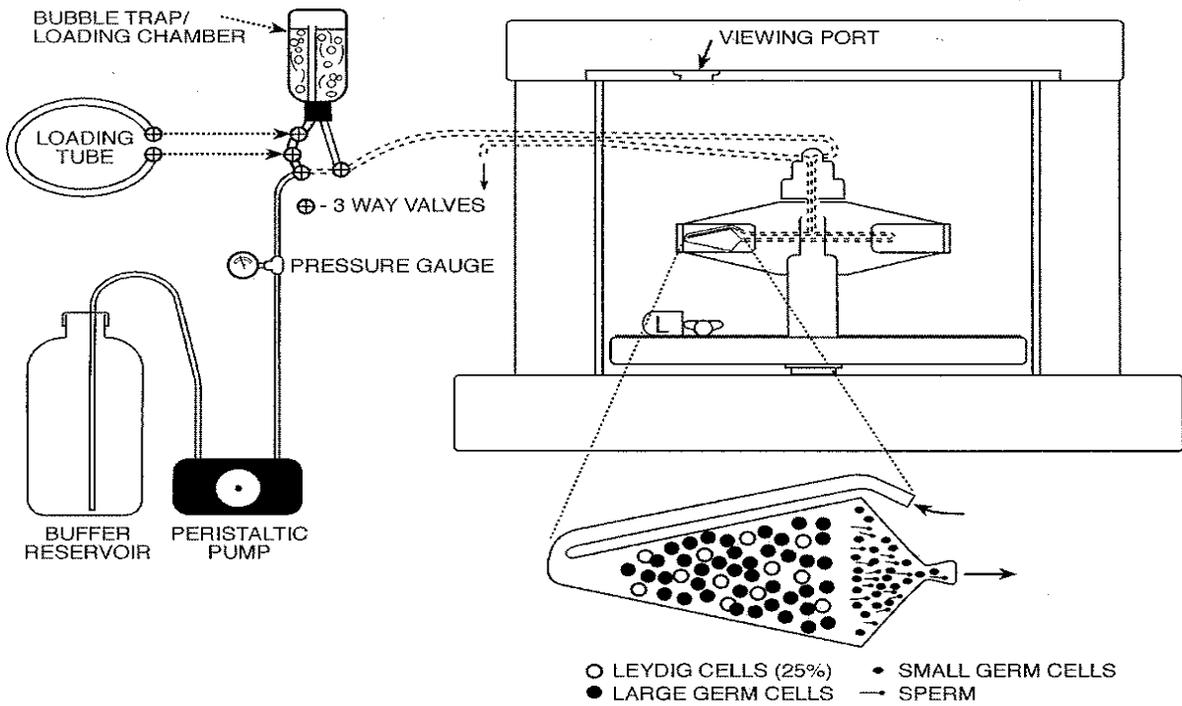


Figure 4-10c. Elutriation System Detail

Source: Klinefelter et al. (1993)

7
8
9
10

1 **Density Gradient Centrifugation.** This step separates Leydig cells from the other cells
2 based on the bouyant densities of the cells. The cell preparation from the previous step are
3 centrifuged and the pellets are resuspended in isotonic Percoll for Percoll density gradient
4 centrifugation. The cell suspensions are centrifuged and the fractions divided. The fraction
5 heavier than 1.068 g/mL contains the purified Leydig cells that are intact and steroidogenically
6 active. The purity of the cell preparation is approximately 98 percent after this step.
7

8 **Culturing.** This step provides an environment that maintains Leydig cell viability for
9 several days. The purified Leydig cell preparation is cultured on Cytodex 3 beads, a porous
10 microcarrier. The beads are coated with a type I denatured collagen, which is used as the
11 substratum for cellular contact. The cells are suspended in media, e.g., DMEM/F12, that
12 contains fetal bovine serum (the source for fibronectin) and incubated at 34EC. The Leydig cell
13 preparation is divided for a yield of approximately 10^6 cells/0.2 mL. The cells are poured into a
14 culture dish, bovine lipoprotein added, and additional media added to bring the final Leydig cell
15 culture volume to 2.0 mL with a cell concentration of 0.5×10^6 cells/mL. The final Leydig cell
16 concentration is critical for optimal testing and must be determined from run to run and lab to
17 lab. The cell cultures are maintained at 34EC in 5 percent CO₂ in air overnight. Additional
18 changes of media are required to keep the cells viable for several days.
19

20 **4.4.1.3 Experimental Design Information.** The purity of the Leydig cell preparation
21 must be high to reduce factors that can confound assessment of steroidogenesis. Red blood cells
22 are removed by perfusion of the testis with media. Perfusion with collagenase also increases
23 Leydig cell yield and steroidogenic Leydig cell response (Klinefelter et al., 1993). Sperm cells
24 are an additional confounding factor, and these cells are removed through centrifugal elutriation.
25 This step also removes smaller and damaged Leydig cells, which is important because
26 steroidogenic activity is altered in the presence of such cells (Klinefelter et al., 1987;
27 Abayasekara et al., 1991). In addition, elutriation removes macrophages. Testicular
28 macrophages release substances that stimulate and inhibit Leydig cell function (Hutson, 1998;
29 Nes et al., 2000).
30

31 The number of Leydig cells used by investigators for a single incubation can vary greatly
32 among investigators. Laskey and Phelps (1991) used an interstitial cell preparation (15 percent
33 Leydig cells) that had 160,000 cells/mL (Laskey and Phelps, 1991). A semi-purified preparation
34 (70 percent Leydig cells) was used with only 10,000 cells/mL (Thoreaux-Manley et al., 1995).
35 The number of cells per unit volume from purified Leydig cell preparations (purities of 95
36 percent Leydig cells) has ranged from as low as approximately 20,000 cells/mL
37 (Klinefelter et al., 1991; 1994) and 50,000 cells/well (Biegel et al., 1995) to as high as 200,000
38 cells/mL (Kelce et al., 1991). All of these preparations were able to show that the substance
39 being tested altered steroid production and secretion.
40

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

1 filtering during the collagenase dispersion step (Salva et al., 2001). This modification did not
2 increase cell purity but did result in a higher concentration of Leydig cell clusters and a higher
3 recovery of greater numbers of Leydig cells.
4

5 Cell viability is important to evaluate in an *in vitro* procedure, and several techniques
6 have been used to ensure that the Leydig cells remain intact. Many investigators actually
7 identify the Leydig cells in the preparation using a histochemical staining technique.
8 Klinefelter, et al. (1987), described how to stain for 3 β -HSD activity, and many investigators
9 now use this technique to demonstrate viability. Other techniques that actually assess cell
10 viability (rather than identify the cell type) include using Trypan blue, which is excluded by
11 intact cells, histological examination of the cells (Klinefelter et al., 1991), quantification of
12 [³⁵S]-methionine incorporation into proteins synthesized *de novo* (Kelce et al., 1991), and a
13 colorimetric assay using tetrazolium salt MTT, which is reduced by succinate dehydrogenase (a
14 mitochondrial enzyme) to formazan (Thoreaux-Manley et al., 1995).
15

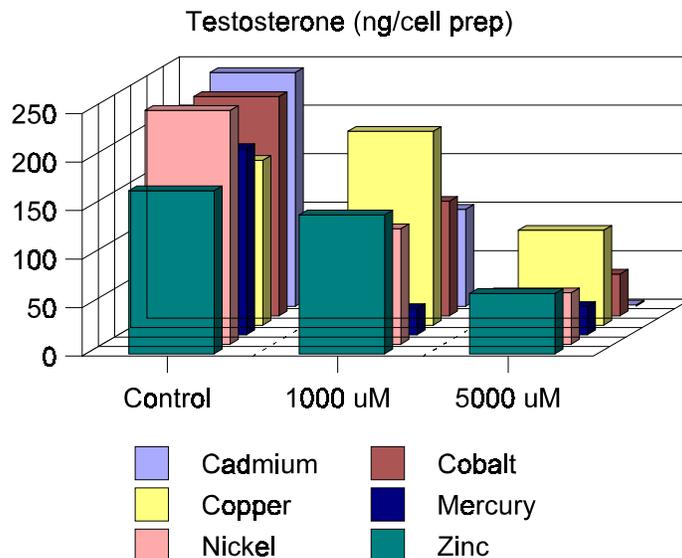
16 Procedures used to culture the cells can affect cell viability. Important factors include
17 screening each lot of collagenase for activity, ensuring that the enzyme concentration is kept
18 relatively constant for all incubations, and using an optimal dispersion technique
19 (Molenaar et al., 1985). In addition, extended culture time is optimized by adding bovine
20 lipoprotein to Leydig cell cultures and using a porous substrate (Cytodex 3 beads) for cellular
21 attachment (Klinefelter et al., 1993). Other factors that extend viability include reduced oxygen
22 and submaximal LH stimulation (Klinefelter and Ewing, 1989).
23

24 An important experimental design consideration is the type and age of the animal used to
25 prepare the isolated and cultured cell preparation. For information regarding this topic, the
26 reader is directed to the experimental design considerations described for the in-life aspects of
27 the *ex vivo* method (Section 4.2.3), where this information is presented.
28

29 Investigators have used a variety of Leydig cell incubation procedures. For example,
30 Biegel et al. (1995) incubated the purified Leydig cells for 2 hours with (treatment) or without
31 (control) the substance being tested, added the stimulant (2 IU hCG), and incubated the cellular
32 preparation an additional 3 hours before discontinuing the incubation and collecting media
33 samples for steroid hormone analysis. Other investigators have mixed the cells, stimulant, and
34 substance being tested all at once and, after a 3-hour incubation, removed the media for steroid
35 hormone analysis (Klinefelter et al., 1991; 1994). Much longer incubation periods have also
36 been utilized. Thoreaux-Manlay et al. (1995) incubated the cells, stimulant, and substance being
37 tested and, after 4 hours, removed the media for analysis. The media were replenished and the
38 cell preparation incubated for an additional 20 hours and, once again, the media samples
39 collected and the media replenished. A final sampling was made 24 hours later (48 hours from
40 initiating the incubation). These examples demonstrate the flexibility of the isolated Leydig cell
41 method and how it can be modified to test for special characteristics of the substance being
42 tested. In addition, the different procedures provide insight into the difficulty of standardizing
43 such a procedure such that it could be used by multiple laboratories and/or at different times (see
44 below for additional issues to be addressed concerning standardization of this procedure).
45

46 **4.4.1.4 Representative Studies from the Literature.** Laskey and Phelps (1991)
47 demonstrated the usefulness of the interstitial cell preparation (15 percent Leydig cells) to

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

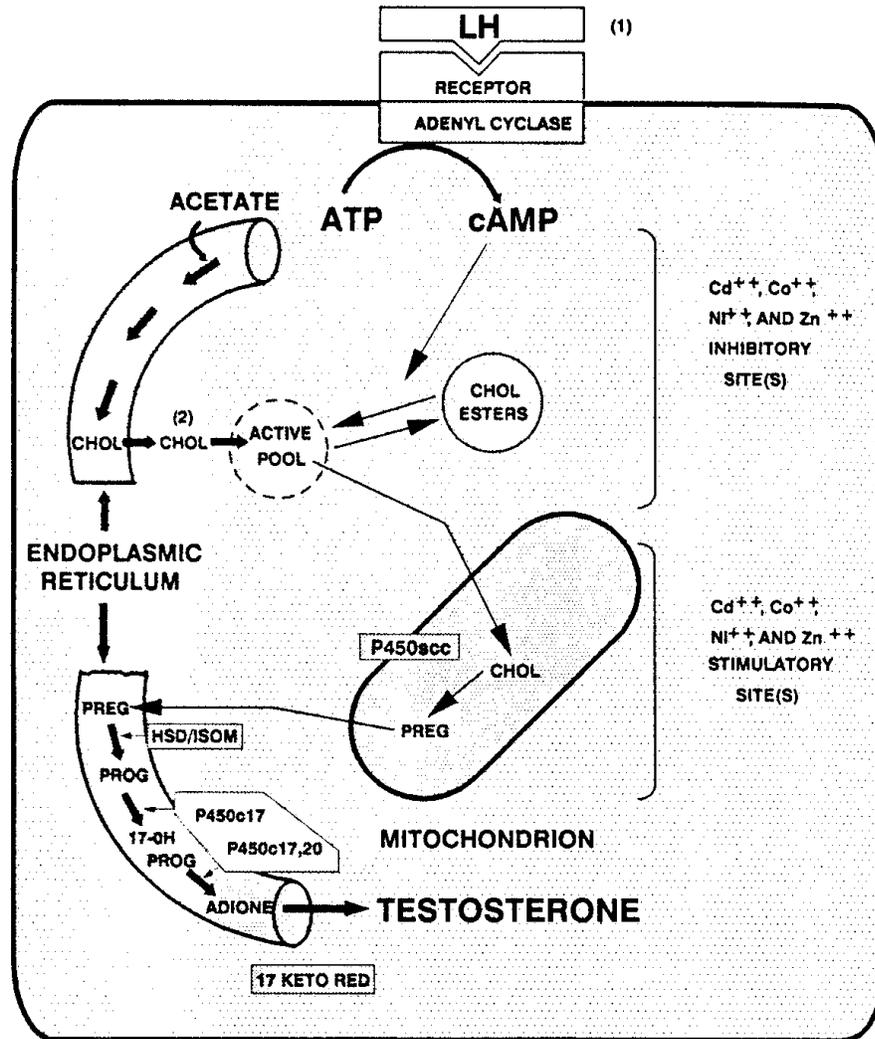


5
 6 **Figure 4-11. Effect of Metal Cation Treatment on hCG-Stimulated Testosterone**
 7 **Production using an *In Vitro* Interstitial Cell Preparation**
 8

9 *Notes: A Leydig cell preparation that was prepared from adult SD rats was used to determine the toxic*
 10 *effects of several metal cations. The Leydig cells were stimulated to produce testosterone by using hCG*
 11 *(simulates LH), cAMP (tests for post LH receptor defects), 20 α -hydroxycholesterol (a substrate for*
 12 *P450_{SCC}), or pregnenolone (a substrate for the isomerase/3 β -hydroxysteroid dehydrogenase enzyme).*
 13 *The metal cations, at concentrations ranging from 1 to 5000 FM, were incubated with the Leydig cells for*
 14 *3 hours in the absence or presence of these stimulating agents or substrates. There was no effect on cell*
 15 *viability. A dose-response depression with hCG- and cAMP-stimulated testosterone production was*
 16 *observed for cadmium, cobalt, copper, mercury, nickel, and zinc. In addition, cadmium, cobalt,*
 17 *and zinc caused a significant increase in testosterone production when the substrates were used.*
 18

19 Source: Laskey and Phelps, 1991, page 300.
 20
 21

22 From the results of this investigation, Laskey and Phelps developed a schematic
 23 representation of the sites of cationic stimulatory or inhibitory action. Their illustration is
 24 provided in Figure 4-12.
 25



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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., 20 α -hydroxycholesterol (HCHOL), pregnenolone (PREG), progesterone (PROG), 17 α -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 perfluorooctanoate @ 100 to 1000 FM/2 IU hCG	9hCG-stimulated testosterone production (IC ₅₀ approx. 200 FM) cytotoxicity \$ 750 FM 9estradiol (0-12 hrs) and 8basal and hCG-stimulated estradiol production	Biegel et al., 1995
Adult male SD rats	lead acetate @ 100, 250, or 500 FM / 50 mIU/mL hCG	9hCG-stimulated testosterone and progesterone production 9Immunohistochemical staining for P450c17, P450 _{SCC} , 3β-HSD distended smooth ER with fewer anastomoses	Thoreux-Manlay et al., 1995
Adult male SD rats	chloroethylmethanesulfonate (CEMS) @ 3 to 10,000 FM/100 mIU/mL hCG ethane dimethanesulfonate (EDS) @ 3 to 10,000 FM/100 mIU/mL hCG	9testosterone production @ \$3000 FM (EC ₅₀ = 2200 FM) 9testosterone production @ \$300 FM (EC ₅₀ = 445 FM)	Klinefelter et al., 1994
Adult male SD rats	EDS @ 100, 200 or 500 FM/100 mIU hCG EDS/5 mM db-cAMP EDS/5 FM 20" - hydroxycholesterol EDS/2 FM pregnenolone	9hCG-stimulated testosterone production (EC ₅₀ = 370 FM) 9db-cAMP-stimulated testosterone production (EC ₅₀ = 370 FM) Maintained testosterone production Maintained testosterone production	Klinefelter et al., 1991
Adult male SD rats	EDS @ 10 to 10,000 Fg/mL /100 ng/mL LH	9LH-stimulated testosterone production (EC ₅₀ = 94 Fg/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 FM 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

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

3
4 **4.4.2.2 Description of the Method.** Like the isolated Leydig cell method, preparation
5 of isolated and cultured granulosa cells is a labor-intensive procedure (Treinen, 1993). Briefly,
6 to prepare rat granulosa cell cultures, 18- to 25-day old female rats are obtained and prepared for
7 implantation of DES implants. The implants are inserted between the skin and subcutaneous
8 tissue of the anesthetized animal. Animals are allowed to recover until they are used 4 to 5 days
9 later. An alternative to the DES implants is daily subcutaneous injections of estradiol or DES
10 (1 mg) for 5 days prior to being used for culturing. Removal of ovaries for culturing involves
11 euthanizing and exsanguinating the animal and removing the ovaries using aseptic techniques.
12 The extraneous tissues are cut away from the ovary, which is then placed into a sterile beaker
13 containing warmed medium. The process is repeated until all ovaries are collected (up to
14 approximately 50 ovaries per beaker can be processed at a time).

15
16 The next steps disperse the granulosa cells from the ovary and prepare the cells for usage.
17 The ovaries are washed and poured onto a flat-bottomed culture plate. Each ovary is teased
18 apart manually with a needle to release the granulosa cells. The medium containing the
19 granulosa cells is transferred to a centrifuge tube, fresh media added, and the suspended cells are
20 centrifuged. The pelleted cells are resuspended in fresh media. An aliquot is removed and
21 checked for viability using the Trypan blue exclusion procedure and a hemocytometer to
22 calculate the number of viable cells in the culture. The cells are then split to obtain
23 approximately 2,500,000 cells/mL and the tubes incubated for 18 to 24 hours to allow the cells to
24 completely attach. Next, the medium is poured off, leaving the granulosa cells in a ring on the
25 bottom of the tube (non-granulosa cells, e.g., theca cells, constitute less than 10 percent of the
26 cells retained). Culture tubes are better than culture plates for steroid production (Carnegie et
27 al., 1988). After 24 hours, the medium is changed at least every 72 hours, antibiotics added, e.g.,
28 penicillin and streptomycin, to reduce bacterial contamination. Viability is assessed with Trypan
29 blue exclusion and ATP measurements.

30
31 **4.4.2.3 Experimental Design Information.** The variety of granulosa cell functions that
32 can be assessed requires consideration of the culture conditions and times for endpoints
33 (Treinen, 1993). For example, FSH, cAMP, or some other stimulant can be added initially in
34 order to evaluate the effect of a substance on steroid hormone, e.g., estradiol or progesterone,
35 production and secretion. The cultures of stimulant and substance being tested are often
36 incubated for 36 to 48 hours in order to get a measurable concentration of the steroid hormone
37 being analyzed. Measurement of FSH-dependent cAMP accumulation by a toxicant can be
38 measured within 24 hours of culturing (Treinen et al., 1990). The stimulant, optimal
39 concentration of stimulant, and time for collection of samples for analysis all must be
40 characterized as has been previously described for a given cell preparation in order to ensure that
41 the cells are viable.

42
43 Cell viability is assessed using a variety of procedures. Most granulosa cell cultures are
44 used within 48 hours; however, some investigators have reported that the culture system is
45 viable for over 10 days (Azhar et al., 1988). Viability has been tested using such procedures as
46 intracellular ATP concentrations, protein determinations, and the Trypan blue exclusion and
47 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.

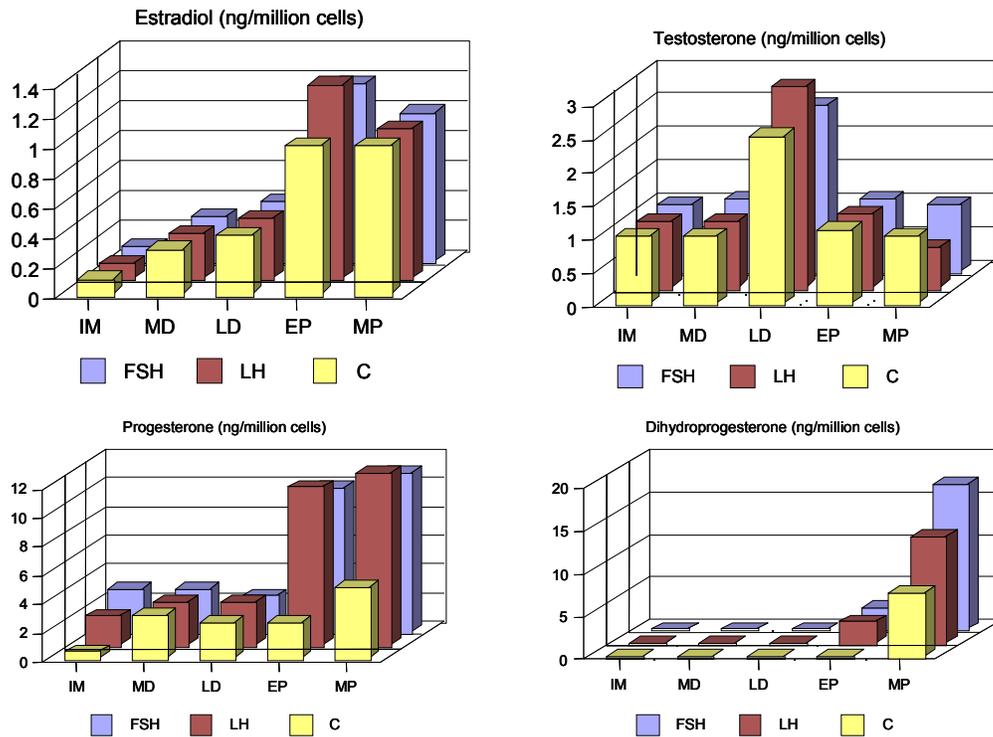


Figure 4-13. Steroid Hormone Production of Rat Granulosa Cells of Follicles at Various Stages of Maturity

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.

1
2 **4.4.2.4 Representative Studies from the Literature.** Other studies that have been
3 conducted using isolated granulosa cells to evaluate substances for estrogenic or anti-estrogenic
4 effects are summarized in Table 4-8.

5
6 **Table 4-8. Representative Studies Using the Isolated Granulosa Cell Preparation**

7

Animal	Treatment	Response	Reference
Adult rat (strain unspecified)	nomegestrol acetate (a synthetic progesterone) @ 0.45, 0.9, or 1.8 mg/L / Stimulated with testosterone (0.5 FM/L)	IC ₅₀ = 6.85 mg/mL 9estrogen production @ \$0.9 mg/mL	Qian et al., 2001
Porcine	cadmium chloride @ 0.2, 10, or 20 ng/mL	9cell membrane, ER, Golgi apparatus complex integrity; 8lysosomes and lipid droplets; and 9progesterone and 17\$-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 FM / FSH @ 100 ng/mL and testosterone @ 10 FM	9FSH- and testosterone-stimulated estradiol production; no effect on progesterone	Milne et al., 1987

17
18
19 **4.4.2.5 Distinguishing Features of the Isolated Granulosa Cell Culture Method.**

20
21 **Stability** Isolated granulosa cell cultures are routinely used for 48 hours, and some
22 investigators have reported cell viability for over 10 days
23 (Azhar et al., 1988).

24
25 **Standardization** This procedure is not standardized and it would be very difficult to do so
26 because of the number of variables. For example, standardization of this
27 method involves many of the same issues that make isolated Leydig cell
28 preparation methods difficult to standardize. Items that are unique to the
29 isolated granulosa cell preparation include the stage of maturity of the
30 follicle and granulosa cells, type and amount of stimulant (DES, estradiol,
31 PMSG), and degree of “contamination” with thecal cells.

32
33 **Sensitivity** The isolation of granulosa cells does not require the use of collagenase or
34 proteolytic enzymes, which can enhance the response of substances, since

1 the membrane-bound receptors and other cellular proteins would not be
2 digested (Treinen, 1993). However, the sensitivity can be compromised in
3 that it does not take into account the interaction that can take place
4 between the surrounding theca interna cells, as well as the oocyte, or
5 paracrine factors in the whole follicle.
6

7 **Specificity**

The granulosa cell preparation steroidogenic response can be antagonized
8 by factors and substances in the serum (Orly et al., 1996; Nothnick and
9 Curry, 1996). Also, residual cells can have an effect. Corpus luteum
10 angiogenic factor, also termed fibroblast growth factor (FGF), inhibits
11 FSH's stimulation of estrogen production and induces LH receptor
12 synthesis. Also, at suboptimal concentrations, FGF enhances the synthesis
13 of progesterone (Baird and Hseuh, 1986).
14

15 **Metabolic Activity**

None.

16 **Equipment**

Standard and specialized laboratory equipment are required. Specialized
17 equipment primarily involves laminar flow hoods for maintaining a sterile
18 work environment when isolating and handling the cells.
19
20

21 **Training.**

Requires general and special laboratory training.
22

23 **Sample Scheduling**

Isolation of the granulosa cells takes 1 day, and then the cells are allowed
24 to incubate and attach to the test tube, which takes another 18 to 24 hours.
25 Afterwards, the cell preparations are usually incubated for 48 hours with
26 the stimulant and the substance being tested before samples are collected
27 for analysis. Investigators prefer using the isolated cells within 48 hours
28 of isolation. Thus, the cell preparation is labor-intensive to obtain, and the
29 cells are generally used promptly after collection for best results.
30

31 **Animal Usage**

The isolation of cells for use as an assay significantly reduces the number
32 of animals used because a number of runs can be conducted from a single
33 set of gonads. The isolated cell assay uses the lowest number animals of
34 the assays that use animals to obtain gonadal organs.
35

36 **4.4.2.6 Conclusions.** The isolated granulosa cell preparation is a sensitive and
37 effective procedure for evaluating the effect of a substance on steroid hormone production and
38 secretion, as well as to investigate the mechanism of action of a substance. However, as with the
39 Leydig cell methods, extended time, relatively high cost, and laborious effort are needed to
40 isolate and purify the granulosa cells. Thus, the procedure needs to be carefully evaluated with
41 respect to the objectives and characteristics of the screen that is desired for identifying
42 substances that alter steroidogenesis.
43

1
2 **4.5 Cell Line Methods (*In Vitro*)**
3

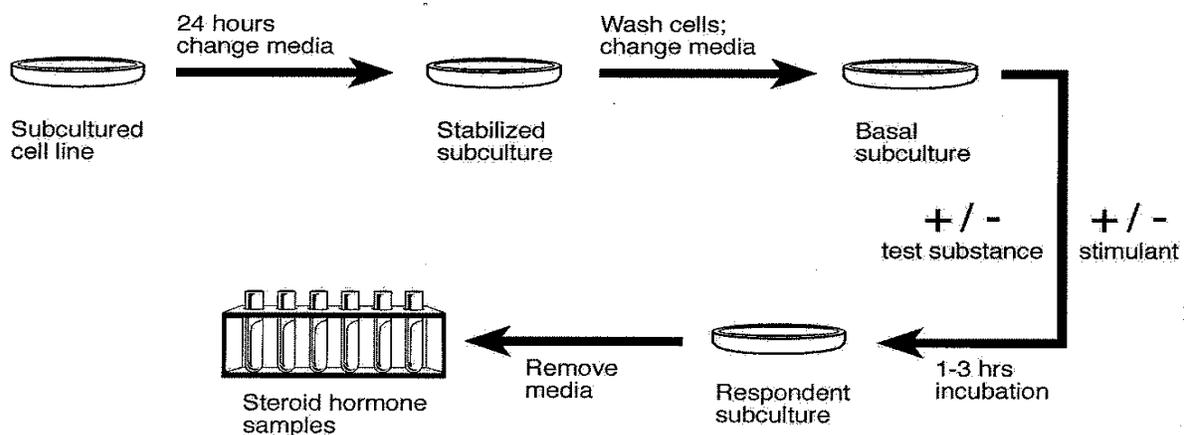
4 Cell lines can be used to measure the effect of a substance on the steroidogenic pathway.
5 This is made possible by the “immortalization” of Leydig cells from Leydig cell tumors.
6 However, cell lines obtained from tumor cells can be different from normal cells. For example,
7 most Leydig cell lines do not retain their capacity to be stimulated by LH/hCG. The use of cell
8 lines requires the researcher to characterize the cell line and evaluate its capacity to simulate the
9 biochemical pathways found in a nontransformed, differentiated cell. Once the differences are
10 characterized and understood, then a given cell line has been shown to prove useful for
11 screening, identification, and characterization of xenobiotics with endocrine-altering properties.
12 This subsection provides an overview of this method.
13

14 **4.5.1 Scope of the Method**
15

16 Cell lines offer another *in vitro* method for studying the effects of substances on the
17 steroidogenic pathway. In regard to steroidogenesis, cell lines have been used to purify and
18 identify proteins that affect steroidogenesis (Boujrad et al., 1995); second messenger activation
19 (Pereira et al., 1987); regulation of receptors (Rebois and Fishman, 1984); gene regulation
20 (Payne et al., 1992); cholesterol transport (Freeman, 1987); paracrine factors that stimulate
21 steroidogenesis (Boujrad et al., 1995); and steroidogenic enzyme characterization
22 (Clark et al., 1994; Walsh et al., 2000b). A review of immortalized Leydig cell lines is provided
23 by Hoelscher and Ascoli (1996).
24

25 **4.5.2 Description of the Method**
26

27 Figure 4-14 shows a schematic diagram for a cell line assay.
28



29
30 **Figure 4-14. Schematic Diagram for a Cell Line Assay**
31

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

11 4.5.3 Experimental Design Information

13 **4.5.3.1 Immortalized Leydig Cell Lines.** Several types of cell lines can be used to study
 14 the effects of substances on steroidogenesis. Most of the cell lines come from rat or mouse
 15 Leydig cell tumors. Some cell lines derived from Leydig tumors and the species/strain from
 16 which they were derived are listed below (Hoelscher and Ascoli, 1996).

18 <u>Cell Line Name</u>	19 <u>Species/Strain</u>	20 <u>Source^a</u>
21 NWL2, NWL15	22 Rat/SD	23 --
24 R2C	25 Rat/Wistar-Furth	26 ATCC
27 LC540	28 Rat/F344	29 --
30 MA-10	31 Mouse/C57Bl/6	32 Dr. M. Ascoli Univ of Iowa, Ames, IA
33 MLTC-1	34 Mouse/C57Bl/6	35 ATCC
36 B-1-A-2	37 Mouse/BALB/c	38 --
39 TM3	40 Mouse/BALB/c	41 ATCC
42 I-10	43 Mouse/BALB/cJ	44 ATCC
45 K-9	46 Mouse/hybrid MA-10 cells and fresh Leydig cells	47 --

48 ^a ATCC = American Type Culture Collection.

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

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

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

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

18
19 **4.5.3.2 Adrenocortical Carcinoma Cell Line.** Steroidogenesis occurs in the adrenal
20 glands, as well as the gonads. Much of the same pathway found in the gonads, which
21 synthesizes cholesterol into androgens and estrogens, is also found in the adrenal gland, which
22 synthesizes cholesterol into glucocorticoids. As described for the steroidogenic pathway in the
23 testis/ovary (Figure 3-6), the glucocorticoid pathway converts cholesterol to pregnenolone via
24 P450_{SCC} and pregnenolone to progesterone via 3 β -HSD and progesterone to 17-
25 hydroxyprogesterone via P450c17. These three enzymes are common to both the
26 androgen/estrogen and glucocorticoid steroidogenic pathways. After formation of 17-
27 hydroxyprogesterone, the glucocorticoid pathway includes two additional reactions involving
28 21-hydroxylase and 11 β -hydroxylase, which results in the formation of 11-deoxycortisol and
29 finally the end-product glucocorticoid-steroid hormone, cortisol. Thus, except for the last two
30 steps, common enzymes and substrates are found in the steroidogenic pathways of the gonads
31 and adrenal glands.

32
33 The commonality of the androgen/estrogen and glucocorticoid steroidogenic pathways
34 is the basis for considering an adrenocortical carcinoma cell line as an alternative to an
35 immortalized Leydig cell line. One such adrenocortical carcinoma cell line is the human NCI
36 H295 cell line. Although the cell line has not been completely characterized with respect to the
37 complete steroidogenic pathway as described in Section 3, investigators have shown that the cell
38 line includes a cyclic-AMP second messenger system, as well as the steroidogenic enzymes
39 P450_{SCC} and P450c17 (Rodriguez et al., 1997; Fassnacht et al., 2000). These components of the
40 pathway have been altered following treatment of the H295 cells with aminoglutethimide,
41 metyrapone, and etomidate (Fassnacht et al., 2000). Endpoints that have been measured to
42 evaluate the effect of a test substance on steroidogenic function of these cells include
43 17-hydroxyprogesterone and cortisol.

1 **4.5.3.3 Cell Line Properties.** A summary of the properties of the cell lines described
 2 above is included in Table 4-9, showing whether the research cited did (T) or did not (x) confirm
 3 the presence of the component in the respective cell line. The properties listed in the table are
 4 those components that comprise the steroidogenic pathway found in the testis/ovary and, for the
 5 H295 cell line, found in the adrenal glands.
 6

7 **Table 4-9. Summary of Immortalized Cell Line Properties ^a**
 8

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)	--	T (Stocco & Chen, 1991)	T (Rebois, 1982)	T (Taylor et al., 1997)	--	--
Signal Transduction							
cAMP	X / T (Stocco, 1992; Freeman, 1996)	T (Pignataro et al., 1992)	T (Stocco & Chen, 1991)	T (Manna et al., 2001a)	T (Taylor et al., 1997)	T (Pignataro et al., 1992)	T (Fassnacht et al., 2000)
Ca ²⁺	--	--	T (Ramnath et al., 1997)	T (Manna et al., 1999)	T (Adebanjo et al., 1998)	--	--
Cl ⁻	--	--	T (Ramnath et al., 1997)	T (Panesar, 1999)	--	--	--
Arachidonic Acid	--	--	T (Wang et al., 2000)	--	--	--	--
Intracellular Cholesterol Storage, Release, and Transport							
cholesterol storage/release	T (Freeman, 1996)	--	T (Dees et al., 2001)	--	--	--	--
StAR protein	T (Stocco et al., 1995)	--	T (Stocco et al., 1995)	T (Manna et al., 2001b)	--	--	--
Enzymes							
P450 _{scc}	T (Stocco et al., 1995)	T (Pignataro et al., 1992)	T (Stocco et al., 1995)	--	--	T (Pignataro et al., 1992)	T (Fassnacht et al., 2000)
3B-HSD	T (Teixeira et al., 1999)	--	T (Teixeira et al., 1999)	T (Manna et al., 2001b)	--	--	--
P450 _{c17}	T (Teixeira et al., 1999)	--	T (Teixeira et al., 1999)	T (Manna et al., 2001b)	--	--	T (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
5 α -reductase	--	--	T (Rommerts et al., 2001)	--	--	--	--
aromatase	T (Doody et al., 1990)	--	--	--	--	--	--
End-Product Hormones							
progesterone	T (Stocco & Chen, 1991)	--	T (Rommerts et al., 2001)	T (Rebois, et al., 1982)	--	T (Taylor et al., 1997)	--
20 α -hydroxy-progesterone	--	--	T (Gocze & Freeman, 2000)	T (Rebois, 1982)	--	--	--
testosterone	--	T (Steinberger et al., 1970)	--	--	--	--	--
estradiol	--	T (Steinberger et al., 1970)	--	--	--	--	--
17-hydroxy-progesterone	--	--	--	--	--	--	T (Fassnacht et al., 2000)
cortisol	--	--	--	--	--	--	T (Fassnacht et al., 2000)

^a No information could be found in the literature for the NWL2, NWL15, and B-1-A-2 cell lines.

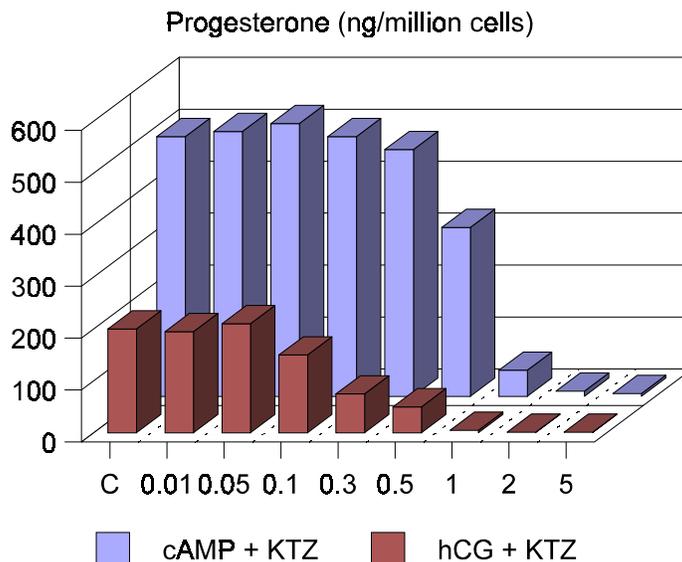
T Information found, and this component is present in the cell line.

x Information found, and this component was not shown to be present in the cell line.

-- No information found.

1
2 **4.5.4 Representative Studies from the Literature**
3

4 Chaudhary and Stocco (1989) used the MA-10 cell line to characterize the inhibition of
5 steroidogenesis by ketoconazole. Some of the results are illustrated in Figure 4-15.
6



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

8
9 *Note: The effect of different concentrations (fM) of ketoconazole (KTZ) on hCG- or cAMP-stimulated*
10 *progesterone production in MA-10 cells was determined during a 3-hour incubation. Cells were grown*
11 *in 12-well tissue culture plates in Waymouth's medium. The concentration of hCG was 34 ng/mL and of*
12 *cAMP was 1 mM. Wells without KTZ were used as control (C). Aliquots were removed after three hours*
13 *from each of the wells and assayed for progesterone by RIA. KTZ inhibited MA-10 cell production of*
14 *progesterone when KTZ concentrations were 0.1 fM in the hCG-stimulated cells and 0.5 fM in the*
15 *cAMP-stimulated cells.*

16
17 Source: Chaudhary and Stocco (1989)

18
19
20 Additional examples of cell lines used in experimental studies for measuring
21 steroidogenesis are summarized in Table 4-10.

22
23 **4.5.5 Distinguishing Features of the Cell Line Method**
24

25 **Stability**

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

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

2

3

4

5

6

7

Cell Line	Treatment	Response	Reference
MA-10 cells	carbonyl cyanide m-chlorophenylhydrazine (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 1Fg/mL conconavalin A @ 0.01 to 50 Fg/mL	8progesterone @ > 0.01 Fg/mL 8progesterone @ > 1 Fg/mL	Boujrad et al., 1995

8

9

10 **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 FM concentrations of a substance (Chaudhary and Stocco, 1989).

11

12

13

14

15

16 **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., AIF, a universal activator of G protein, do not initiate intracellular biochemical pathways, which is attributed to receptor density.

17

18

19

20

21

22

23 **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).

24

25

26

27

28 **Metabolic Activity**

None.

29

30 **Equipment**

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

31

32

33

34 **Training**

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

35

1 **Animal Usage** This assay does not use animals.

2 3 **4.5.6 Conclusion**

4
5 Leydig tumor cell lines have been used to study the effects of substances on steroid
6 hormone production. These cell lines do not express the complete steroidogenic pathway.
7 Although incomplete, the pathway that is present is similar in many ways to the steroidogenic
8 pathway in the normal Leydig cell. For this reason, it can be used to assess the effect of a
9 substance on steroid hormone production when stimulated with LH, hCG, cAMP, etc.

10 11 **4.6 Basis for Selection of a Steroidogenic Screening Method**

12
13 The first objective of this DRP is to determine whether a method can be selected, which
14 could serve as the most promising screen, so as to identify substances that have inhibitory or
15 stimulatory effects on the production of testicular and ovarian steroidogenic hormones. To
16 achieve this end, the methods that are used to identify such substances, examples of data
17 generated by each of the methods, and distinguishing features of each method were reviewed
18 (Subsections 4.1 to 4.5). The information that was presented about each method has been
19 compared with criteria that define an optimal screen. Out of this comparison and critique, one of
20 the methods was selected for recommendation to be used as a screening tool. The following
21 subsections provide the basis for this selection.

22
23 Several comparisons were made in order to narrow down the choices and select the most
24 promising assay to be used as the screen. These comparisons include:

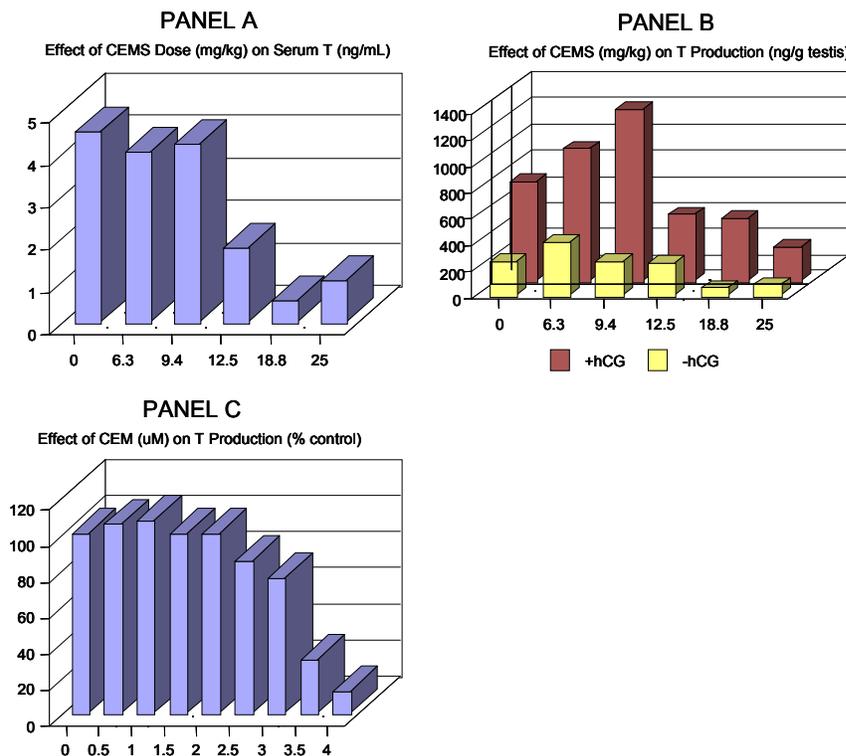
- 25
- 26 ● Method type comparisons, i.e., *In Vivo* vs. *Ex Vivo* vs. *In Vitro*
- 27 ● Gender comparisons, and
- 28 ● Different method-subtype comparisons, e.g., sectioned testes vs. isolated/cultured
29 Leydig cell preparation.

30 31 **4.6.1 Method Type Comparisons**

32
33 The purpose of this subsection is to provide a review of the literature for some of those
34 studies that tested the same substance for its effect on steroidogenesis using two or more of the
35 methods described in the preceding subsections. In this way, a side-by-side comparison of the
36 relative outcome can be made for the various methods and the strengths/weaknesses of each
37 method relative to one another will be made more apparent. Although many different
38 investigators have tested a given substance using the different methods, the information
39 presented here focuses on those studies where the same investigator tested the same substance
40 but used different methods. In so doing, a better comparison of the methods' outcome (rather
41 than the differences between laboratories) can be made. The examples presented below will also
42 identify some of the strengths and weakness of each method and thereby allow some conclusions
43 to be drawn regarding the relative benefits of each method.

44
45 **4.6.1.1 *Ex Vivo* vs. *In Vitro*.** Numerous studies are available in the literature that make
46 it possible to compare the outcome of *ex vivo* and *in vitro* methods. In fact, the natural
47 progression of a scientific investigation involves using both methods in order to evaluate the

1 response in an intact animal followed by an attempt to characterize the test substance's
 2 mechanism and site of action using isolated organs. Klinefelter et al. (1994) used an *ex vivo*
 3 method (four-day in-life phase followed by isolation and testing sectioned testis) and an *in vitro*
 4 method (purified Leydig cell preparation) to evaluate the effects of chloromethylmethane-
 5 sulfonate (CEMS) on steroidogenesis. The results are illustrated in Figure 4-16.
 6



7
 8 **Figure 4-16. Example of *Ex Vivo* vs *In Vitro* Data**
 9

10 Source: Klinefelter et al. (1994)

11
 12
 13 As can be seen from the figures, a dose-related decrease in serum testosterone was
 14 observed at dosages \$12.5 mg/kg of CEMS (Panel A). Likewise, hCG-unstimulated and
 15 hCG-stimulated testosterone production using sectioned testes was reduced at dosages \$18.8 and
 16 25 mg/kg of CEM, respectively (Panel B). Agreement with these *ex vivo* results was observed
 17 when CEMS was incubated with isolated and cultured Leydig cells. In the *in vitro* purified
 18 Leydig cell preparation, a dose-dependent decrease in hCG-stimulated testosterone production
 19 was measured (Panel C). It was from the significant dose-related decrease in serum testosterone
 20 (*ex vivo*) that the investigators deduced that the chemical was affecting steroidogenesis. They
 21 confirmed this finding with the *in vitro* method. In addition to characterizing the mechanism and
 22 site of action of CEMS, the investigators were able to identify differences in sensitivity. For
 23 example, serum testosterone decreased significantly at 12.5 mg/kg, which was a lower dose than
 24 that required to significantly decrease testosterone production from the testis (18.8 to 25 mg/kg).
 25 The authors concluded that LH-stimulation of Leydig cells is compromised *in vivo*. Finally,

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

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

20
21 **4.6.1.2 In Vivo vs. Ex Vivo vs. In Vitro.** Investigators have utilized *in vivo*, *ex vivo*,
22 and *in vitro* methods to characterize the steroidogenic effects of a substance.
23 Biegel, et al. (1995), investigated the effect of ammonium perfluorooctanoate (C8) following a
24 14-day in-life exposure phase (*in vivo*). They also treated the animals with the same dosing
25 regimen and, at termination, isolated and cultured purified Leydig cells (*ex vivo*). Finally, these
26 investigators isolated and cultured purified Leydig cells from untreated animals for incubation
27 with C8 (*in vitro*). Some of the results for this study are illustrated in Figure 4-17.

28
29 These results show that C8 produced no effect on serum testosterone but decreased
30 testicular interstitial fluid testosterone based on the *in vivo* method (Panels A and B). Using the
31 *ex vivo* method, C8 increased testosterone production in hCG-stimulated cells (Panel C).
32 Finally, in the *in vitro* experiment, C8 produced a dose-dependent decrease in hCG-stimulated
33 testosterone production (Panel D). By using all three methods, these investigators were able to
34 show that C8 directly inhibits release of testosterone from Leydig cells (*in vitro*). Furthermore,
35 using the *ex vivo* method, they were able to demonstrate that the effect of C8 was reversible.
36 The *in vivo* data supported their hypothesis that estradiol may modulate growth factor expression
37 in the testis.

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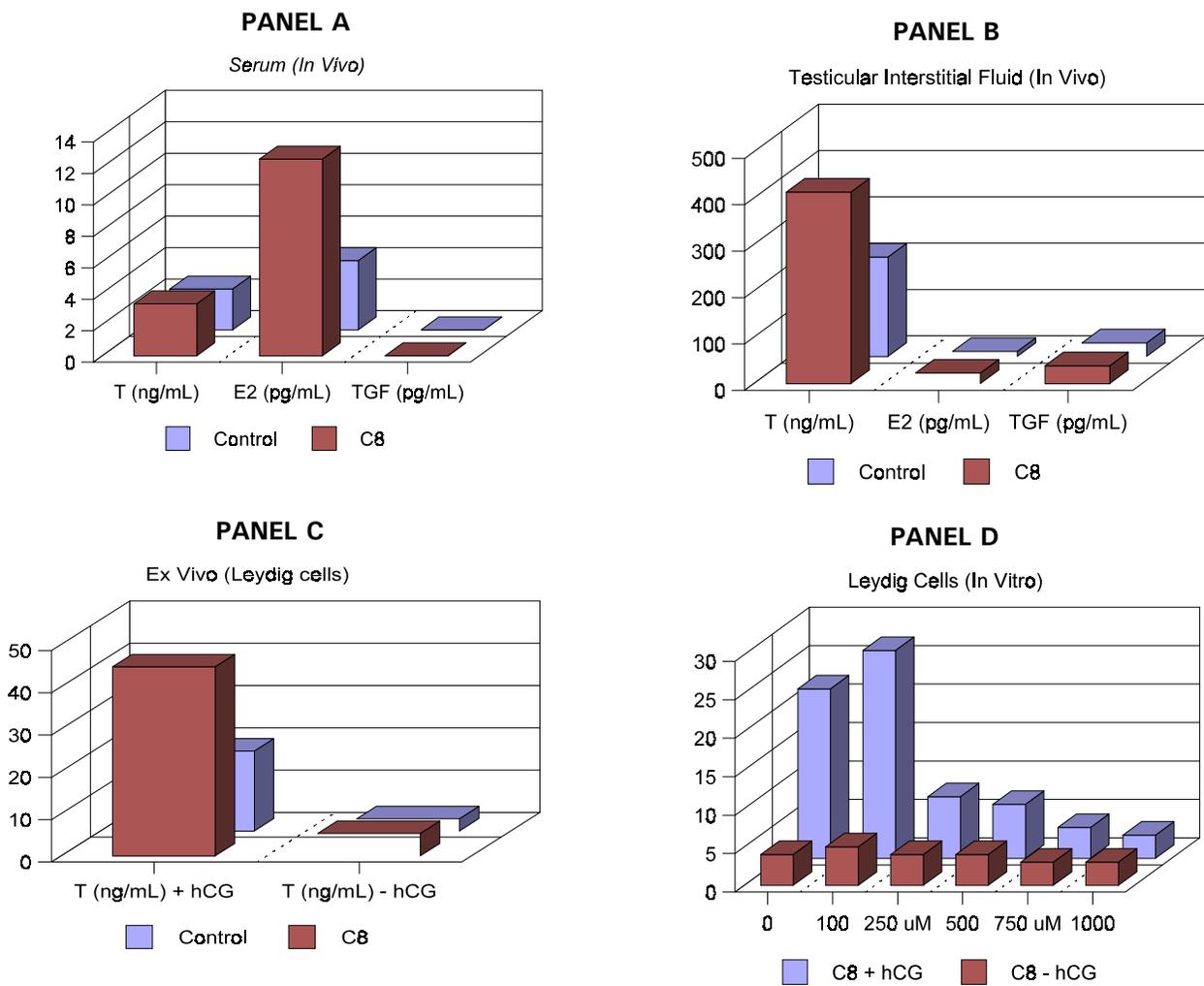


Figure 4-17. *In Vivo* vs *Ex Vivo* vs *In Vitro* Results for C8

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 EC₅₀ values obtained for EDS by using these different *in vitro* procedures were:

<u><i>In Vitro</i> Procedure</u>	<u>EC₅₀ (FM EDS)</u>
Sectioned Testis	336
Perfused Testis	430
Purified Leydig Cells	370 to 430

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

11
 12 **4.6.1.4 Conclusions Regarding Method-Type Comparisons.** The *in vivo*, *ex vivo*, and
 13 *in vitro* methods all provide a means to evaluate steroid hormone production in the testis or
 14 ovary following exposure to a substance. However, while these methods complement each other
 15 and together provide more information than any one method alone, each method also has unique
 16 attributes that favor characterization of the substance in a way that the other method is not able
 17 to do. Distinct advantages can be enumerated for the different types of methods. The following
 18 list is a compilation of the attributes of *in vivo* and *in vitro* methods (EDSTAC, 1998).
 19 Obviously, the *ex vivo* method includes the attributes from both lists.

<i>In Vivo</i>	<i>In Vitro</i>
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

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

1 The characteristics of a screen must be considered in order for the most promising
2 method to be chosen. A screen is not used to establish a dose response, describe the mechanism
3 of action, or determine the adverse response of a substance on development and/or the
4 reproductive system (Gray et al., 1997). Rather, to evaluate a method for its capacity to be a
5 screen, the following list of criteria can be used:
6

7 Inexpensive (low cost)	Predictive level of age, sex, and mammalian systems
8 Degree of representation of the organ 9 function (cytoarchitecture)	Numerous endpoint options
10 Short time needed to set-up the method	Reduced animal usage
11 Fast sample throughput	Standard laboratory technical training required
12 Standard laboratory equipment required	Specific
13 Sensitive	Minimal inter-/intra-laboratory variability
14 Stable preparation (viability)	No chemical solubility/permeability limitations
15 Can be standardized	Acceptable to the scientific community
16 Metabolic activation	Minimization of false negatives, i.e., absent
17 “Acceptable level” of false positives 18 (active <i>in vitro</i> but not <i>in vivo</i>)	Metabolic activation
19 Quick and easy to perform	

20
21
22 A decision can now be made that reduces the number of possible methods for
23 consideration. Based upon the attributes of *in vivo* and *in vitro* methods and the criteria of a
24 screen, the *in vitro* method is in better agreement with the criteria for a screen. The information
25 presented in the preceding sections supports this conclusion. For example, all of the *in vivo*
26 assays are more labor-intensive, costly, and require more time and special laboratory skills to
27 conduct, than most of the *in vitro* method assays or procedures. **Thus, based on these criteria
28 and their better alignment with the *in vitro* methods, assays using the *in vitro* method will
29 be given further consideration for selecting one as a screen.**
30

31 **4.6.2 Gender Comparisons**

32

33 The *in vitro* methods can be adapted to male and/or female animals. For every procedure
34 designed to evaluate testicular function, there is a co-procedure designed to evaluate ovarian
35 function. For example, a perfusion apparatus exists for the testis as well as the ovary. A
36 sectioned testis procedure exists for the male and a minced ovary procedure for the female.
37 However, with respect to selecting a screen method, it may not be necessary to have both a male
38 and a female screen. Is there a basis for selecting a method from one gender over the other, or
39 are methods from both genders required? This subsection attempts to provide a basis for
40 demonstrating that an *in vitro* method using a single gender may be reasonable.
41

1 Distinguishing characteristics of the male and female steroidogenic pathways may be
2 useful for excluding a gender from consideration as a screen. One such distinction occurs with
3 regard to location or cell type. In the male, the steroidogenic pathway is located in the Leydig
4 cell of the testis. The initial substrate (cholesterol) to the primary terminal hormone
5 (testosterone), together with the intermediate substrates and enzymes, are in this cell type
6 (Griffin and Wilson, 1994). There are two steps of the pathway not located in the Leydig cell:
7 the conversions of testosterone to DHT by 5 α -reductase and to estradiol by aromatase. These
8 reactions occur in peripheral tissues, as was reviewed in Section 3.
9

10 In the female, the steroidogenic pathway is in the ovary but distributed in and among
11 different cell types – the follicular cell, which contains the granulosa and theca cells, and the
12 interstitial cells (Carr and Wilson, 1994). The synthesis of various hormones can differ by cell
13 type at various times. In short, while steroidogenic hormone production occurs in most cell
14 types, each cell type can produce varying amounts of a given enzyme and hormone. This results
15 in some cell types producing more of one hormone than another at varying times, which is
16 associated with the estrus cycle. Thus, the cyclicity of the female is another complicating factor
17 in using the ovary for evaluating the effect of a substance on the steroidogenic pathway.
18 Therefore, since the male steroidogenic pathway is also representative of the female
19 steroidogenic pathway, it may be reasonable to consider using the testis to screen for effects of a
20 substance.
21

22 Organ isolation and preparation steps must also be given consideration when considering
23 a screen method. Regarding collection of the organ, the testis is a much simpler organ to remove
24 than the ovary. This is important considering the number of organs that would be needed. In
25 addition, the size of the testis is much larger than the ovary, thereby providing more organ mass
26 per animal for screening substances. This is very advantageous, as it would allow more
27 experiments to be conducted with fewer animals.
28

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

31 **4.6.3 Different Method Sub-Types**

32 The *in vitro* methods described for the male include several different assays or
33 procedures. The male *in vitro* methods included the whole or sectioned testis, perfusion,
34 perfusion, isolated crude and purified Leydig cell preparations, and cell lines. Narrowing these
35 assays down to the most promising one to be used as a screen should, at this point, be based on
36 the criteria for a screen as listed above. Therefore, each of these criteria is evaluated for each of
37 the *in vitro* assays used to assess steroidogenesis. This information has been summarized in
38 Table 4-11 presented at the end of Section 4. (The criteria are further described, defined, and
39 discussed in Appendix C).
40
41
42

43 Comparison of the assay's relative attributes with the criteria for a screen points out
44 apparent differences among the assays (Table 4-11). Regarding **cost**, the least expensive assays
45 are the simple whole testis, sectioned testis, and cell line assays. A cost estimate for running a
46 steroidogenic assay in sectioned testis is estimated to be less than \$12K per test substance
47 (EDSTAC, 1998).

1 A second criterion, **time**, should be given high-level consideration. Two aspects of this
2 parameter were evaluated for comparison—time required for the initial set-up and time to
3 conduct a single experiment (includes organ removal, preparation, testing and sample
4 collection). The whole testis simple incubation, sectioned testis, and crude Leydig cell assays
5 require a relatively short investment in time to obtain the supplies and set-up the laboratory. In
6 contrast, the other assays require specialized supplies, which can take weeks to months to obtain
7 (some perfusion equipment requires customized manufacturing) and, once obtained, require
8 additional time to set up and ensure that they are operational. Furthermore, the actual time
9 needed to conduct the assay and the number of testes preparations and substances that can be
10 tested per day favor the whole or sectioned testes, and crude Leydig cell assays. Of these three,
11 the sectioned testes and crude Leydig cell assays use the organ more efficiently than the whole
12 organ preparation, e.g., 4 times more if using quartered sections, while requiring only a small
13 additional time and effort. Finally, the sectioned testis assay uses less time for preparation
14 relative to the crude Leydig cell preparation.
15

16 A comparison of the **laboratory parameters** (level of training and type of equipment)
17 was made. The whole testis simple incubation, sectioned testis, and crude Leydig cell assays can
18 be performed by broad-based, experienced, laboratory-trained technicians and in laboratories
19 equipped with standard supplies and instrumentation for general biology experiments. For the
20 perfusion, perfusion, purified Leydig cells, and cell lines, specialized training and equipment are
21 needed, e.g., laminar flow hoods, special apparatus and training to set up and operate the
22 perfusion or perfusion assays.
23

24 **Standardization** of the assays has not occurred. Standardization of the assay selected
25 for screening would need to be performed by optimization or consensus regardless of which
26 assay was chosen. However, the level of difficulty involved in standardizing the assay is
27 important to consider. As described earlier in Section 4 for each method, the assays have
28 varying numbers of variables that must be controlled in order to generate data that can be
29 compared among and between the laboratories. The least difficult assays to standardize are the
30 whole and sectioned testis assays, as they involve the fewest number of procedures and factors to
31 standardize.
32

33 **Animal usage** is replaced by cells if a cell line assay were to be used. This assay offers
34 the best approach to achieving the goal of reducing, refining, or replacing animals for use in
35 toxicity studies. Fewer animals are used in the Leydig cell preparations and the sectioned testes
36 assay than the other assays.
37

38 The degree that the organ's **cytoarchitecture** is maintained varies considerably among
39 the different *in vitro* assays. There is little difference in the cytoarchitecture of the organ in the
40 whole testis simple incubation, perfusion, perfusion, and sectioned testis assays. However, the
41 cytoarchitecture of the Leydig cell preparations is extensively altered during the cellular
42 isolation steps that involve collagenase incubation, elutriation, and Percoll density gradient
43 centrifugation. Of course, the cytoarchitecture of the cell line is even further reduced when
44 compared to the organ/cells of the other assays, since cells from cell lines are tumor cells,
45 thereby necessitating consideration of not only the absence of *in situ* intercellular communication
46 but also intracellular and biochemical pathway differences.
47

1 As for **stability**, all of the preparations remain stable for approximately 3 to 6 hours.
2 Stability data were based on the length of time that the preparation would respond to stimulation,
3 e.g., hCG, with a linear increase in steroid hormone production. Some preparations have been
4 reported to be used for 24 to 48 hours, i.e., Leydig cells, perfusion. The studies using the
5 preparation for this prolonged length of time did (Thoreaux-Manlay et al., 1995) or did not
6 (Rommerts et al., 1988) show linear steroid hormone production.
7

8 In regard to **sensitivity**, there is little disparity among the assays based on the very
9 limited information available to date that can be used to assess sensitivity. None of the assays
10 introduce factors that compromise the sensitivity of the RIA method to measure a steroid
11 hormone in the medium, perfusate, etc. Measurement of sensitivity from the viewpoint of
12 responding to LH or hCG may distinguish the assays one from another. However, only the cell
13 line assay would be different from the other assays in that some cell lines do not respond to
14 stimulation, e.g., I-10, although others can, e.g., MA-10, MLTC-1.
15

16 The **specificity** of the *in vitro* assays is a difficult parameter to assess without
17 experimental data specifically designed to evaluate this parameter. The most relevant
18 information available comes from those studies where the same investigators tested the same
19 substance using different assays (e.g., Kelce, Klinefelter, and Laskey tested EDS using sectioned
20 testis, perfused testis, and purified Leydig cells in the adult rat). In general, all of the methods
21 are able to detect a substance-induced change in steroid hormone production at similar
22 FM concentrations of the test substance.
23

24 **Metabolic activation** is not present to any appreciable extent in the testicular
25 preparations. However, it may be possible to include an S9 fraction with the purified Leydig cell
26 and cell line assays.
27

28 **Endpoints** for the *in vitro* assays primarily involve measuring steroid hormone
29 production and secretion. Based on the literature reviewed for this DRP, the whole testis simple
30 incubation and cell line assays used the fewest endpoints, i.e., enzyme activity and single-point
31 steroid hormone, respectively. The perfused testis assay analyzed the most steroid hormones,
32 i.e., 11 (Chubb and Ewing, 1979b). Purified Leydig cells were used to determine the most
33 different endpoints, i.e., steroid hormones, enzyme activity, and histology (light and electron
34 microscopy). The other assays, sectioned testis and crude Leydig cells, also had several different
35 steroid hormones (5) used as endpoints.
36

37 The other parameters that were not listed in the table for comparison were believed to be
38 similar for all the assays, i.e., sample throughput, chemical solubility/permeability, and
39 predictive capacity (age, sex, and species), or there was believed to be insufficient information to
40 comment, i.e., inter-/intra-laboratory variability, acceptance by the scientific community, and
41 frequency of false positives/false negatives.
42

1 **4.6.4 Recommended Steroidogenic Screen Assay**
2

3 Based on the information presented above, a recommendation can be made for
4 selecting—from the various methods and assays—the single most promising assay to further
5 pre-validate as a screen for substances that alter steroidogenesis. **The *in vitro* sectioned testis**
6 **assay is recommended as a screen for measuring disruption of steroidogenesis.** The most
7 salient features of this assay are that it identifies substances that alter steroid hormone production
8 and can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment
9 and basic laboratory training. All of these are important features for a screen. In addition, the
10 assay is stable (hours), relatively sensitive and specific, maintains the cytoarchitecture of the
11 organ, uses a reduced number of animals (quartered sections), will be relatively easy to
12 standardize (by optimization or consensus), has well-defined and multiple endpoints. A protocol
13 for the sectioned testis assay is included in Appendix D.
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Table 4-11. Continued

	Whole Testis (simple incubation)	Perfused Testis	Perifused Testis	Sectioned Testis	Isolated & Cultured Leydig Cells (crude)	Isolated & Cultured Leydig Cells (purified)	
1	Metabolic Activation	None	None	None	None	Add an S9 fraction (evidence is equivocal)	Add an S9 fraction (evidence is equivocal)
2	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) 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 EC₅₀. 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 (ED₅₀) or to inhibit the maximal response by 50 percent is the inhibitory concentration (IC₅₀).

Table 5-1. Sectioned Testis Assay Results for EDS

Response ^a	Reference
EC ₅₀ = 2250 FM ^b	Gray et al., 1995
EC ₅₀ = 336 FM	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 IC₅₀ of 320 Fg/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 EC₅₀ 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).

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

13
 14 **Table 5-2. Cumulative mean concentration and standard error of the mean by hour and**
 15 **study.**
 16

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	--	--	--

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

- 26 a. F = Fail et al., (1994); Gr = Gray et al., (1995); Gu = Gurthler and Donatsch (1979); and L = Laskey et al., (1994).
 27 b. The degrees of freedom associated with each study is somewhere between n-1 and 2n-1, depending on whether one or
 28 two testes per animal were used and the degree of correlation between testes from the same animal. We conservatively
 29 assume n-1 degrees of freedom.
 30 c. No SEM reported. Also, not used for ANOVA calculation.
 31

1
 2 The results of this comparison showed that for each hour for which several studies
 3 reported cumulative testosterone concentrations there was wide variation of mean concentrations
 4 across studies. In each case there was strong indication of significant statistical differences
 5 among studies, particularly after 2 hours and 3 hours.
 6

7 For each hour where more than one study reported results, the total variance of the
 8 cumulative mean values among studies was divided into variance between studies and variance
 9 within studies. The variance within studies was estimated as the average of the squares of the
 10 within study standard errors of the mean, based on those studies for which standard errors were
 11 reported. The variance between studies was estimated as the variance of the mean values among
 12 studies minus the variance within studies. Table 5-3 displays the standard deviations among the
 13 means between studies and the standard errors of the means within studies after 1, 2, and
 14 3 hours. The standard deviation of the means between studies is approximately 3.8 to 6.8 times
 15 the standard errors of the means within studies. This agrees with the results shown in Table 5-2.
 16 The variation among study means far exceeds that which is due to within study variation.
 17

18 **Table 5-3. Standard deviations of between studies and within studies components of**
 19 **variance by hour.**
 20

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

21
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 23
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 27 **5.1 Flow Diagram of the Sectioned Testis Assay**
 28

29 Figure 5-1, which also appeared in Section 4.3, illustrates the procedure for the sectioned
 30 testis assay.
 31
 32

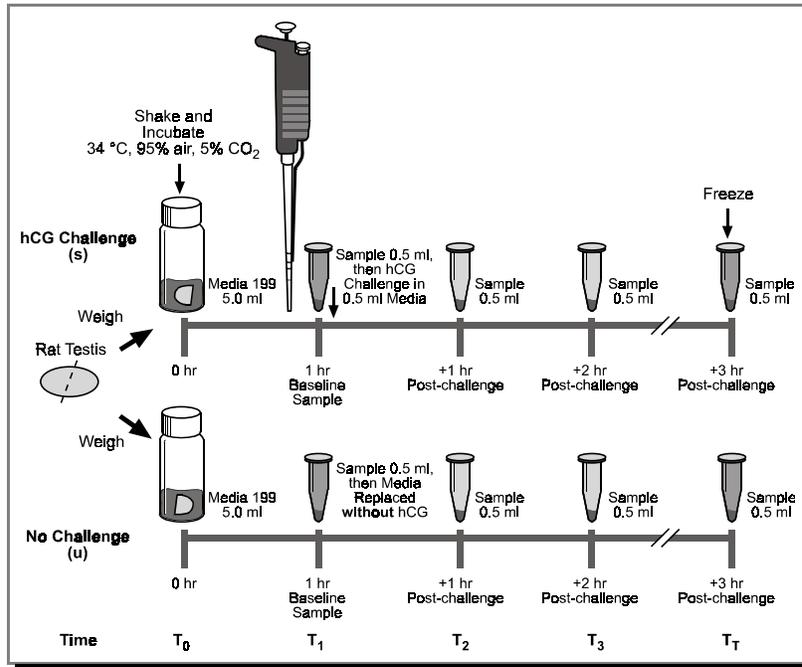


Figure 5-1. Technical Flow Diagram of the Sectioned Testis Assay

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 of this document.

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 (CrI: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.

1 The age of the rat is an important consideration. As described in Section 4, steroidogenic
2 hormone production in the Leydig cells changes as the cells undergo sexual development.
3 Testes from adult male rats, 10 to 12 weeks of age (250 to 275 g BW), will be used in the assay.
4 Each testis weighs approximately 1 g and, when both are quartered, will provide eight organ
5 sections, each weighing approximately 250 mg. (Although the 250 mg section is the most
6 commonly cited amount of testis used according to the literature, it is not known if smaller
7 amounts could be used; therefore, Section 6 describes a prevalidation experiment that would
8 determine the minimal optimum amount of testis to use.)
9

10 Specific animal husbandry conditions will be followed. Upon receipt, animals are
11 quarantined for at least 7 days. During the quarantine period, the animals are housed singly in
12 solid-bottom polycarbonate cages fitted with stainless steel wire lids. Sani-Chips® is used as
13 bedding. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) is made available *ad libitum*.
14 The source of drinking water is the municipal water supply, which is made available *ad libitum*
15 via automatic water delivery systems or water bottles. Animal rooms are maintained on a 12
16 hours on and 12 hours off light cycle. Target conditions for temperature and relative humidity
17 are 64 to 79EF (18 to 26EC) and 30 to 70 percent, respectively. Room air change rate is 10 to
18 15 times per hour.
19

20 Testes will be collected with adherence to limiting discomfort or injury. Animals are
21 humanely terminated by carbon dioxide inhalation. A possible alternative to termination is to
22 use the testis from animals that are used on studies that require castrated animals, e.g.,
23 Hershberger assay. In such studies, animals are anesthetized and the testes removed. If the
24 testes are not being used directly in the study that requires castrated animals, then there may be a
25 way to keep the testes viable long enough to use them for the *in vitro* steroidogenesis assay.
26 Conversely, if fresh testes are determined to be necessary, then after the testes have been
27 removed for the *in vitro* steroidogenesis assay, the animals could be saved and used later on the
28 study that requires castrated animals. This latter procedure may be the most pragmatic as
29 animals require a recovery period after castration in order for hypothalamic and pituitary
30 hormone levels to become stable. Only then can they be used in reproductive toxicity studies.
31

32 The assay is initiated by properly removing and preparing the testes. After the animal is
33 anesthetized, the testes are removed by cutting open the scrotum and cutting away connective
34 tissue. The freed testes are cleaned of any residual tissue, e.g. epididymus, fat, etc., and then the
35 whole testis weight is obtained (weighed to the nearest 1 mg). The connective tissue capsule
36 surrounding the testis (tunica albuginea) is removed and cut along the longitudinal axis in order
37 to provide four similar portioned sections. The testis sections are weighed (to the nearest
38 0.1 mg). Each section is placed in its own scintillation vial that contains media. At this point, a
39 given testicular section is ready for testing.
40

41 **5.2.2 Test Substance Evaluated**

42

- 43 ● The test substance (chemical, product, test article) to be evaluated will be fully
44 characterized prior to use in the sectioned testis assay. The compound will be identified
45 by name and, if a mixture, information on all components will be provided. The CAS

1 registry number, chemical and/or product class, and supplier/source will also be
2 identified. Physical and chemical characteristics directly related to test performance, e.g.,
3 water and lipid solubility, pH, pK_a, stability of the test material in the test medium at low
4 and high concentrations, and purity and stability of the bulk substance will also be
5 provided.

6 7 **5.2.3 Method of Exposure**

8
9 A stock solution of the substance being tested will be dissolved in an appropriate solvent.
10 The solvent will be the medium used to incubate the testicular sections. The medium is modified
11 Medium 199 (GIBCO BRL, Life Technologies, Inc., Grand Island, NY) with 0.1 percent bovine
12 serum albumin, 8.5 mM sodium bicarbonate, 8.8 mM HEPES, and 0.0025 percent soybean
13 trypsin inhibitor, at pH 7.4. Medium 199 without phenol red (an indicator) will be used. In
14 addition, it will be necessary to check the solubility after the hCG and any other components
15 have been added, to ensure that the mixture remains in solution. If a solvent other than the
16 medium is used to prepare the formulation for mixing the substance with the sectioned testis,
17 then the formulation should be checked for solubility in the medium. If the substance being
18 tested is not soluble in the media, then it will need to be dissolved in another solvent for addition
19 to the media. Other solvents that could be considered are ethanol or DMSO, to name a few.
20

21 **5.2.4 Incubation Concentration Selection Procedures and Number of Replicates**

22
23 The sectioned testis screening assay will be conducted using three concentrations of the
24 test substance. Examples of the concentrations that could be tested are 5, 50, and 500 FM (final
25 concentration). It is important to note that the objective of a screen is not to characterize a
26 concentration-response relationship between the concentration and the response. Rather, the
27 most important objective is to test a concentration range that identifies a concentration of the
28 substance that will initiate at least a single event expressed as an increase or a decrease in the
29 steroid hormone production. If in the course of identifying a concentration that produces a
30 measurable effect there is also a graded response over the concentration range tested, such a
31 finding represents the advantage of using this particular assay for screening substances. Along
32 those lines, if an effective concentration (EC) response curve is desired, then more
33 concentrations could be tested so that an EC₅₀ can be determined. The concentration range for
34 these full concentration-response studies could be performed at concentrations ranging from
35 1 nM (10⁻⁹ M) to 1 mM (10⁻³ M), depending on solubility limitations of the substance being
36 tested. Three replicates should be performed at each concentration.
37

38 **5.2.5 Controls**

39
40 The assay is performed using vehicle, negative, and positive controls. The basic vehicle
41 control is the medium. A second type of vehicle control would be included if the test substance
42 requires a solvent other than the medium. In such an instance, an additional vehicle control
43 group would be added to evaluate the effect of the additional solvent(s). The vehicle control(s)
44 is/are conducted to identify whether the incubation medium and its components could confound

1 the response being produced by the substance being tested. Theoretically, the medium and/or
2 solvents could increase or decrease hCG-stimulated or non-stimulated steroid hormone
3 production.

4
5 The assay also includes testing positive or negative controls. These terms require further
6 explanation and clarification. The unique feature of the sectioned testis assay is that it is
7 performed without hCG (basal or unstimulated steroid hormone production) and with hCG
8 (stimulated steroid hormone production). Looking at just the basal condition to make a point,
9 the term “positive control” could have two different meanings. First, it could mean the
10 substance being used as the positive control increases hCG-stimulated or non-stimulated steroid
11 hormone production or, second, that it inhibits hCG-stimulated or non-stimulated steroid
12 hormone production, thereby having a “positive” effect. In contrast, a negative control would
13 have no effect on hCG-stimulated or non-stimulated hormone production. Thus, these terms will
14 be defined and used as follows:

15
16 **Positive control** - a substance used at a non-cytotoxic concentration that increases or
17 decreases the production of the steroid hormone(s) in the presence and absence of hCG
18 stimulation.

19
20 **Negative control** - a substance that has no effect on the production of the steroid
21 hormone(s) in the presence and absence of hCG stimulation.

22
23 In addition, the testicular section is evaluated for its capacity to respond by treating the sections
24 with hCG, thereby demonstrating that the tissue is viable.

25
26 Based on these definitions, the substances and their expected affects in the various
27 incubation conditions are:

28
29

<u>Condition</u>	<u>Positive Control</u>	<u>Negative Control</u>
30 <u>Basal</u>	Aminoglutethimide (\uparrow steroid hormone prod.)	Finasteride (no effect - inhibits 5 α -reductase)
31 <u>hCG-Stimulated</u>	Aminoglutethimide (\uparrow steroid hormone prod.)	Finasteride (no effect)

32
33

34 Each of the controls is tested at a single concentration. The amount of hCG added is 50 to
35 100 mIU, and the concentrations of aminoglutethimide and finasteride are 250 FM and 10 μ M.
36
37

1 **5.2.6 Test Conditions**

2
3 The animals are euthanized and the testes removed, sectioned, and weighed (see 5.2.1).
4 The sections are placed in 15 mL glass scintillation vials containing 5.0 mL of medium (see
5 below). Vials are kept refrigerated (4EC) until the assay is initiated. The various groups and
6 replicates per group are summarized in Table 5-4.

7
8 Vials are prepared to correspond with the groups in Table 5-4, except that the hCG is not
9 added to any of the vials at this stage. The vials are placed in the incubator (34EC on a shaker in
10 5% CO₂/95% air) and, after the first period of incubation, i.e., 1 hour, an aliquot of medium
11 (0.5 mL) is collected. The sample is added to a small tube, the tube centrifuged, the sample
12 removed and frozen. This is the baseline secretion sample.

13
14 **Table 5-4. Test Condition Matrix, Showing Number of Replicates per Group**

15
16

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

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36 One half of the replicates are then challenged with hCG (0.5 mL) and the other half are
37 given media without hCG (0.5 mL). After the designated time intervals, i.e., 2, 3, and 4 hours,
38 aliquots (0.5 mL) are removed, processed as described above, the samples frozen, and retained
39 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.

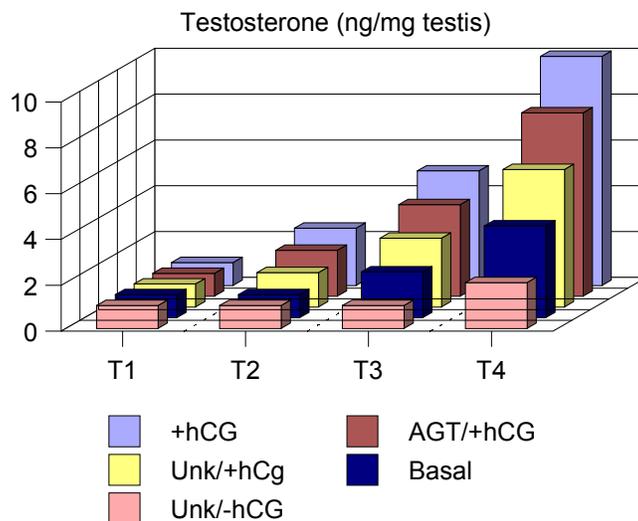
Based on the experimental design described above, the total number of samples to be analyzed for testosterone will be 624 (78 with and 78 without hCG, in duplicate, at four collection intervals).

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

1 The following illustration shows a possible scenario that could occur as a result of performing
 2 the assay with a single unknown (Unk):
 3



4
 5
 6 Based on this simulation, hCG produced a linear and proportional increase in testosterone
 7 production over the entire collection period. The positive control, aminoglutethimide (AGT) and
 8 hCG, reduced the hCG-stimulated testosterone production as can be seen by comparing the
 9 results to hCG alone. As for the unknown substance, it was tested with and without hCG-
 10 stimulation. The unknown substance inhibited hCG-stimulated testosterone production even
 11 more than the positive control. Furthermore, when it was tested in the absence of hCG,
 12 testosterone production was less than that observed under basal conditions. These simulated data
 13 can be used to describe the relevance of the data collected and associated endpoints.

14
 15 A number of individual and group comparisons can be made from such data sets.
 16 Suggested comparisons include:

- 17
- 18 ● For a given group, the concentration at T1 is compared with the following
 19 concentrations at T2, T3, and T4. If T1 is a sample taken after equilibration and just
 20 prior to addition of a stimulant or test substance, then it could be considered a
 21 baseline measurement. If stimulation occurs, then the concentration at T1 would be
 22 different from the concentrations at the later time points. If no stimulation occurs or
 23 there is inhibition, then the concentration at T1 may be similar to the concentrations
 24 at the later time points.
- 25
- 26 ● The baseline value for all groups could be compared. The optimal outcome would be
 27 for all groups to have similar baseline values so that effects after adding the stimulant
 28 and/or substance being tested could be compared directly. This value could also be
 29 used as a quality control standard by determining the degree of variation that occurs

1 in this value under standardized assay conditions, and then use that value and its
2 determined degree of variability as the standard for accepting results from future
3 studies and to ensure comparable work is performed from laboratory to laboratory.
4

- 5 ● The slopes of the lines for each group could be determined and differences between
6 groups could be determined by comparing the lines using linear regression analysis.
7 An unknown could stimulate or inhibit testosterone production and this could be
8 evaluated by comparing the slope of the line produced by the unknown to see if it is
9 steeper (stimulant) or flatter (inhibitor) than the line obtained using hCG alone or
10 under basal conditions. The slopes of the lines for the hCG-stimulated and basal
11 condition groups could also be used as quality control standards.
12

13 **5.2.9 Known False Negatives and False Positives**

14

15 The assay will almost certainly produce false negative results. As for false negatives, this
16 is most likely to occur for those test substances that require metabolic activation, since the testes
17 do not include pathways for metabolism. Other examples of false negatives involve those
18 instances when a substance evokes an indirect effect on steroidogenesis, e.g., site of action is at
19 the hypothalamus or pituitary gland. Finally, if the effect of the toxicant is delayed for a time
20 greater than the duration of the incubation period, then a false negative result will occur. An
21 example of a delayed effect was observed when lead was tested for its effect on steroidogenesis,
22 which inhibited steroid hormone production 4 hours after initiation of the incubation
23 (Thoreux-Manlay et al., 1995).
24

25 There are no known false positive instances to report at this time.
26

27 **5.2.10 Sensitivity of the Assay and Lowest Level of Detection**

28

29 The sectioned testis assay has measured hCG-stimulated testosterone production and
30 secretion to be 200 ng/g of testis/hour (Gray et al., 1995). The lowest concentration of a
31 substance tested is in the 1 to 10 FM range. Actual effects on the production of testosterone
32 have been reported for substances at the 50 to 100 FM range.
33

34 **5.2.11 Statistical Methods**

35

36 First, a test will be performed on suspected outliers. If examination of pertinent study
37 data do not provide a plausible, technologically sound reason for inclusion of the data flagged as
38 “outlier,” the data will be excluded from summarization and analysis and will be designated as
39 outliers. For all statistical tests, $p \# 0.05$ (one- or two-tailed) will be used as the criterion for
40 significance.
41

42 Second, the data are expressed as ng testosterone/mg testes/hour for each replicate in the
43 incubation vial. (Each replicate determination will be the mean of two replicates.) All group

1 data are reduced to mean and standard deviation values. Treatment groups will be compared to
2 the concurrent control group (within time) using either parametric ANOVA under the standard
3 assumptions or robust regression method, which does not assume homogeneity of variance or
4 normality.

5
6 The homogeneity of variance assumption will be examined via Levene's test, which is
7 more robust to the underlying distribution of the data than the traditional Bartlett's test. If
8 Levene's test indicates lack of homogeneity of variance ($p < 0.05$), then a log 10 conversion of the
9 data will be made. These values will be tested for normalcy and an ANOVA applied to test all
10 treatment effects. If Levene's test does not reject the hypothesis of homogeneous variances,
11 standard ANOVA techniques will be applied for comparing the treatment groups. The GLM
12 procedure in SAS® will be used to evaluate the overall effect of treatment and, when a
13 significant treatment effect is present, to compare each exposed group to control via Dunnett's
14 Test. A two-tailed test (i.e., Dunnett's test) will be used for all pairwise comparisons to the
15 vehicle control group.

16
17 If the assumptions of ANOVA are not met, then robust regression methods will be
18 applied. The robust regression methods use variance estimators that make no assumptions
19 regarding homogeneity of variance or normality of the data. They will be used to test for overall
20 treatment group differences, followed by individual tests for exposed vs. control group
21 comparisons (via Wald Chi-square tests), if the overall treatment effect is significant. The
22 presence of linear trends (over the time points) will be analyzed by GLM procedures for
23 homogeneous data or by robust regression methods for nonhomogeneous data. Standard
24 ANOVA methods, as well as Levene's test, are available in the GLM procedure of SAS® and
25 the robust regression methods are available in the REGRESS procedure of SUDAAN®.

26 27 **5.3 Strengths of the Sectioned Testis Assay**

28
29 The *in vitro* sectioned testis assay is optimal as a screen because it is reliable, easy to
30 set up, relatively simple to conduct, and has short conduct-time requirements. In addition, the
31 assay preserves the cytoarchitecture of the testis, uses standard laboratory equipment, requires
32 only general laboratory training, and the sections remain viable for a sufficiently long period of
33 time.

34
35 The method of exposure allows for an exact concentration to be achieved by dissolving
36 an accurately measured amount of test substance in a particular vehicle and adding it to the
37 testicular preparation. The recommended exposure period is 4 hours, but this can be varied to
38 improve the sensitivity or reduce test substance and/or product degradation.

39
40 The complete steroidogenic pathway from signal transduction to end-hormone production
41 is present in the testicular section. This preparation also includes receptors for stimulation,
42 e.g., LH, hCG, as well as the receptor-second messenger system for an evaluation of the
43 receptor-intracellular coupling mechanism.

1 In some sense, this assay does provide some information about the mechanism of action
2 of a substance in that if it alters gonadal steroid hormone production, then it is reasonable to
3 consider the gonads as a target organ, thereby identifying a site of action at the organ level.
4

5 This assay is able to identify substances that either increase or decrease steroid hormone
6 production. Thus, it can identify inhibitors or stimulants of the steroidogenic pathway.
7

8 The assay minimizes the number of animals used for study. In addition, if organs from
9 another study are able to be used in the steroidogenesis assay, then the assay will contribute to
10 the goal of reducing, refining, and replacing animals.
11

12 The assay lends itself to multiple endpoints, i.e., intermediate hormones. Also, the media
13 can be collected and stored, which allows additional hormones to be analyzed at a later date.
14

15 **5.4 Weaknesses and/or Limitations of the Sectioned Testis Assay**

16
17 The primary limitation of the testis section assay is its lack of metabolism. Test
18 substances that require activation will not alter steroid hormone production in this *in vitro* assay,
19 whereas they would in an *in vivo* assay.
20

21 The assay, when used as a screen with a single endpoint, does not provide complete
22 information regarding the site or mechanism of action. However, the sectioned testis assay can
23 be used for additional endpoints, if so desired, which will make the assay a more mechanistic
24 type assay.
25

26 The assay may be compromised by a substance that is insoluble in an aqueous medium.
27 Based on the solubility of the substance in appropriate solvents, the range of concentrations that
28 can be tested could be limited.
29

30 **5.5 Test Method Performance and Test Method Reliability**

31
32 The performance of this assay is assessed as part of the experimental design. First, the
33 sectioned testes are checked for viability by measuring the testosterone production over time
34 before and after the addition of hCG, which stimulates the steroidogenic pathway to synthesize
35 steroid hormones. When the organ preparation remains viable, then hCG will stimulate the testis
36 to produce and secrete testosterone in a linear fashion. The production and secretion of
37 testosterone at a rate of 200 ng/g testis/hour was measured using the sectioned testis assay
38 (Gray et al., 1995). Second, the assay evaluates an active substance by blocking hCG-stimulated
39 testosterone production, if it is an inhibitor of the pathway, or it may induce an increase in
40 non-stimulated testosterone production, if it is an inducer of the pathway. The response curves
41 will be evaluated using statistical tools that compare treated responses to the control.
42

1 The endpoint of the assay is reliable. Measurement of testosterone in the media is a very
2 well-established analytical endpoint. The analytical method used is a RIA, which is accurate and
3 precise. Concentrations as low as 0.07 ng/mL are routinely determined.
4

5 A limited number of examples exist in the literature for evaluating the sectioned testis
6 assay as a screen of substances. It is not reasonable with the present information to claim with
7 assurance that the assay will be sufficiently rigorous, sensitive, and reproducible. The assay is,
8 however, the most promising assay from the number of competing assays. Even with the limited
9 number of studies to draw from, these studies show that the assay identifies substances that alter
10 steroid hormone production by altering enzyme activity or substrate availability. Also, this assay
11 has been used by a number of different laboratories, which allows some estimate of the assay
12 variability to be assessed. Assay variability, sensitivity, and reproducibility, will become better
13 understood once additional substances are screened using an assay that has been standardized,
14 either by optimization or consensus of various experimental design considerations.
15

16 **5.6 Implementation Considerations**

17 **5.6.1 Establishment of Assay**

18 The *in vitro* sectioned testis assay was selected as a screen in part because it is relatively
19 simple to set up and conduct. The materials, supplies, and equipment needed to conduct this
20 assay are standard for any biology laboratory. In addition, the training needed to conduct the
21 assay requires skills that are fairly common among general biology laboratory technicians. A
22 general list of the materials, supplies, and equipment needed and skills required are listed below:
23
24
25

26 **Materials and Equipment**

26 **Skills**

27 Centrifuge (low and high speed)

General necropsy

28 Analytical balance

Radioimmunoassay (RIA)

29 Incubator

Incubation/*in vitro* organ preparation

30 Shaker/water bath

Animal husbandry

31 pH meter

32 Gamma or scintillation counter(s)

33 Glassware, test tubes, vials

34 Pipettes, automatic pipettors
35
36

1 **5.6.2 Cost/Time Required**
2

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

10
11 The time required to conduct the assay described here and in Appendix D would be
12 2 days, which would cover the time between the removal of the testes to the time that the
13 medium samples were collected. Additional time would be needed to analyze the medium
14 samples, summarize the data, interpret the results, and write the report.

15
16 **5.6.3 Animal Welfare Considerations**
17

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

20
21 A possible alternative to using animals solely for this assay would be to utilize animals
22 from other studies that require castrated animals. Such a proposition has not been checked to
23 determine whether such a plan is feasible. It remains to be determined how long a testis will
24 remain viable after it has been removed and whether any practical storage conditions can extend
25 its viability. If feasible, then the use of animals for this assay would be less of an issue, as it
26 would involve using discarded organs from other studies already being conducted.
27 Alternatively, the testis could be collected fresh, and the animals used at a later date in the
28 studies that require castrated animals. Often times such studies require a 3 to 5 week recovery
29 period, which if scheduled properly may allow the same animals to be used for both the *in vivo*
30 and *in vitro* studies.
31
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1 **6.0 DEVELOPMENTAL STATUS OF THE ASSAY AND RECOMMENDATIONS**
2 **FOR PREVALIDATION STUDIES**

3
4 **6.1 Current Status**

5
6 The endpoint included in the sectioned testes assay has been evaluated in other studies.
7 However, the protocol itself has not been validated. Pending a final decision on the study
8 design, the protocol would be ready to enter the prevalidation phase.

9
10 **6.2 Recommendation for Optimization of the Sectioned Testis Assay Protocol**

11
12 **6.2.1 Testicular Preparation Issues**

13
14 Optimization of the assay could be determined for the amount of testis actually needed to
15 obtain a given level of sensitivity. For example, a single testis from an adult SD rat weighs
16 approximately 1 g. If such a testis were quarter sectioned, then each section would weigh
17 approximately 250 mg, which is the weight of the sample generally described by investigators
18 who have used quartered sections of testis. However, no documentation was found that
19 demonstrates whether smaller sections would give similar results. Thus, it would be
20 advantageous to conduct a study that investigates whether the sensitivity of the preparation is
21 affected by the amount of testicular tissue used and, if so, if there is an optimal and/or threshold
22 amount to use. The weight of the sections to be tested could range from the customary amount
23 used, i.e., 250 mg, down to an amount of tissue that represents a practical minimum, e.g., 5 to
24 10 mg.

25
26 It would also be useful to explore storage and viability of the testis and/or sections. One
27 possible scenario to assist in meeting an ICCVAM and U.S. EPA objective to reduce, refine, and
28 replace animal usage would be to use testes from animals in a separate study, that requires
29 castration in the experimental design, e.g., Hershberger studies. It may be possible to store testes
30 after removal in such a way that they remain viable, can be shipped to various locations, and are
31 used at a later date for the *in vitro* sectioned testis steroidogenesis assay. A storage condition,
32 stability, and viability study could be designed and tested.

33
34 **6.2.2 Endpoint Issues**

35
36 The importance of measuring progesterone could be evaluated. For example, it is
37 possible that the stoichiometric molar relationship for progesterone and testosterone is not 1:1.
38 If such a relationship exists and a substance inhibits progesterone production but the “pool” of
39 progesterone is sufficiently large such that the production of testosterone is not affected, then the
40 assay would not detect an effect on steroidogenesis if only testosterone were measured.
41 However, this effect would be observed if progesterone were measured. This possibility could
42 be determined during the initial experiments used to optimize the assay.

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

7
8 Another experimental design factor that could be optimized by experimental
9 determination is the number of collection time points. The current study design includes four
10 time points. Media samples are collected at 1, 2, 3, and 4 hours after the incubation is initiated.
11 The possibility exists that other time points are better suited to characterize the effect of a
12 substance on steroid hormone production. Along those same lines, perhaps fewer time points are
13 equally as useful to measure an effect. Statistical analysis could be used to determine whether
14 concentrations measured at 1 and 4 hours provide no more or no less information than that
15 obtained by measuring samples at four different time points. Such information could be used to
16 reduce extraneous collection and analysis steps.

17 18 **6.2.3 Stimulation Factor Issues**

19
20 The initial studies need to optimize the concentration of stimulant added to the testicular
21 preparation. The stimulant planned for use is hCG. The amount of stimulant used is important
22 because it can affect whether a steady proportional increase in steroid hormone production
23 occurs over the entire duration of the incubation period. Also, a measure of the variability of
24 different lots of hCG could be determined during such experiments. This would serve to provide
25 needed information about factors that affect the variability of the assay.

26 27 **6.3 Recommendation for Sectioned Testis Assay Prevalidation Studies**

28
29 Prevalidation studies following the ICCVAM validation process should be initiated.
30 Prevalidation studies should include evaluation of six to eight substances to establish the
31 database for the validation studies. It is recommended that the study be performed using test
32 substances with different chemical classifications, as well as varying sites and/or mechanisms of
33 action, which will aid in the development of the prevalidation database for the assay. The
34 recommended positive and negative control test substances were selected based on their sites of
35 action, i.e., aminoglutethimide inhibits P450_{SCC} and finasteride inhibits 5 α -hydroxylase, which is
36 not found in the testes. Other test substances of interest that are recommended for testing in the
37 prevalidation studies include:

- 38
- 39 ● bisphenol A (inhibits steroidogenic signal transduction)
- 40 ● lindane (inhibits signal transduction and the StAR protein)
- 41 ● ketoconazole (a weak imidazole anti-fungal; inhibits P450_{SCC} and aromatase)
- 42 ● genistein (a weak phytoestrogen/flavonoid; inhibits 3 β -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 of the EPA 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.

1 **Database Search Strategies**

2
3 English Language Articles

4 Foreign language articles with English abstracts

5
6 **Keywords and phrases considered**

- 7
8 C steroidogenesis C minced testis
9 C testosterone C minced ovary
10 C dihydrotestosterone C P450 isoforms/isozymes
11 C testis C Leydig cells
12 C ovary C Luteinizing hormone (LH)
13 C aromatase C adrenal cortex
14 C 5-alpha reductase C fetal steroidogenesis
15 C cholesterol

16
17 Storage of electronic database information in Reference Manager format

18
19 Results of the online search:

- 20
21 C The search term “steroidogenesis” was used; the term was limited to appear
22 in at least the titles or descriptors of relevant records; this search set was then
23 combined with the search set on the term “vitro.” This resulted in
24 7008 records.
- 25 C This search then combined a search on the terms “method or methods or
26 methodology or assay* or test or tests or testing or protocol* or guideline*.”
27 This reduced the set to 887 records. [Note: The asterisk denotes the use of a
28 truncation symbol to gather plurals or alternate endings of terms.]
- 29 C The term “testosterone” was added to this second set and limited the results
30 to be in the English Language only. After removing duplicate records,
31 179 items remained.
- 32 C Next, I added the term “dihydrotestosterone” to the second set. After limiting
33 by English Language only and removing duplicate records, 11 items
34 remained.
- 35 C Going back to the second set (method or methods), duplicate records were
36 removed from that set to see how many unique records were there. This was
37 also limited to English Language only. This reduced the set from 887 to
38 607 records. From this set, a sample of 12 items was retrieved for initial
39 review to determine appropriateness of journals retrieved. Based upon
40 examination of the sample records, it was apparent that the terms “method or
41 methods” were problematic. It was decided to limit those terms to appear in
42 only the descriptors of relevant records. This resulted in 110 items from
43 search Set 2 having the terms “method or methods” appearing in the
44 descriptors.

- 1 C The second search was repeated by initially leaving out the terms “method or
2 methods” and then searching them separately with Set 1. This search was
3 then combined with our previous search, limited by English Language only
4 and removed duplicate records, resulted in 530 records remaining, down from
5 607 records.
- 6 C A sample of records from this set of 530 were then reviewed. After review of
7 this sample, a decision was made to include the concept “procedure*,” in
8 doing so, this search was combined with Set 1, resulting in 111 records
9 retrieved. The term “procedure*” was limited to appear in at least the titles or
10 descriptors of relevant records. After removing duplicate records and limiting
11 to English Language, only 6 records remained.
- 12 C Upon investigation of the titles of the 6 records, it was concluded that they
13 were on target. However, during examination of other sample records, the
14 terms, “test, tests, or testing” were also problematic. Articles where the terms
15 “test, tests, or testing” appeared only in the abstract were removed, leaving a
16 total of 420 articles remaining. This set was combined with the 6 from the
17 “procedure” search, making a total of 426 records.
- 18 C From this set of 426 records, a online review of approximately 12 articles was
19 conducted to determine whether identified articles met research expectations.
20 It was concluded that this set of articles closely matched the research
21 objectives of this task. However, to ensure adequate coverage of the
22 published literature on steroidogenesis, a slightly different retrieval approach
23 was used to find additional records.
- 24 C Another search was conducted on the term “steroidogenesis,” limiting this
25 term to appear in at least the titles or descriptors of relevant records, and
26 combining that with the phrases “minced ovary or minced testes or minced
27 testis.” After removing duplicate records from the above set of 426, 9 items
28 remained. This set was then combined with the previous set for a grand total
29 of 432 records. All 432 records were downloaded in a tagged format.

30

Literature Evaluation Process

31

32

33

Criteria for Literature Selection

34

C Appropriateness of methods for measuring endpoints of interest,

35

C method clearly described

36

C appropriate use of controls

37

C data adequately reported

38

C appropriate statistical analysis

39

Literature Review

40

1 Summary of the Review Process

2 Literature describing *in vitro* studies of mammalian reproduction and/or endocrine
3 disruption, and/or applicable steroidogenesis test protocol evaluation was retrieved.
4 Approximately 232 of the 432 references were reviewed for relevancy. The references
5 were given a number from the reference list. These references were divided into
6 ovarian, testicular, and adrenal steroidogenesis. Twenty-eight papers regarded adrenal
7 steroidogenesis and were not used in this paper. Twenty-two were not in mammals and
8 were not included in the ovarian steroidogenesis descriptions of methods. The ovarian
9 references were divided into categories such as human IVF granulosa cell cultures (32),
10 by species and compounds, and by methods used to determine steroidogenesis.
11 Approximately 11 papers were purely mechanistic in nature. The references were then
12 read by the authors and placed in the following categories:

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

20
21 Each article in each group was then evaluated according to the criteria described.
22 Summaries of the best articles that illustrated the usefulness of an *in vitro* exposure
23 protocol for detection of endocrine-disrupting activity of the test compound are
24 presented below.

25
26 Hazard- and risk-based study designs may examine different endpoints, but if these
27 endpoints will be used for risk assessment, they must first be shown to be robust,
28 reproducible, appropriately sensitive, biologically plausible, and relevant to the adverse
29 outcomes of concern. Definitions of the attributes of such endpoints are as follows:

30
31 **Reproducible:** These endpoints must be reliable; the same findings occur under
32 the same conditions within the initial reporting laboratory (intra-laboratory) and
33 among other laboratories (inter-laboratory). If the results from endpoints are not
34 reproducible, they cannot form the basis for future research and are most likely
35 not useful for risk assessment.

36
37 **Robust:** These endpoints must be present after comparable routes of exposure
38 (e.g., whole organism as well as cell or organ culture). Different effects may be
39 observed by different exposure routes, different species, or different neurological
40 controls. The findings from routes unrelated to human or environmental
41 exposures may not be useful for risk assessment. These findings must also be
42 present at the same routes and doses over time.

1 **Sensitive:** These endpoints should not be dependent on unique conditions,
2 especially those which are not relevant to the species at risk. These endpoints
3 should not exhibit high variability (insensitive) or be greatly affected by
4 confounders (too sensitive).
5

6 **Relevant:** These endpoints must be biologically plausible and related to adverse
7 effects of interest/concern. If there are no adverse effects at the
8 dose/duration/route evaluated, these endpoints should be predictive of other
9 adverse effects at higher doses, after longer exposure duration, and/or by
10 different routes, etc.
11

12 **Consistent:** These endpoints must occur in the presence of effects in other
13 related, relevant endpoints, if possible, at the same dose, timing, duration, routes
14 of exposure, etc.
15

16 The literature was evaluated in terms of how well the study design was described.
17 Studies that were described in such a way that they could be repeated in the authors'
18 laboratory only on the basis of information contained in the manuscript were further
19 evaluated for scientific soundness, and for the likelihood that the results could be
20 reproduced by repeating the experiment.
21

22 The literature was evaluated for the use of appropriate controls. In *in vitro* studies, the
23 use of a concurrent control sample (media) that is under the same culture conditions as
24 the treated samples is essential for valid statistical analysis.
25

26 Data and results were considered to be adequately reported if the data followed the
27 study design in a logical manner, and all of the animals or samples could be tracked
28 through the description of the results and placed appropriately in their data groups. In
29 addition, it was necessary that the data appear to be consistent and realistic within
30 groups and compared to the concurrent control.
31

32 Data analysis was evaluated with regard to the use of appropriate statistical methods,
33 appropriate comparisons with the control group, tightness of data, and appropriate
34 interpretation of the statistical results.
35

36 This appendix contains the entire "online" search results in chronological fashion such
37 that one can discern the number of articles indexed for given key word combinations
38 and phrases that were encountered during the online search conducted in April 2001.
39 This section also includes the title and abstract for all articles retrieved.
40

41 **Summary of Literature Retrieved** 42

43 The purpose of this section is to numerically categorize and organize the articles (hard
44 copy retrieved articles) for the steroidogenesis reference manager database. The
45 criteria used are species, test species sex, tissue type, chemicals or steroids used, and
46 various components of experimental design. The experimental design is further
47 categorized into those studies involving the investigation of a certain chemical on the

1 steroidogenesis process, along with those studies that simply investigate the general
2 biological function of the steroidogenesis process. Many of the papers in this database
3 explore several different species, tissues, chemicals, and methods. Therefore, the
4 numbers displayed in the following tables do not add up to the total number of papers
5 retrieved.
6

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

9 **Table A-1. Summary of Database Count**

10

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

11
12

13 The papers in this database that were retrieved were categorized into two different
14 types. The first type included all papers that investigated the basic biological function of
15 a system. This included papers describing alterations in hormone levels and the
16 biological activity of naturally produced hormones and steroids etc. The second type
17 included those papers that investigated the effects of an outside substance on the
18 steroidogenic process or system.
19

20
21 **Table A-2. Breakdown of Paper Objectives**

22

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

23
24
25

26 This database contained papers studying multiple species of organisms. As stated
27 previously, many papers included more than one species of organism in their study, so
28 the values given will not add up to the total number of papers in the database.
29
30
31

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.

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18

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.

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

2
3 **Note:** Indication of use does not imply that the study
4 only examined one chemical, but rather that it
5 was involved somewhere in the process.
6

7

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

31

32 Table A-11 lists the specific chemicals studied in the papers describing the effects of an
33 outside chemical on a specific system.
34
35
36

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:

- C Rat and human steroidogenic systems were studied most frequently when investigating the steroidogenic process.
- C 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.
- C Radioimmunoassay was by far the most commonly used method of ascertaining the presence and amounts of specific hormones.

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7

**APPENDIX B:
INTERVIEWS**

1 **APPENDIX B: INTERVIEWS**

2
3 **Interviews with Principal Investigators—Published and Unpublished Studies**
4 (See Appendix 1)

5
6 **Researchers contacted:**

7
8 Gary R. Klinefelter, Ph.D.
9 Reproductive Biologist and Toxicologist
10 Reproductive Toxicology Division, MD#72
11 National Health and Environmental Effects Research Laboratory
12 U.S. Environmental Protection Agency
13 Research Triangle Park, NC 27711
14 Phone: (919) 541-5779
15 Fax: (919) 541-4017
16 E-mail: klinefelter.gary@epa.gov
17

18 Jerome M. Goldman, Ph.D.
19 Endocrinology Br. MD-72, Reproductive Toxicology Div.
20 National Health & Environmental Effects Research Laboratory
21 Office of Research and Development
22 U.S. Environmental Protection Agency
23 Research Triangle Park, NC 27711
24 Phone: (919) 541-2320
25 Fax: (919) 541-4017
26 E-mail: goldman.jerome@epa.gov
27

28 Patricia A. Fail
29 Manager, Laboratory of Reproductive and Endocrine Toxicology
30 Research Triangle Institute
31 P.O. Box 12194, 3040 Cornwallis Road
32 Research Triangle Park, NC 27709-2194
33 Phone: (919) 541-6079
34 Fax: (919) 541-5956
35 E-mail: patf@rti.org
36 Reproductive and endocrine toxicologist
37

38
39 David T. Armstrong
40 E-mail: david.armstrong@adelaide.edu.au
41

42 John W. Laskey
43 7017 Branton Drive
44 Apex, NC 27502
45 Phone: (919) 362-3945
46 Fax: (919)362-3946

1 E-mail: JohnL45198@aol.com
2 Reproductive and endocrine toxicologist
3 National Health & Environmental Effects Research Laboratory
4 U.S. Environmental Protection Agency
5 Consultant
6

7 **Clarification of Study Design, Interpretation, and Data Analysis of Studies Through**
8 **Interviews with These Scientists**
9

10 The interviewer identified him/herself, identified the contract, and indicated the title and
11 objectives/goals of this particular work assignment. He/she briefly described the study
12 designs under consideration (in vitro culture of ovarian or testicular cells). The
13 questions were designed to be open-ended and to encourage discussion; and follow-up
14 questions were asked, as appropriate. The questions were modified to suit each
15 interviewee's experience, as appropriate.
16

17 The written text will become part of the permanent record.
18

19 At a minimum, the questions provided in the following template should be answered.
20 Other ideas, opinions, thoughts, suggestions, tangents, or anecdotal type of information
21 will be welcomed and will become part of the permanent record. Other information
22 should be recorded on this form under the "Open Dialogue" heading.
23
24

1 Template for Steroidogenesis Interviews

2
3 Chemicals that interfere with the androgen and estrogen systems can act via a number
4 of direct mechanisms in addition to those that directly involve the steroid hormone
5 receptors. One prominent mechanism of endocrine disruption is interference with the
6 synthesis of hormones themselves. Such interference is increasingly thought to occur
7 by up-regulation or down-regulation of the genes responsible for producing the enzymes
8 in the steroid synthesis pathway. In some cases, it may be by direct interaction and
9 inhibition of the enzymes themselves. The purpose of this work assignment is to
10 prepare a detailed review paper to survey and investigate the status of various *in vitro*
11 methods that have been used to identify chemicals that affect (i.e., inhibit or enhance)
12 steroidogenesis.

13
14 **Name of Interviewer:** Patricia A. Fail, PhD

15
16 **Name of Interviewee:** Dr _____

17
18 **Date:** _____

19
20
21 **Have the questions been responded to in writing by interviewee (circle or omit**
22 **one)?** Yes/No

23
24 **If so was a follow up phone call made to discuss any of the major points (circle**
25 **one)?** Yes/No

26
27 **Note to reviewer:** Please feel free to add more space or modify
28 question as needed. Please put your modifications
29 in italics.

30 --Thanks, Pat Fail
31

1 **QUESTIONS**

2
3 **Q1. Do you know of any references that you would recommend that investigate**
4 **the status of various *in vitro* methods that have been used to identify**
5 **chemicals that affect (i.e., inhibit or enhance) steroidogenesis?**
6

7 Specific comment(s) to individual "expert" from me, such as, "Jerry, I have
8 the early ones for ovarian steroidogenesis from John Laskey's work. Are
9 there any of yours we can include or have you published the follicle
10 method yet?"
11

12 In our experiments (for the EPA CA with Earl Gray) we used both the ovarian
13 steroidogenesis and testicular steroidogenesis methods. With regards to these
14 assays or your own, please respond to the following question to the best of your
15 ability.
16

17 Each form was customized for the specific scientific "expert."
18

19 **Ovary**
20

21 **Q2. What are the limitations of this method?**
22

- 23 - Whole follicle
- 24 - Minced ovary
- 25 - Other
- 26

27 **Q3. What are the strengths?**
28

- 29 - Whole follicle
- 30 - Minced ovary
- 31 - Other
- 32

33 **Q4. What would you recommend to further enhance this method or what**
34 **changes would you recommend (if any)?**
35

- 36 - Whole follicle
- 37 - Minced ovary
- 38 - Other
- 39

40 **Q5. Do you know of any other published literature that corroborates or refutes**
41 **the findings for these methods?**
42

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

- 1 - Whole follicle
- 2 - Minced ovary
- 3 - Other

4
5 **Q7. Quality Control: What are the quality control measures to be included in**
6 **these assays that you favor?**

- 7
8 **a. Are there specific or special circumstances when additional quality**
9 **control measures need to be added?**

10
11 **Q8. Based upon your expertise and experience, what endpoints would be most**
12 **appropriate for elucidating the effects of chemicals on**
13 **testicular/ovarian/adrenal steroidogenesis when investigating an in vitro**
14 **study design?**

15
16 **Q9. Based on your experience, what chemicals, routes, duration, and doses**
17 **would you recommend to be used to validate an in vitro steroidogenesis**
18 **assay/protocol?**

- 19
20 **a. What specific step of steroidogenesis or organ of steroidogenesis does**
21 **each access?**

22
23 **Q10. Do you have any unpublished data relevant to these assays that you**
24 **would be willing to share? If so, are there any restrictions?**

25
26 **Q11. Are there variations of the assay that should be considered that you did not**
27 **have time to validate or access? Anything you would change about the**
28 **assay to increase it's sensitivity, efficiency, relevancy, or robustness?**

29
30 **Q12. Is there anyone else you can think of that we should contact? Yes/No**

- 31
32 **a. If so, whom?**
33 **b. Can we mention your name when we contact him/her? Yes/No**

34
35 **Q13. Open Dialogue**

36
37 **Testes**

38
39 **Q14. What are the limitations of this method?**

40
41 **Q15. What are the strengths?**

42
43 **Q16. What would you recommend to further enhance this method or what**
44 **changes would you recommend (if any)?**

45
46 **Q17. Do you know of any other published literature that corroborates or refutes**
47 **the findings in this paper?**

1 **Q18. In running this method/procedure are there any steps that are especially**
2 **difficult that require special attention (i.e., lessons learned that come after**
3 **numerous runs that you would like to share) or is there any special set-up**
4 **strategy you would recommend that would save time or resources that**
5 **come from experience in running the assay?**
6

7 **Q19. Quality Control: What are the quality control measures to be included in**
8 **these assays that you favor?**
9

10 a. **Are there specific or special circumstances when additional quality**
11 **control measures need to be added?**
12

13 **Q20. Based upon your expertise and experience, what endpoints would be most**
14 **appropriate for elucidating the effects of chemicals on**
15 **testicular/ovarian/adrenal steroidogenesis when investigating an *in vitro***
16 **study design?**
17

18 **Q21. Based on your experience, what chemicals, routes, duration, and doses**
19 **would you recommend to be used to validate an *in vitro* steroidogenesis**
20 **assay/protocol?**
21

22 a. **What specific step of steroidogenesis or organ of steroidogenesis does**
23 **each access?**
24

25 **Q22. Do you have any unpublished data relevant to these assays that you**
26 **would be willing to share?**
27

28 a. **If so, are there any restrictions?**
29

30 **Q23. Are there variations of the assay that should be considered that you did not**
31 **have time to validate or access?**
32

33 **Anything you would change about the assay to increase its sensitivity,**
34 **efficiency, relevancy, or robustness?**
35

36 **Q24. Is there anyone else you can think of that we should contact? Yes/No**
37

38 a. **If so, whom?**

39 b. **Can we mention your name when we contact him/her? Yes/No**
40

41 **Q25. Open Dialogue**
42

1 **Detailed Review Paper on Steroidogenesis**

2 **EPA Contract Number 68-W-01-023**

3 **Work Assignment # 1-6**

4
5 **Interviewee**

6
7 Patricia A. Fail, PhD
8 Manager
9 Laboratory of Reproductive and Endocrine Toxicology
10 Research Triangle Institute
11 P.O. Box 12194 (RTI)
12 Durham, NC 27709
13 Phone: (919) 541-6079; 1 800 334-8571 ext. 6079
14 Fax: 919 541-7208

15
16 Instruction for interviewing researchers for input regarding procedures, methods, and
17 lessons learned regarding Steroidogenesis (minced testes, ovary, adrenal leydig cells,
18 or granulosa cells etc). Use a separate template to capture information for each person
19 interviewed. This information can be scribed by hand during the interview or you may
20 ask the person being interviewed if he or she would like to provide his or her own written
21 response to the questions to be followed up with a phone call.

22
23 The written text will become part of the permanent record.

24
25 At a minimum the questions provided in the template below will be asked. All
26 impromptu questions, thoughts, suggestions, tangents or anecdotal type of information
27 will be captured and become part of the permanent record. This information will be
28 recorded on this form under the "Open Dialogue" heading.
29

1 **Steroidogenesis Interviews**

2
3 Chemicals that interfere with the androgen and estrogen systems can act via a number
4 of direct mechanisms in addition to those that directly involve the steroid hormone
5 receptors. One prominent mechanism of endocrine disruption is interference with the
6 synthesis of hormones themselves. Such interference is increasingly thought to occur
7 by up regulation or down regulation of the genes responsible for producing the enzymes
8 in the steroid synthesis pathway. In some cases it may be by direct interaction and
9 inhibition of the enzymes themselves. The purpose of this work assignment is to
10 prepare a detailed review paper to survey and investigate the status of various in vitro
11 methods that have been used to identify chemicals that affect (i.e., inhibit or enhance)
12 steroidogenesis.

13
14 **Name of Interviewer:** Carol S. Sloan, MS

15
16 **Name of Interviewee:** Patricia A. Fail, PhD

17
18 **Date:** August 2 and 10, 2001

19
20 **Have the questions been responded to in writing by interviewee?** Yes

21
22 **If so was a follow-up phone call made to discuss any of the major points?** Yes

23
24 **Q1. Do you know of any references that you would recommend that investigate**
25 **the status of various in vitro methods that have been used to identify**
26 **chemicals that affect (i.e., inhibit or enhance) steroidogenesis?**

27
28 A1. Laskey et al., 1994 and many others. See Literature Search and Methods in
29 Toxicology Vol. 3a and 3b for technical methods. Also see attached list of
30 abstracts and reports from my own CV.

31
32 In your papers and reports you describe methods (or assays)—the testicular
33 steroidogenesis and ovarian steroidogenesis assessments *in vitro*—in regards to
34 these papers please respond to the following question to the best of your ability.

35
36 **Q2. What are the limitations of these methods? [All answers apply to both**
37 **assays unless otherwise stated]**

38
39 A2. High variability when assessing individual animal's testis. See Table M-5 of
40 document for a measure of variation. A general test.

41
42 **Q3. What are the strengths?**

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

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

5
6 A nice compromise might be a crude Leydig Cell isolation.

7
8 **Q4. What would you recommend to further enhance this method or what**
9 **changes would you recommend (if any)?**

10
11 A4. Use of ED 50 or ED75 (of hCG) but no more than ED100, for the challenge test.

12
13 Variability must be addressed by using triplicate determinations (or more) per test
14 point. I also recommend that the variation be summarized for these two specific
15 methods within and between labs and compared with variation in other methods
16 (in literature).

17
18 I believe that further reduction in costs are possible by downsizing it—using a
19 smaller volume of media and a decreased amount of testis used per evaluation
20 replicate. Klinefelter et al are now using 50 mg testis/ml media. This can be
21 accomplished by using 1.5 ml vials rather than the 20 ml vials or by using a
22 24-well plate.

23
24 **Q5. Do you know of any other published literature that corroborates or refutes**
25 **the findings in this paper?**

26
27 A5. All HEERL EPA literature from Laskey et al., Klinefelter et al., and Goldman et
28 al. J. W. Laskey and others taught us to use these assays in a cooperative
29 research program.

30
31 **Q6. In running this method/procedure are there any steps that are especially**
32 **difficult that require special attention i.e., lessons learned that come after**
33 **numerous runs that you would like to share...or is there any special set up**
34 **strategy you would recommend that would save time or resources that**
35 **come from experience in running the assay?**

36
37 A6. I believe that further reduction in costs are possible by using 96 well plate assay
38 and decrease amount of testis used per evaluation replicate.

39
40 **Q7. Quality Control: What are the quality control measures to be included in**
41 **these assays that you favor?**

42
43 A7. Always include negative and positive controls (two compounds) at one or more
44 doses. Doses for the positive control should be on the straight part of the dose
45 response curve. Both the positive and a negative controls should be easily
46 obtainable and reasonable inexpensive. Always include procedural controls (e.
47 g., blank media that receives all treatments).

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

3
4 A7a. For the RIA being used—testosterone, estradiol, and/or progesterone—the test
5 chemicals should be tested for cross reactivity. That is, do these chemicals
6 themselves bind to the antibody used in the RIA?

7
8 All labs doing interlab validation should use same RIA kit source or have RIAs
9 done at a central facility. Storage time and temperature should be controlled.

10
11 **Q8. Based upon your expertise and experience, what endpoints would be most**
12 **appropriate for elucidating the effects of chemicals on**
13 **testicular/ovarian/adrenal steroidogenesis when investigating an in vitro**
14 **study design?**

15
16 A8. First and foremost, the endproducts of steroidogenesis in the system being
17 tested. For the whole testis—testosterone; for the whole or minced
18 ovary—estradiol. Androstenedione and progesterone are also easily assayed
19 and could be included in the first analyses or as a secondary triggered measure if
20 the primary product is affected.

21
22 **Q9. Based on your experience, what chemicals, routes, duration, and doses**
23 **would you recommend to be used to validate an in vitro steroidogenesis**
24 **assay/protocol?**

25
26 A9. See Table in Goldman interview, incorporated into the text.

27
28 **Q9a. What specific step of steroidogenesis or organ of steroidogenesis does**
29 **each access?**

30
31 A9a. Each targets one or more aspects of gonadal steroidogenesis or the effect of
32 gonadal steroid on the target organ (pituitary and accessory sex organ's
33 receptors). If I understand the major recommendation of EDSTAC, it is to rapidly
34 access the overall synthesis of steroidogenesis. Thus the overall process in
35 ovary (estradiol and or progesterone) or testes (testosterone) should be tested
36 initially. Then if desirable the specific parts or place [of steroidogenesis affected]
37 can be evaluated. The overall processes include the integrity of the LH receptors,
38 P450 enzyme systems, cholesterol uptake (and possible biosynthesis), labile
39 protein synthesis (e.g., STAR), bioactivity of specific steroidogenic enzymes,
40 and precursor uptake. These steps can be tested only if the "pure" mechanism
41 of action(s) are known. I doubt that they do for many toxicants. Do not use
42 cytotoxicants such as EDS.

43
44 **Q10. Do you have any unpublished data relevant to theses assays that you**
45 **would be willing to share? If so, are there any restrictions?**
46

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

8
9 **LIST OF TABLES ATTACHED TO DRP**

10
11 Table M-1: Effects of daily administration of methoxychlor on testicular
12 testosterone (ng/g testis) In f0 male Long-Evans rats: in vitro incubation

13
14 Table M-2: Preliminary studies to define the dose and time response in testicular
15 cultures: in vitro testosterone at 0, 1, 2, and 3 hours after hCG challenge

16
17 This is an example of an in vitro study for a dose response to hCG. Any (or
18 several) toxicant(s) would be substituted in appropriate doses

19
20 Table M-3: Experimental design: Endocrine toxicity of a toxicant (xxx) on rat
21 testes after in vivo exposure for 2, 7, and 14 days. An example of a definitive
22 study design with *in vitro* testing after in vivo exposure

23
24 Table M-4: Preliminary Studies to Define Dose and Time Response in Adult
25 Male Sprague Dawley Rats: Plasma and Testicular Testosterone at 1, 3, or 6
26 Hours Post-hCG Challenge

27
28 Table M-5: Characteristics of Radioimmunoassays Validated for Determination
29 of Testosterone in Adult Male Sprague Dawley Rats

30
31
32 **Q11. Are there variations of the assay that should be considered that you did not**
33 **have time to validate or access. Anything you would change about the**
34 **assay to increase it's sensitivity, efficiency, relevancy, or robustness?**

35
36 A11. Differences in aged or prepubertal males; differences in females in different
37 stages of cycle; use of the prepubertal PMSG primed females (also see Laskey's
38 interview); use of ovarian whole follicles (see goldman interview).

39
40 **Q12. Is there anyone else you can think of that we should contact? Yes**

41
42 **a. If so, whom?**

43 **b. Can we mention your name when we contact him/her?**

1
2
3
4
5
6
7
8

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

Q13. Open Dialogue

1 **REFERENCES**

2
3
4 **Publications**

5
6 Fail, P.A., and R.P. Reynolds (1987). Influence of cortisol on prostaglandin synthesis
7 by fetal membranes, placenta and uterus of pregnant rabbits, *Biol. Reprod.*, 37, 47-54.

8
9 Fail, P.A., S.A. Anderson, S.W. Pearce, R.W. Tyl, G. Klinefelter, and L.E. Gray, Jr. (in
10 preparation). Reproductive and endocrine toxicity of vinclozolin administered in corn oil
11 to male Long-Evans hooded rats using the alternative reproductive test protocol, *Tox.*
12 *Sci.*

13
14 Fail, P.A., S.A. Anderson, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. (in preparation).
15 Endocrine and reproductive toxicities of methoxychlor administered in corn oil to male
16 Long-Evans hooded rats using the alternative reproductive test protocol, *Tox. Sci.*

17
18 Fail, P.A., S.A. Anderson, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. (in preparation).
19 Two-generation study of endocrine and reproductive toxicities of methoxychlor
20 administered in corn oil to female Long-Evans hooded rats using the alternative
21 reproductive test protocol, *Tox. Sci.*

22
23 **Abstracts (Poster or Platform Presentations)**

24
25 Noden, P.A., and J.F. Roux (1981). Prostaglandin-forming cyclooxygenase in rabbit
26 amnion, yolk sac splanchnopleure, placenta, decidua and uterus at 20 to 30 days
27 gestation, Society for Gynecological Investigation, St. Louis, MO, March: 89.

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

32
33 Stroud, C., J.M. Whitsett, and P.F. Noden (1984). 2-bromo-a-ergocryptine decreases
34 serum prolactin, flank gland diameter, total accessory sex gland weight, serum
35 testosterone, and testicular weight in prepubertal male golden hamsters, *Biol. Reprod.*,
36 30: Suppl. 1: 171.

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39 testosterone and LH response to human chorionic gonadotropin or
40 gonadotropin-releasing hormone in male Long-Evans hooded rats. *Biology of*
41 *Reproduction*, 50, Suppl. 1, p. 102 (Abstract 206).

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

1 Fail, P.A., S.W. Pearce, S.A. Anderson, R.W. Tyl, and L.E. Gray, Jr. (1995). Endocrine
2 and reproductive toxicity of vinclozolin (VIN) in male Long-Evans hooded rats, *The*
3 *Toxicologist*, 15: p. 293 (Abstract 1570).

4
5 Anderson, S.A., S.W. Pearce, P.A. Fail, B.T. McTaggart, R.W. Tyl, and L.E. Gray, Jr.
6 (1995). Validation of the alternative reproductive test protocol (ART) to assess toxicity
7 of methoxychlor in rats, *The Toxicologist*, 15: p. 164 (Abstract 871).

8
9 Anderson, S.A., P.A. Fail, S.W. Pearce, R.W. Tyl, and L.E. Gray, Jr. (1995). Testicular
10 and adrenal response in adult male Long-Evans hooded rats after antiandrogen
11 vinclozolin exposure, *J. Andrology*, 15 (Suppl. 1), p. P-43 (Abstract 78).

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

18
19 Fail, P.A., S.W. Pearce, S.A. Anderson, R.W. Tyl, and L.E. Gray, Jr. (1996).
20 Estrogenicity of methoxychlor in two generations of Long-Evans hooded rats.
21 Presented at the IBC International Environmental Congress of Endocrine Disruptors:
22 Advances in Measuring and Analyzing Their Effects, October 7-8, 1996, Washington,
23 DC.

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25 Fail, P.A., S.W. Pearce, S.A. Anderson, R.W. Tyl, and L.E. Gray, Jr. (1996).
26 Vinclozolin-induced endocrine toxicity in adult male Long-Evans hooded rats via
27 antiandrogenicity. Presented at the IBC International Environmental Congress of
28 Endocrine Disruptors: Advances in Measuring and Analyzing Their Effects, October
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31 Fail, P.A. (1998). Assessing the estrogenicity and other endocrine activity of chemicals.
32 Presented at 1998 IBC's Third Annual International Congress on Endocrine Disruptors:
33 An Unbiased Examination of the Impact Recent Scientific Developments Will Have on
34 Industry, April 14-15, 1998, Washington, DC.

35
36 **Study Reports - Commercial Client**

37
38 Toxicity Testing of a Fungicide, XXXX, in Adult Male CD® Sprague Dawley Rats. P.A.
39 Fail, S.A. Anderson, and S.W. Pearce, Project Number 65C-5703, October 20, 1994,
40 Confidential Client.

41
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43 Adult Male CD® Sprague Dawley Rats Exposed to XXXX In Vivo. P.A. Fail, S.A.
44 Anderson, and S.W. Pearce. Project No. 65C-6169, September 16, 1996, Confidential
45 Client.

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

5
6 **Study Reports - Government Client**

7
8 Two-Generation Study of Methoxychlor Administered in Corn Oil to Long-Evans Hooded
9 Rats Using the Alternative Reproductive Test Protocol. P.A. Fail, S.A. Anderson, S.W.
10 Pearce, R.W. Tyl, and L.E. Gray, Jr. Project No. 65U-5456, June 3, 1996, U.S. EPA.

11
12 Two-Generation Study of Vinclozolin Administered in Corn Oil to Long-Evans Hooded
13 Rats Using the Alternative Reproductive Test Protocol. Volume I: Males, Volume II:
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15 No. 65U-5456, February 28, 1997, U.S. EPA.

16
17 Synthesis and Testing of New Antiprogestational Agents. Final Report. C.E. Cook,
18 Y.-W. Lee, P.A. Fail, R.D. Bagwell, J.M. O'Reilly, G. Bartley, S.W. Pearce, P.S. Raje,
19 and R.S. Shetty. Project No. 60U-6413, Contract No. N01-HD-3238, September 19,
20 1998, NICHD and several others dating back to 1986.

1 **Detailed Review Paper on Steroidogenesis**
2 **EPA Contract Number 68-W-01-023**
3 **Work Assignment #1-6**
4

5 **INTERVIEWEE:** Testicular steroidogenesis
6

7 Gary R. Klinefelter, Ph.D.
8 Reproductive Biologist and Toxicologist
9 Reproductive Toxicology Division, MD#72
10 National Health and Environmental Effects Research Laboratory
11 U.S. Environmental Protection Agency
12 Research Triangle Park, NC 27711
13 Ph: (919) 541-5779
14 Fax: (919) 541-4017
15 E-mail: klinefelter.gary@epa.gov
16

17 The written text will become part of the permanent record.
18

19 At a minimum the questions provided in the following template should be answered.
20 Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information
21 will be welcomed and will become part of the record. Other information should be
22 recorded on this form under the "Open Dialogue" heading.
23
24

1 **Template for Steroidogenesis Interviews**

2
3 Chemicals that interfere with the androgen and estrogen systems can act via a number
4 of direct mechanisms in addition to those that directly involve the steroid hormone
5 receptors. One prominent mechanism of endocrine disruption is interference with the
6 synthesis of hormones themselves. Such interference is increasingly thought to occur
7 by up regulation or down regulation of the genes responsible for producing the enzymes
8 in the steroid synthesis pathway. In some cases it may be by direct interaction and
9 inhibition of the enzymes themselves. The purpose of this work assignment is to
10 prepare a detailed review paper to survey and investigate the status of various in vitro
11 methods that have been used to identify chemicals that affect (i.e., inhibit or enhance)
12 steroidogenesis.

13
14 **Name Interviewer:** Patricia A. Fail, PhD

15
16 **Name of Interviewee:** Dr. Gary Klinefelter

17
18 **Date of Response:** August 1, 2001; follow -up interview on October 11, 2001

19
20 **Have the questions been responded to in writing by interviewee (circle or omit**
21 **one) Yes**

22
23 **If so was a follow up phone call made to discuss any of the major points (circle**
24 **one) Yes/No**

25
26 **NOTE to reviewer:** Please feel free to add more space or modify question as needed.
27 Please put your modifications in italics. Thanks, Pat Fail

1 **QUESTIONS**

- 2
- 3 **1) Do you know of any references that you would recommend that investigate**
4 **the status of various in vitro methods that have been used to identify**
5 **chemicals that affect (i.e., inhibit or enhance) steroidogenesis?**

6

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

10

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

15

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

19

20 Klinefelter, G.R., Kelce, W.R. A Comparison of Leydig Cell Responsiveness to
21 Hormonal and Nonhormonal Factors in Vivo and In Vitro. In: The Leydig Cell,
22 (Payne A.H., Russel L. and Hardy M., eds.), pp 535-553, Cache River Press,
23 1996.

- 24
- 25 **2) In our experiments [for the EPA CA with Earl] we used both the ovarian**
26 **steroidogenesis and testicular steroidogenesis methods. With regards to**
27 **these assays, please respond to the following question to the best of your**
28 **ability.**

29

30 **Testis**

- 31
- 32 **a. What are the limitations of this method?**

33

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

- 36
- 37 **b. What are the strengths?**

38

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

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

3
4 For testicular assessments, consider going away from minced testis parenchyma
5 incubations and moving forward with modifications of Leydig cell purification that
6 permit a yield of enriched Leydig cells from an individual animals that is sufficient
7 for steroidogenic profile assessment (see Salva et al., J. Androl., In Press).

8
9 For ovarian assessments, consider use of preovulatory follicles and in vivo
10 challenges for experimental correlate. Regardless of organ/cell type ensure that
11 linear steroid production is achieved [over time], and that assessments are made
12 within the linear response range.

13
14 **d. Do you know of any other published literature that corroborates or refutes**
15 **the findings in this paper?**

16
17 **e. In running this method/procedure are there any steps that are especially**
18 **difficult that require special attention i.e., lessons learned that come after**
19 **numerous runs that you would like to share...or is there any special set up**
20 **strategy you would recommend that would save time or resources that**
21 **come from experience in running the assay.**

22
23 **f. Quality control**

24
25 **1. what are the quality control measures to be included in these assays**
26 **that you favor?**

27
28 Tests for linearity of steroid production and cell viability. For testis, include
29 3B-HSD histochemistry at end of assessment period.

30
31 Incubations with intermediate substrates as well as hCG.

32
33 An *in vivo* experimental correlate. For example challenge in vivo with hCG in
34 control and exposed animals to determine changes in hCG responsivity,
35 examining steroid in serum as well as in intersitial fluid (testis).

36
37 **2. Are there specific or special circumstances when additional quality**
38 **control measures need to be added?**

39
40 **3) Based upon your expertise and experience, what endpoints would be most**
41 **appropriate for elucidating the effects of chemicals on**
42 **testicular/ovarian/adrenal steroidogenesis when investigating an in vitro**
43 **study design?**

44
45 Degree of LH/hCG responsivity, i.e. to what degree does stimulation with hCG
46 enhance steroid production. If only 2 fold over baseline, cells aren't happy.

47

1 Ability to respond to a greater degree to intermediate substrates of steroid
2 production. Does stimulation with Cholesterol increase steroid production over
3 that achieved with hCG stimulation ?
4

5 **4) Based on your experience, what chemicals, routes, duration, and doses**
6 **would you recommend to be used to validate an in vitro steroidogenesis**
7 **assay/protocol?**
8

9 Chemicals such as aminoglutithemide, and other suicide substrates for P450
10 enzyme activity. Chemicals that inhibit membrane signal transduction and
11 cholesterol trafficking (i.e. StAR) should also be evaluated.
12

13 Duration [of incubation] should be within the linear range of steroid production.
14

15 What specific step of steroidogenesis or organ of steroidogenesis does each
16 access.
17

18 **5) Do you have any unpublished data relevant to theses assays that you**
19 **would be willing to share? If so, are there any restrictions?**
20

- 21 **6) a. Are there variations of the assay that should be considered that you did**
22 **not have time to validate or access?**
23 **b. Anything you would change about the assay to increase it's sensitivity,**
24 **efficiency, relevancy, or robustness?**
25

26 **7) Is there anyone else you can think of that we should contact?**
27

- 28 **a. If so, whom?**
29 **b. Can we mention your name when we contact him/her?**
30

31 **8) Open Dialogue**
32
33

1 **DETAILED REVIEW PAPER ON STEROIDOGENESIS**
2 **EPA CONTRACT NUMBER 68-W-01-023**
3 **WORK ASSIGNMENT #1-6**
4

5
6 **INTERVIEWEE:** Ovarian Steroidogenesis
7

8 Jerome M. Goldman, Ph.D.
9 Endocrinology Br. MD-72, Reproductive Toxicology Div.
10 National Health & Environmental Effects Research Laboratory
11 Office of Research and Development
12 U.S. Environmental Protection Agency
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14 (919) 541-2320 FAX: (919) 541-4017
15 email: goldman.jerome@epa.gov
16

17 The written text will become part of the permanent record.
18

19 At a minimum the questions provided in the following template should be answered.
20 Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of information
21 will be welcomed and will become part of the record. Other information should be
22 recorded on this form under the "Open Dialogue" heading.
23
24

1 **Template for Steroidogenesis Interviews**

2
3 Chemicals that interfere with the androgen and estrogen systems can act via a number
4 of direct mechanisms in addition to those that directly involve the steroid hormone
5 receptors. One prominent mechanism of endocrine disruption is interference with the
6 synthesis of hormones themselves. Such interference is increasingly thought to occur
7 by up regulation or down regulation of the genes responsible for producing the enzymes
8 in the steroid synthesis pathway. In some cases it may be by direct interaction and
9 inhibition of the enzymes themselves. The purpose of this work assignment is to
10 prepare a detailed review paper to survey and investigate the status of various in vitro
11 methods that have been used to identify chemicals that affect (i.e., inhibit or enhance)
12 steroidogenesis.

13
14 **Name Interviewer:** Patricia A. Fail, PhD
15 **Name of Interviewee:** Dr Jerome Goldman
16 **Date:** August 1, 2001

17
18 Have the questions been responded to in writing by interviewee (circle or omit one) Yes
19 If so was a follow up phone call made to discuss any of the major points (circle one) Yes

20
21 **NOTE to reviewer:** Please feel free to add more space or modify question as needed.
22 Please put your modifications in italics. Thanks, Pat Fail

23
24 **QUESTIONS**

- 25
26 **1. Do you know of any references that you would recommend that investigate**
27 **the status of various in vitro methods that have been used to identify**
28 **chemicals that affect (i.e., inhibit or enhance) steroidogenesis.**

29
30 Off-hand, I don't know of an article that compares the different ovarian
31 approaches for use in a Tox Study (if that is what you are asking).

32
33 A discussion of ovarian perfusion in Tox studies, along with a presentation of the
34 method, is in: Peluso, JJ, Pappalardo, A. (1993). Ovarian perfusion culture: A
35 tool to assess ovarian toxicity. In: J.J. Heindel, R.E. Chapin (Eds.), Female
36 Reproductive Toxicology (Methods in Toxicology, Vol. 3B). Academic Press: San
37 Diego. pp. 180-193 and in Brannstrom, M. In vitro Perfused rat ovary (same text
38 as above, pp. 160-179). Chapters discussing methods for ovarian cell isolations
39 are in the same text.

40
41 Jodi Flaws and Pat Hoyer have a chapter in Vol. 10 of the Comprehensive
42 Toxicology series (1997) that provides a discussion of follicular isolations as a
43 technique (Flaws, JA, Hoyer, PB. (1997). A new direction in experimental
44 approaches: Follicular isolations. In: P. Hoyer (Ed.), Reproductive and Endocrine
45 Toxicology, Vol. 10, Section II, Female Reproductive Toxicology (Comprehensive
46 Toxicology, I.G. Sipes, C.A. McQueen, A.J. Gandolfi, eds.-in-chief). Pergamon
47 Press: Oxford. pp. 373-377.).

1
2 Our lab has used in vitro exposures of pre-ovulatory follicles under
3 hCG-stimulated and non-stimulated conditions (Balchak et al., *Repro. Toxicol.* 14:
4 533, 2000). A second manuscript has been submitted for publication using our
5 improved approach, but exploring sites of toxic impact along the early portions of
6 the steroidogenic pathway.
7

8 In our experiments (for the EPA CA with Earl Gray) we used both the ovarian
9 steroidogenesis and testicular steroidogenesis methods. With regards to these
10 assays or you own, please respond to the following questions to the best of your
11 ability.
12

13 **OVARY**

14 **2. What are the limitations of this method?**

15 **- Whole follicle**

16
17 This is not a pure cell population. Isolations (of follicles) are more technically
18 demanding than a Laskey-type minced ovary approach.
19

20 **- Minced ovary**

21
22 Minced ovaries are much more heterogeneous than either isolated cell preps
23 or follicular preps. Ovarian perfusions, as described by Peluso/Pappalarda
24 and Brannstrom are reasonably time-consuming and more technically
25 demanding, but would generate more reliable data than simply placing
26 minced ovaries in a tube without controlling the oxygen content of the
27 medium.
28

29 **- Other?**

30
31 Isolated cell preparations (i.e., granulosa cells or theca cells). Loss of
32 architectural structure and interactions among different cell types that would
33 normally occur.
34

35
36 Likely require longer baselines to characterize "normal" cells.
37 Viability should be tested.
38

39 **3. What are the strengths?**

40 **- Whole follicle**

41
42 Maintains follicular structure and intercellular communication
43 (theca-granulosa).
44
45
46
47

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

4
5 Better tissue penetration of test compound than with minced ovaries.

6
7 Can obtain multiple follicles per rat and randomly assign follicular pairs among
8 treatment conditions to reduce any variability between animals/individual
9 ovaries. Also allows a reduction in the number of animals used per study.

10
11 - **Minced ovary:**

12 Laskey type incubations- Minimum of preparation time; easy to obtain.

13 Ovarian perfusions or perifusions- obtain a picture of the dynamics of steroid
14 secretion under different experimental conditions.

15
16 - **Other?**

17
18 Isolated cell preparations (i.e., granulosa cells or theca cells)

19 Homogeneous cell types may have advantage in mechanistic studies and
20 improve consistency of hormonal data.

21 Better penetration of test compound for in vitro exposures.

22
23 **4. What would you recommend to further enhance this method or what**
24 **changes would you recommend (if any)?**

25
26 - **Whole follicle**

27
28 Besides whole follicles, isolated corpora lutea (from rats made pseudo
29 pregnant) can be used as well. Choice would depend on the nature of the
30 questions asked (pregnancy maintenance/focus on progesterone production,
31 maintenance of cyclicity. Also both can be used in a perfusion system
32 (We've done some unpublished work with CLs. Came out pretty well, but we
33 never took the approach further).

1 - **Minced ovary**

2
3 This really depends on which approach to ovarian incubations you mean.

4
5 - **Other?**

6
7 **5. Do you know of any other published literature that corroborates or refutes**
8 **the findings for these methods?**

9
10 I don't understand. The utility of the approaches?

11
12 **6. In running this method/procedure are there any steps that are especially**
13 **difficult that require special attention i.e., lessons learned that come after**
14 **numerous runs that you would like to share...or is there any special set up**
15 **strategy you would recommend that would save time or resources that**
16 **come from experience in running the assay?**

17
18 For any of the in vitro procedures using steroid secretory endpoints, use of teflon
19 vials/tubes/tubing is the ideal, since the sex steroids (particularly progesterone)
20 adhere quite readily to plastic, less so to glass (see Bruning et al. J. Steroid
21 Biochem. 14:553, 1981; Higuchi and Espey. J. Reprod. Fertil. 87:821, 1989).

22
23 Use well-oxygenated media for incubations.

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

28
29 - **Whole follicle:**

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

31
32
33 - **Minced ovary:**

34
35 For the perfusion or perifusion approaches, practice and confirmation of a
36 marked increase in steroid secretion under stimulation (e.g., cAMP). This
37 would also be true for a simple minced ovarian prep (a general check on
38 viability). In fact, under experimental conditions, it is always informative to
39 include comparisons of baseline release under control and treatment levels in
40 addition to a response to stimulation (hCG, cAMP, etc.) to evaluate hormonal
41 release when the tissue is "pushed".

42
43 - **Other?**

44
45 **7. Quality Control: What are the quality control measures to be included in**
46 **these assays that you favor?**

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

3
4 Discussion with Fail: Likely should characterize how often pH should be
5 measured, or how long it will hold in validation experiments. With oxygenated
6 follicle sealed in flasks, it was stable up to 4 hours.

7
8 For most cultures, an atmosphere of 95% Oxygen 5% CO2 works nicely.

9
10 **Are there specific or special circumstances when additional quality control**
11 **measures need to be added?**

12
13 Although it is not generally done, it would be helpful to be able to sample the
14 dissolved oxygen content of the incubation media. However, this can be tricky to
15 do under most circumstances without a more elaborate setup. For some data on
16 the effects of oxygenation on follicular steroid secretion, see Roby KF, Terranova
17 PF. (1990). Effects of tumor necrosis factor-alpha in vitro on steroidogenesis of
18 healthy and atretic follicles of the rat: theca as a target. Endocrinology 126:
19 2711-2718.

20
21 **8. Based upon your expertise and experience, what endpoints would be most**
22 **appropriate for elucidating the effects of chemicals on testicular/ovarian/**
23 **adrenal steroidogenesis when investigating an in vitro study design?**

24
25 Depends on the general focus. For toxicant effects on pregnancy maintenance,
26 it would be more appropriate to focus on P4, using corpora lutea. For general
27 effects on ovarian steroidogenesis, the most obvious endpoints for whichever
28 cell/tissue approach employed are E2 and P4 release. Supplementation of the
29 media with known concentrations of hormones such as pregnenolone or
30 testosterone may provide some information about the synthetic capacity of the
31 system under toxic insult and indications of effects on the particular enzymes
32 (3b-HSD, P450arom, or others) involved.

33
34 **9. Based on your experience, what chemicals, routes, duration, and doses**
35 **would you recommend to be used to validate an in vitro steroidogenesis**
36 **assay/protocol?**

37
38 Give me a call to discuss. August 3, 2001 Discussion

39
40 Table x.x. Chemical Candidates for Controls

41			
42		MOA	hormone
43	Flutamide	receptor	antiandrogen
44			
45	Ethinl Estradiol	receptor	estrogenic
46			
47	Tamoxifen	??receptor	antiestrogen

1			
2	Ketoconazole	general p450, depress	Testosterone
3		? not specific	
4			
5	Mthoxychlor	?receptor	estrogenic
6			
7	Vinclozolin	receptor	antiandrogen
8			
9	Methyl Testosterone	receptor	ANDROGENIC
10			
11	Dibutylphthalate	Sertoli cell?	testosterone
12		General cell toxicity	
13		Solubility problems	
14			
15	Testosterone Proprionate	receptor	androgenic
16			
17			
18	DDE	?receptor	antiestrogen
19			
20	Genestein		
21			
22	Finasteride	enzyme	% alpha reductase
23			
24	Dibromo acetate	TBD	Decreased Progesterone
25			in CL fragments
26			

27

28

29 **What specific step of steroidogenesis or organ of steroidogenesis does**

30 **each access?**

31

32 See above, authors, this aspect needs additional attention. Up to 40 hours could

33 be easily used selecting appropriate controls, but after the assays are selected.

34 PAF August 3, 2001

35

36 **10. Do you have any unpublished data relevant to theses assays that you**

37 **would be willing to share? If so, are there any restrictions?**

38

39 Maybe. Contact me to discuss circumstances.

40

41 **11. Are there variations of the assay that should be considered that you did not**

42 **have time to validate or access? Anything you would change about the**

43 **assay to increase it's sensitivity, efficiency, relevancy, or robustness?**

44

45 There are a variety of things to try when issues of sensitivity are considered.

46 Robustness of the assays is always affected by cell/tissue viability.

1 If the minced ovarian tissue is to be used one should consider using PMSG
2 primed immature female rat to get similar follicles (size and status). The
3 pseudopregnant females could be used to donate ovaries with coprae lutea
4 dominate status.
5

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

7
8 **a. If so, whom?**

9 **b. Can we mention your name when we contact him/her?**

10
11 **13. Open Dialogue**

12
13 The Effective dose issue is an important one. When stimulating tissues, it is
14 important not to overwhelm the system, but the challenge dose must be high
15 enough to evoke a response. In our follicle cultures we add the hCG and the
16 toxicant at the same time and incubate for two hours. That avoids opening the
17 sealed vials to take a sample.
18

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

22
23 Specific comment(s) to individual "expert" from me, such as, "Jerry, I have the
24 early ones for ovarian steroidogenesis from John Laskey's work. Are there any
25 of yours we can include or have you published the follicle method yet?"
26

27 In our experiments (for the EPA CA with Earl Gray) we used both the ovarian
28 steroidogenesis and testicular steroidogenesis methods. With regards to these
29 assays or you own, please respond to the following question to the best of your
30 ability.
31

1 **OVARY**

2
3 **15. What are the limitations of this method?**

- 4
5 - Whole follicle:
6 - Minced ovary
7 - Other?
8

9 **16. What are the strengths?**

- 10 - Whole follicle:
11 - Minced ovary
12 - Other?
13

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

- 15 - Whole follicle:
16 - Minced ovary
17 - Other?
18
19

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

21
22
23 **19. In running this method/procedure are there 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?**

- 24 - Whole follicle:
25 - Minced ovary
26 - Other?
27
28

29
30
31
32 **20. Quality Control: What are the quality control measures to be included in these assays that you favor?**

33
34
35 **Are there specific or special circumstances when additional quality control measures need to be added?**
36
37

38 **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?**
39
40
41

42 **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?**
43
44
45

1 **What specific step of steroidogenesis or organ of steroidogenesis does**
2 **each access?**

3
4 **23. Do you have any unpublished data relevant to these assays that you**
5 **would be willing to share? If so, are there any restrictions?**

6
7 **24. Are there any variations of the assay that should be considered that you did not**
8 **have time to validate or access? Anything you would change about the**
9 **assay to increase its sensitivity, efficiency, relevancy, or robustness?**

10
11 **25. Is there anyone else you can think of that we should contact?**

12
13 **a. If so, whom?**

14 **b. Can we mention your name when we contact him/her?**

15
16 **26. Open Dialogue**
17
18

1 **DETAILED REVIEW PAPER ON STEROIDOGENESIS**
2 **EPA CONTRACT NUMBER 68-W-01-023**
3 **WORK ASSIGNMENT #1-6**
4
5

6 The written text will become part of the permanent record.
7

8 At a minimum the questions provided in the following template should be
9 answered. Other ideas opinions, thoughts, suggestions, tangents or anecdotal type of
10 information will be welcomed and will become part of the record. Other information
11 should be recorded on this form under the "Open Dialogue" heading.
12
13

1 **Template for Steroidogenesis Interviews**

2
3 Chemicals that interfere with the androgen and estrogen systems can act via a
4 number of direct mechanisms in addition to those that directly involve the steroid
5 hormone receptors. One prominent mechanism of endocrine disruption is interference
6 with the synthesis of hormones themselves. Such interference is increasingly thought
7 to occur by up regulation or down regulation of the genes responsible for producing the
8 enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction
9 and inhibition of the enzymes themselves. The purpose of this work assignment is to
10 prepare a detailed review paper to survey and investigate the status of various in vitro
11 methods that have been used to identify chemicals that affect (i.e., inhibit or enhance)
12 steroidogenesis.

13
14 Name Interviewer Patricia A. Fail, Ph.D.
15 Name of Interviewee : Dr John W. Laskey
16 Date of Response 08/12/2001

17
18 Have the questions been responded to in writing by interviewee (circle or omit one) Yes
19 or NO?
20 If so was a follow up phone call made to discuss any of the major points (circle one) Yes
21 or NO?

22
23 **QUESTIONS**

- 24
25 **1. Do you know of any references that you would recommend that investigate**
26 **the status of various in vitro methods that have been used to identify**
27 **chemicals that affect (i.e., inhibit or enhance) steroidogenesis.**

28
29 Check the References included in my CV – sent separately

30
31 John, I am especially interested in the reference(s) for the testicular assay. I have
32 the ovarian references. Pat - in the listed references there should be several
33 references for the testicular assay G. Klinfelter and I have considered the
34 possible problems with this assay and in consultation with Dr. L. Ewing have
35 concluded that this minced testicular assay is consistent with the isolated Leydig
36 cell assay.

37
38 In our experiments (for the EPA CA with Earl Gray) we used both the ovarian
39 steroidogenesis and testicular steroidogenesis methods. With regards to these assays
40 or you own, please respond to the following question to the best of your ability.

1 **OVARY**

2
3 **2. What are the limitations of this method?**

- 4
5 i. For all these responses check the attached freelance file Pathway.jpg or
6 Pathway.pre file.
7
8 ii. The steroidogenic pathway in humans is somewhat different than in
9 laboratory species probably due to the order of the order of the enzymatic
10 reactions .
11
12 iii. The stage of the ovarian cycle (estrus, diestrus, . . . pregnancy) makes a
13 great deal of difference in the response to stimulation and/or inhibition.
14
15 iv. Blocking of the study to insure that day to day changes in assay conditions
16 (technical differences, media prep, stimulant prep, inhibitor prep, sample
17 origin, etc.) can be statistically corrected.
18

19 **3. What are the strengths?**

- 20
21 i. Ease of sample preparation.
22 ii. With good quality/technical control there is excellent reproducibility.
23

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

- 26
27 i. With normal laboratory precautions this method doesn't require any changes.
28

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

- 31
32 i. I haven't been following the literature for the past five years.
33

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

- 39
40 i. See above
41

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

3
4 i. See above

5
6 **Are there specific or special circumstances when additional quality control**
7 **measures need to be added?**

8
9 i. See above

10
11 **8. Based upon your expertise and experience, what endpoints would be most**
12 **appropriate for elucidating the effects of chemicals on testicular/ovarian/**
13 **adrenal steroidogenesis when investigating an in vitro study design?**

14
15 a. Responses to appropriate hormone/stimulation.

16
17 **9. Based on your experience, what chemicals, routes, duration, and doses**
18 **would you recommend to be used to validate an in vitro steroidogenesis**
19 **assay/protocol?**

20
21 **What specific step of steroidogenesis or organ of steroidogenesis does**
22 **each access?**

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

26
27 **11. Are there variations of the assay that should be considered that you did not**
28 **have time to validate or access? Anything you would change about the**
29 **assay to increase it's sensitivity, efficiency, relevancy, or robustness?**

30
31 **12. Is there anyone else you can think of that we should contact?**

32
33 a. If so, whom?

34 b. Can we mention your name when we contact him/her?

35
36 **13. Open Dialogue**
37
38
39
40
41

1 **Template for Steroidogenesis Interviews**
2

3 Chemicals that interfere with the androgen and estrogen systems can act via a
4 number of direct mechanisms in addition to those that directly involve the steroid
5 hormone receptors. One prominent mechanism of endocrine disruption is interference
6 with the synthesis of hormones themselves. Such interference is increasingly thought
7 to occur by up regulation or down regulation of the genes responsible for producing the
8 enzymes in the steroid synthesis pathway. In some cases it may be by direct interaction
9 and inhibition of the enzymes themselves. The purpose of this work assignment is to
10 prepare a detailed review paper to survey and investigate the status of various in vitro
11 methods that have been used to identify chemicals that affect (i.e., inhibit or enhance)
12 steroidogenesis.
13

14
15 Name Interviewer: Patricia A. Fail, PhD

16
17 Name of Interviewee: Dr. D. T. Armstrong

18
19 Date: Sept 7, 2001
20

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

22
23 NO Not all of them,
24

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

26
27 NO
28
29

30 **QUESTIONS:**
31
32

- 33 **1. Do you know of any references that you would recommend that investigate**
34 **the status of various in vitro methods that have been used to identify**
35 **chemicals that affect (i.e., inhibit or enhance) steroidogenesis.**
36

37 There are a lot of papers that use in vitro methods to study regulation of various
38 aspects of steroidogenesis. Many of them could be potentially useful in
39 identification of chemicals that affect the specific steroidogenic outcomes being
40 investigated. I don't know of a specific critical review of the status of such
41 methods for your purposes. It would be a good topic for someone to write;
42 perhaps an honours student with interests along these lines.
43

44
45 I wrote a review which touched on some of these ideas some years ago.
46 Armstrong DT 1986 Environmental stress and ovarian function. Biology of
47 Reproduction 34: 29-39.

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

3
4 With regards to these assays, please respond to the following question to the
5 best of your ability.
6

7 **OVARY**

- 8 **a. What are the limitations of this method?**
9 **b. What are the strengths?**
10 **c. What would you recommend to further enhance this method or what**
11 **changes would you recommend (if any)?**
12

13 Our research deals primarily with regulation of ovarian steroidogenesis and its
14 relationship to oocyte competence and ovulation. We have used a variety of
15 ovarian cell systems and end points. Studies with isolated systems of single cell
16 types have the limitation that they are able only to identify actions that involve the
17 cell type under study. Since a lot of the more subtle effects of compounds that
18 influence ovarian regulation involve cellular interactions, e.g. between oocytes
19 and granulosa cells, or thecal cells and granulosa cells, their effects may be
20 missed by restricting study of a single cell type. In addition, many agents that
21 alter steroidogenesis do so through extra-ovarian actions such as the
22 hypothalamus or pituitary (or even the liver, as in agents that affect steroid
23 clearance and hence alter feedback regulation of the ovary).
24

25 The strength of in vitro methods is their sensitivity, as well as the ability to identify
26 sites and mechanisms of action. In addition to the limitations alluded to above, in
27 vitro methods usually have to make assumptions as to the probable site of action
28 of a given compound. Therefore they may be inappropriate for screening of
29 compounds suspected to affect reproductive functions but for which the site or
30 mechanism of action has not yet been identified. This limitation may be
31 overcome by a sequential strategy that includes a series of tests such as:
32

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

35
36 whole ovary or isolated whole follicle culture in which steroids are measured
37 in culture media;

38
39 strategic cell combinations, such as thecal and granulosa cell co-culture;
40 oocyte-granulosa cell co-cultures; macrophage-luteal cell co-culture; isolated
41 single cell types;

42
43 subcellular components that enable study of a single component, e.g
44 hormone receptor, signal transduction molecule, specific intracellular reaction
45 (enzymatic, transport mechanism)
46

- 1 d. Do you know of any other published literature that corroborates or
2 refutes the findings in this paper?
3
4 e. In running this method/procedure are there any steps that are especially
5 difficult that require special attention i.e., lessons learned that come
6 after numerous runs that you would like to share...or is there any
7 special set up strategy you would recommend that would save time or
8 resources that come from experience in running the assay.
9
10 f. Quality control
11
12 1. what are the quality control measures to be included in these assays
13 that you favor?
14 2. Are there specific or special circumstances when additional quality
15 control measures need to be added?
16 3. Based upon your expertise and experience, what endpoints would be
17 most appropriate for elucidating the effects of chemicals on
18 testicular/ovarian/adrenal steroidogenesis when investigating an in vitro
19 study design?
20
21

22 There are important linkages and interactions between germ cell and somatic
23 cell compartments of the ovary that have not been explored as much as
24 warranted, and that could undoubtedly be exploited for design of specific test
25 systems that could be of interest to your group. Thus, in addition to
26 steroidogenesis end points, oocyte end points could also be used to assess
27 effects of chemicals. Specific oocyte end points that have proven useful in our
28 studies include effects (of regulatory agents) on meiotic maturation in vitro
29 (both spontaneous and gonadotropin-induced), ability to undergo normal
30 fertilization in vitro, and developmental competence after fertilization or
31 artificial activation. All of these end points depend on or are influenced by
32 input from follicular somatic cells, and hence would have potential for
33 development of assay systems that could prove useful for your purposes.
34

- 35 4. Based on your experience, what chemicals, routes, duration, and doses
36 would you recommend to be used to validate an in vitro steroidogenesis
37 assay/protocol?
38
39

40 What specific step of steroidogenesis or organ of steroidogenesis does
41 each access?
42

- 43 5. Do you have any unpublished data relevant to these assays that you
44 would be willing to share? If so, are there any restrictions?
45
46 6. Are there variations of the assay that should be considered that you did
47 not have time to validate or access. Anything you would change about

1 **the assay to increase it's sensitivity, efficiency, relevancy, or**
2 **robustness?**

- 3
4 **7. Is there anyone else you can think of that we should contact?**
5 **a. If so, whom?**
6 **b. Can we mention your name when we contact him/her?**

7
8 Professor Fulvio Gandolfi
9 Istituto di Anatomia degli Animali Domestici
10 via Trentacoste 2
11 I - 20134 Milano. Italy
12 Tel: (+39) 02-2154-036
13 Fax: (+39) 02-2140-745
14 Email: gandolfi@imiucca.csi.unimi.it

15
16 **8. Open Dialogue**

17
18 Pat, I'm not sure whether I've addressed this in a way that is useful. It will be
19 evident that our research over the years has not been directly aimed at ovarian
20 regulation from the standpoint of chemicals that disrupt/modify ovarian function.
21 Rather it has attempted to better understand normal (physiological) ovarian
22 regulatory processes. New concepts and details are being discovered in this
23 field at an unprecedented rate, as increasingly powerful methodology is focussed
24 on the topic. As our understanding of normal regulatory processes in the ovary
25 increases in both depth and breadth, it should be possible to use this information
26 to design approaches to study specific actions of chemicals such as those that
27 your institute wishes to investigate.

28
29 **TESTES:**

- 30 **a. What are the limitations of this method?**
31 **b. What are the strengths?**
32 **c. What would you recommend to further enhance this method or what**
33 **changes would you recommend (if any)?**
34
35 **d. Do you know of any other published literature that corroborates or**
36 **refutes the findings in this paper?**
37
38 **e. In running this method/procedure are there any steps that are**
39 **especially difficult that require special attention i.e., lessons learned**
40 **that come after numerous runs that you would like to share...or is**
41 **there any special set up strategy you would recommend that would**
42 **save time or resources that come from experience in running the**
43 **assay.**

44
45
46
47 **f. Quality control**

1
2
3
4
5
6
7
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11
12

- 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?**

1
2
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APPENDIX C:
PARAMETERS FOR COMPARISON SUMMARY OF
***IN VITRO* METHODS IN TABLE 4-11**

1 **Appendix C: Parameters for Comparison Summary of**
2 ***in Vitro* Methods in Table 4-11**
3
4

5 **Cost** – The overall cost of doing the screen including labor, cost of animals, and supplies. The
6 relative scale used in the table is defined as: (\$) - inexpensive; (\$\$) - moderately expensive;
7 (\$\$\$) - very expensive. The cost of conducting a steroidogenic assay using sectioned testes was
8 estimated to be \$11,600 per chemical (EDSTAC). This was considered an (\$) inexpensive assay.
9

10 **Time** - Under this parameter, the time necessary for the initial set-up and time to conduct an
11 assay were evaluated and compared. As for the initial set-up, the evaluation was based on the
12 laboratory equipment and apparatus needed for the assay. Also, the time needed to set-up the
13 apparatus and verify that it was functioning properly. In regard to the time needed to conduct the
14 assay, information from the literature was reported (when ever possible) using the number of
15 preparations that could be used for testing in a day. It does not include the time needed to
16 process samples since that would vary based on the selected endpoints, as well as would not be a
17 distinguishing factor between the assays.
18

19 **Laboratory** - Under this parameter, the level of training and type of equipment were compared
20 for each *in vitro* assay. In regard to the level of training and type of equipment, the *in vitro*
21 methods were designated as either general or specialized. General was used to describe those
22 assays that can be conducted with standard laboratory training and equipment. Specialized
23 describes those *in vitro* methods that require unique equipment, as well as additional and
24 advanced laboratory training in order to be able to perform the assay.
25

26 **Sensitivity**–The ability of the assay to detect an effect. The more sensitive the screening
27 bioassay, the less likely it will be that it fails to detect the positive action of a compound. Failure
28 to detect a positive effect (whether that effect increases or decreases some component of
29 steroidogenesis) is a false negative (a type II error). In the radioimmunoassays (RIAs) or other
30 determination of the hormone concentration, sensitivity is defined as the minimum detectable
31 amount of that hormone for a given set of assay conditions.
32

33 **Specificity**–Specificity refers to the ability to detect very well defined or specific activities. In
34 the case of the steroidogenesis screen, it is more desirable to rank any chemical that interferes
35 with any step of steroidogenesis as having positive activity. It is desirable then for the bioassay
36 to be less specific. For example, any substance that affects the P450 enzymes should cause an
37 altered hormone secretion in the assay. In RIAs, the antibody (detector) used must be
38 characterized to define which if any related substances it is detecting. For example, in a
39 testosterone RIA it is important to know if only testosterone is being detected or if the antibody
40 also recognizes dihydrotestosterone, androstenedione, or other androgens.
41

42 **Multiple endpoints** – The assays were evaluated for their capacity to foster multiple endpoints.
43 The steroidogenic pathway's hormones are the most frequently measured endpoints. The
44 number of different steroid hormones measured are included in the table (in parenthesis) along
45 with the reference. In addition, enzyme activities and architectural changes observed by
46 microscopic examination are also used as endpoints, but less frequently.

1 **Metabolic Activation** - An evaluation of whether the assay has an endogenous capacity to
2 metabolize pro-xenobiotics into active toxicants. Also, whether the assay lends itself to
3 exogenous addition of a metabolic fraction, i.e. S9.
4

5 **Stability** - A measure of the time that the preparation has been used by investigators to
6 characterize a steroidogenic response in the presence of a substance being tested. In addition,
7 the times listed in the table were based on papers in the literature that reported some measure of
8 viability. Viability was assessed using data about linear steroid hormone production in the
9 presence of a stimulating agent, e.g. hCG, as well as other viability type tests, e.g. Trypan Blue.
10

11 **Cytoarchitecture** - Describes the degree that the organ remains intact once removed from the
12 animal and is prepared for testing. Preparation of the organ for treatment, e.g. treatment with
13 collagenase in order to isolate cells, then the cytoarchitecture is changed, which may affect how
14 closely the preparation mimics the *in situ* environment.
15

16 **Repeatability (Inter-assay variability)**—Chemicals that test positive (or negative) in the subject
17 bioassay will do so repeatedly and with the same degree of effect. That is, the initial assay is
18 predictive of the same or similar results when repeated in another assay of the same type but set
19 up on different dates. In this case the variation associated with the repeated evaluation is
20 actually characterized (e.g. 10+ 0.5 %; Mean and SEM, n=y dates). For example, Chemical X
21 results in depressed testosterone secretion in a bioassay using adult male rat testes each time it is
22 tested in sequential evaluations and the amount (quantitative) of that suppression is similar. That
23 amount is calculated and reported as a characteristic of the bioassay.
24
25
26

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8

**APPENDIX D:
RECOMMENDED PROTOCOL**

DRAFT GENERIC PROTOCOL	[LABORATORY]	Page 1
---------------------------------------	---------------------	---------------

1 EPA Contract No.:
2 Lab Contract No.:
3 Lab Study Code:
4 Lab Master Protocol No.:

DRAFT: 5/12/02

7 TITLE: Testicular Steroidogenesis Bioassay Screening Protocol

10 SPONSOR:

14 TESTING FACILITY:

19 PROPOSED STUDY IN-LIFE DATES: _____

22 AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			
5			

30
31
32

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1 **1.0 OBJECTIVE AND BACKGROUND**
2

3 Steroid hormones produced by the gonads affect most of the organs in the body including
4 bone, muscle, brain, and reproductive organs. It is for this reason that the EDSTAC
5 recommended the *in vitro* steroidogenesis assay in testicular tissues as a component of the Tier 1
6 Screening (T1S) battery. The objective of this assay is to detect disruption of the steroidogenic
7 pathway *in vitro*. It may: (1) be used as one of the protocols recommended by EDSTAC for the
8 Tier 1 screening battery, (2) serve as a follow-up test for certain substances for which additional
9 data are required or desired, and/or (3) predict the likelihood that steroidogenesis and
10 downstream biologically dependent processes would be affected by the same or similar
11 substances *in vivo*. The endpoints were selected for their potential to detect toxicant-induced
12 alterations of steroidogenesis in gonadal tissue.
13

14 The Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996
15 required the EPA to develop and implement a screening program for determining the potential in
16 humans for estrogenic (and anti-estrogenic) effects from pesticides. This program has been
17 expanded on the advice of the EDSTAC to include androgenic (and anti-androgenic) effects and
18 effects from thyroid-hormone (TH)-like (or anti-TH) substances. The EDSTAC, assembled by
19 the EPA in 1996, believed, to the best of its knowledge, that the recommended Tier 1 screening
20 battery, if validated, would have the necessary breadth and depth to detect any currently known
21 disruptors of estrogen, androgen, and thyroid (EAT) hormones.
22

23 The suggested T1S protocols are being tested within the Endocrine Disruptor Screening
24 Program (EDSP) “to characterize the nature [and] likelihood of a dose-response relationship of
25 endocrine disruption in humans and wildlife” (EDSTAC, 1998). To this end, the EPA has
26 requested the development of a screening protocol that identifies compounds having the potential
27 to affect steroidogenesis.
28

29 The testis steroidogenesis bioassay was selected as a component of the Tier 1 screening
30 (T1S) by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to
31 evaluate the potential toxicity of substances on the endocrine system (EDSTAC, 1998). The T1S
32 tests were selected to obtain minimum yet sufficient estimates of potential endocrine disrupting
33 activity. The Committee stated that these tests should be relatively inexpensive, quick, and
34 technically easy to perform. Furthermore, they should be sensitive and specific, capture multiple
35 endpoints, and be predictive across species, gender, and age. Finally, they should be validated
36 and standardized as soon as possible (EDSTAC, 1998).
37

38 The *in vitro* testis steroidogenesis bioassay assesses non-receptor mediated effects on P450
39 steroidogenic enzymes. This assay has been used with fetal, neonatal, and adult testis, and is not
40 limited to mammalian species, having been used to assess steroidogenesis in fish, reptile, avian,
41 and amphibian systems as well. Thus, the steroidogenesis bioassay as a component in the T1S
42 phase should be broadly understood to screen for any disruption of the overall steroid
43 biosynthetic pathway. Both synthesis and release can be tested in gonads from normal animals.
44

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1 The goal of the *in vitro* steroidogenesis Tier 1 screen is to evaluate simultaneously all of
2 the processes involved with gonadal synthesis of steroid hormones (receptor binding, signal
3 transduction, transcription, translation, and cellular secretion of the steroids). A number of
4 compounds can inhibit the synthesis of various steroid hormones. These compounds inhibit one
5 or more enzymatic steps in the biosynthetic pathway of steroidogenesis (e.g., aminoglutethimide,
6 cyanoketone, finasteride, ketoconazole). Estrogen biosynthesis can be inhibited by exposure to
7 aromatase inhibitors such as the fungicide fenarimol (Hirsch et al., 1987). In addition, a number
8 of other steps in gonadal steroidogenesis might be disrupted, such as binding of LH to the
9 receptor on the cell membrane, cholesterol synthesis, and cholesterol intracellular transfer.

10
11 Antiandrogens and antiestrogens act via a number of direct mechanisms in addition to
12 those that directly involve the steroid hormone receptors (on the target organs). One prominent
13 mechanism of antihormonal activity is inhibition of hormone synthesis by inhibiting the activity
14 of P450 enzymes in the steroid pathway. Such activity will be detected with this *in vitro*
15 procedure using testicular tissue obtained from adult male rats. It will, however, detect only
16 pesticides and other substances that are active without metabolism, that is, the parent material is
17 active. The assay will also detect other mechanisms that alter gonadal steroid synthesis via the
18 LH receptor, cholesterol biosynthesis, and intracellular cholesterol transfer.

19
20 Substances that interfere with steroidogenesis primarily by inhibiting cytochrome P450
21 enzymes in the steroid pathway include two major classes of herbicides, the imidazoles and the
22 triazoles (Taton et al., 1988). They inhibit P450 enzymes in the sterol synthesis pathway for
23 lanosterol, a vital precursor of cholesterol (required for steroidogenesis and a component of
24 fungal membranes). Cytochrome P450 inhibitors tend to be nonspecific, and these fungicides
25 can also inhibit other P450 enzymes such as those required for mammalian steroid hormone
26 synthesis (Murray and Reidy, 1990). Inhibition of mammalian steroid synthesis can potentially
27 result in a broad spectrum of adverse reproductive effects *in vivo*, including abnormal serum
28 hormone levels, pregnancy loss, delayed parturition, demasculinization of male pups, lack of
29 normal male and female mating behavior, altered estrous cyclicity, and altered reproductive
30 organ weights.

31
32 Measures of not only testosterone, but also other intermediate hormones of the pathway
33 can be determined from media collected during the incubation period. Aromatase, another P450
34 enzyme, is also present, albeit at low concentrations, in the testes. Thus, it may also be possible
35 to utilize this assay for determination of aromatase disruption by measuring estradiol
36 concentrations. In support of this notion, Ammonium Perfluorooctanoate increased estradiol
37 production in isolated Leydig cell cultures (Biegel et al., 1995). Thus, while testosterone is the
38 ultimate endpoint as it is the end-product hormone, the assay has the advantage of having
39 multiple endpoints that can be used to assess effects at various sites of the pathway.

40
41 The purpose of this protocol is to outline a procedure for the quantitation of steroidogenic
42 hormone production from rat testicular tissue. The hormone measured – testosterone – is, if
43 altered, indicative of altered gonadal enzyme activity. This generic protocol is written for 250
44 mg testicular sections with a focus upon the components that are to be included in a validation

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1 protocol.

2
3 **2.0 MATERIALS AND METHODS**

4
5 **2.1 Test Substances**

6
7 **2.1.1 Test Substance–A Negative Control**

8
9 Common Name:

10 Chemical Name:

11 Synonyms:

12 CAS No.:

13 Molecular Formula:

14 Molecular Weight:

15 Appearance:

16 Odor:

17 Melting Point:

18 Density/Specific Gravity:

19 Solubility:

20 Vehicle:

21 Supplier:

22 Batch/Lot Number:

23 Purity:

24 Storage Conditions:

25
26 **2.1.2 Test Substance–A Positive Control**

27
28 Common Name:

29 Chemical Name:

30 Synonyms:

31 CAS No.:

32 Molecular Formula:

33 Molecular Weight:

34 Appearance:

35 Odor:

36 Melting Point:

37 Density/Specific Gravity:

38 Solubility:

39 Vehicle:

40 Supplier:

41 Batch/Lot Number:

42 Purity:

43 Storage Conditions:

44

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1 **2.1.3 Test Substance—Each Tested Unknown and Each Stimulant**

2
3 Unknown test substances will be selected by the client. All information as listed above
4 will be inserted.

5
6 **2.1.4 Standard Substance—Each Radioimmunoassay Standard**

7
8 Radioimmunoassay Standards of the highest purity will be obtained and prepared. The
9 following standard curves can be used: Testosterone 0.07 to 500 ng/mL.

10
11 **2.2 Chemical Safety and Handling**

12
13 An MSDS for each substance used will be attached.

14
15 **2.3 Dose Formulation and Analysis**

16
17 Each test substance, as well as the positive and negative controls, should have known
18 purity, stability in bulk, as well as stability and homogeneity in solution (in the range to be used).
19 The solubility of the test substance in the media to be used must be known. It has been standard
20 procedure to mix the substance in the incubation media. If it is necessary to use a special solvent
21 such as DMSO as a vehicle to enhance solubility in media, then that solvent should be tested
22 alone (as a vehicle control) for its effect in the assay system.

23
24 The formulation will be prepared at a frequency determined by stability tests performed
25 prior to the start of the study. Suspensions will be prepared and stored in wide-mouth, amber
26 bottles. The test materials will be suspended in appropriate vehicles or media, with the
27 concentration determined by the experimental design. At least three concentrations of the
28 unknowns should be tested.

29
30 An aliquot of each concentration per formulation will be analyzed. The formulation
31 bottles will be identified by a five-digit, random number Rx code and a color code. Personnel,
32 other than the Laboratory Supervisor, Project Toxicologist, and Study Director, will not be
33 informed of the test substances or formulation concentrations until all laboratory work is
34 completed (i.e., the study technicians will be “blind” for substance and dose). Aliquots from the
35 formulations will be collected on the day of treatment of the tissues and will be analyzed.

36
37 **2.4 Animals**

38
39 **2.4.1 Species and Supplier**

40
41 The proposed test animals will be the Sprague Dawley Derived Outbred Albino Rat
42 Crl:CD®(SD) IGS BR supplied by Charles River Laboratories, Inc., Raleigh, NC.

1 **2.4.2 Live Animals and Species Justification**

2
3 The use of tissues from live animals has been requested by the Sponsor. Alternative test
4 systems are not available for the assessment of effects of substances on reproduction and
5 development in intact mammals for determining the potential risk for humans from endocrine-
6 mediated effects of pesticides and other substances. The Charles River CD® rat has been the
7 subject of choice on reproductive and developmental toxicology contracts, and has been used for
8 other reproductive toxicology studies with this test material. Large historical data bases for
9 reproductive performance and prevalence of spontaneous malformations in control rats are
10 available from the supplier (Charles River, 1988). This strain of rat has been proven to have
11 robust fertility and fecundity, and does not present any unusual endocrine patterns. This study
12 does not unnecessarily duplicate any previous study.

13
14 **2.4.3 Total Number, Age, and Weight**

15
16 Number of Males: 10
17 Age on Receipt: ~10-12 weeks

18
19 (no less than 8 weeks of age to ensure maturity)

20
21 Animal Wt. Range: 250-275 g

22
23 NOTE: The number of animals needed will depend upon the size of the bioassay. Ten
24 male rats are sufficient to provide tissue for 80 incubations. Minimum sample size requirements
25 should be verified (based on assay variation). Estimates may be made from existing data. This is
26 essential to providing adequate power for statistical comparison of data among treatment groups
27 and will provide information needed to determine the number of testicular sections assigned to
28 each dose group.

29
30 **2.4.4 Quality Control** (tests of animals to verify antibody free status)

31
32 Serological evaluation of animals for fecal or blood viral, bacterial, or protozoan
33 antibodies is not deemed necessary for this protocol as they will not be housed at the laboratory
34 for more than 1 or 2 weeks.

35
36 **2.4.5 Quarantine**

37
38 Upon receipt, animals will be quarantined for 7 days. They will be observed daily for
39 general health status and ability to adapt to husbandry conditions. They will be released from
40 quarantine by the attending veterinarian or his/her designate.

1 **2.5 Animal Husbandry**

2
3 **2.5.1 Housing, Feed, and Water**

4
5 During the quarantine period, animals will be randomly assigned to cages. Animals will
6 be singly housed in solid-bottom polycarbonate cages (8"x19"x10.5") fitted with stainless steel
7 wire lids (Laboratory Products, Rochelle Park, NJ). Sani-Chip® cage bedding (P.J. Murphy,
8 Forest Products, Inc., Montville, NJ) will be used in all solid-bottom cages. Pelleted feed (No.
9 5002 Purina Certified Rodent Chow®) will be available *ad libitum* for the rats and tap water
10 from the water system will be filtered and available *ad libitum* to all animals via an automatic
11 water delivery system (Edstrom Industries Inc., Waterford, WI). Water is also available in
12 plastic bottles with stainless steel sipper tubes *ad libitum*. The analysis of the rodent chow for
13 chemical composition and possible chemical contamination and analysis of city water will be
14 provided by the suppliers and maintained in the study records if deemed necessary. It is
15 anticipated that contaminant levels will be below certified levels for both feed and water and will
16 not affect the design, conduct, or conclusions of this study. Rat chow will be stored at
17 approximately 60-70EF, and the period of use will not exceed 6 months from the milling date.
18 At all times, animals will be housed, handled, and used according to the NRC Guide (NRC,
19 1996).

20
21 **2.5.2 Environmental Conditions**

22
23 Environmental conditions in the laboratory facility will be continuously monitored,
24 recorded, and controlled during the course of the study by an automated system. Animal rooms
25 used for this study will be maintained on a 12:12 hour light:dark cycle. Target conditions for
26 temperature and relative humidity in the animal rooms will be between 64 and 79EF (18 and
27 26EC) and 30 and 70%, respectively, with 10 to 15 air changes per hour (NRC, 1996).
28 Temperature and/or relative humidity excursions will be documented in the study records and
29 the final report.

30
31 **2.5.3 Animal Identification**

32
33 During quarantine, animals will be individually identified by a cage card. They will not
34 be tattooed or given ear tags. Data generated during the course of this study will not be tracked
35 by these numbers.

36
37 **2.5.4 Limitation of Discomfort**

38
39 Discomfort or injury to animals will be limited, in that any animal will be humanely
40 terminated by CO₂ inhalation.
41

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.

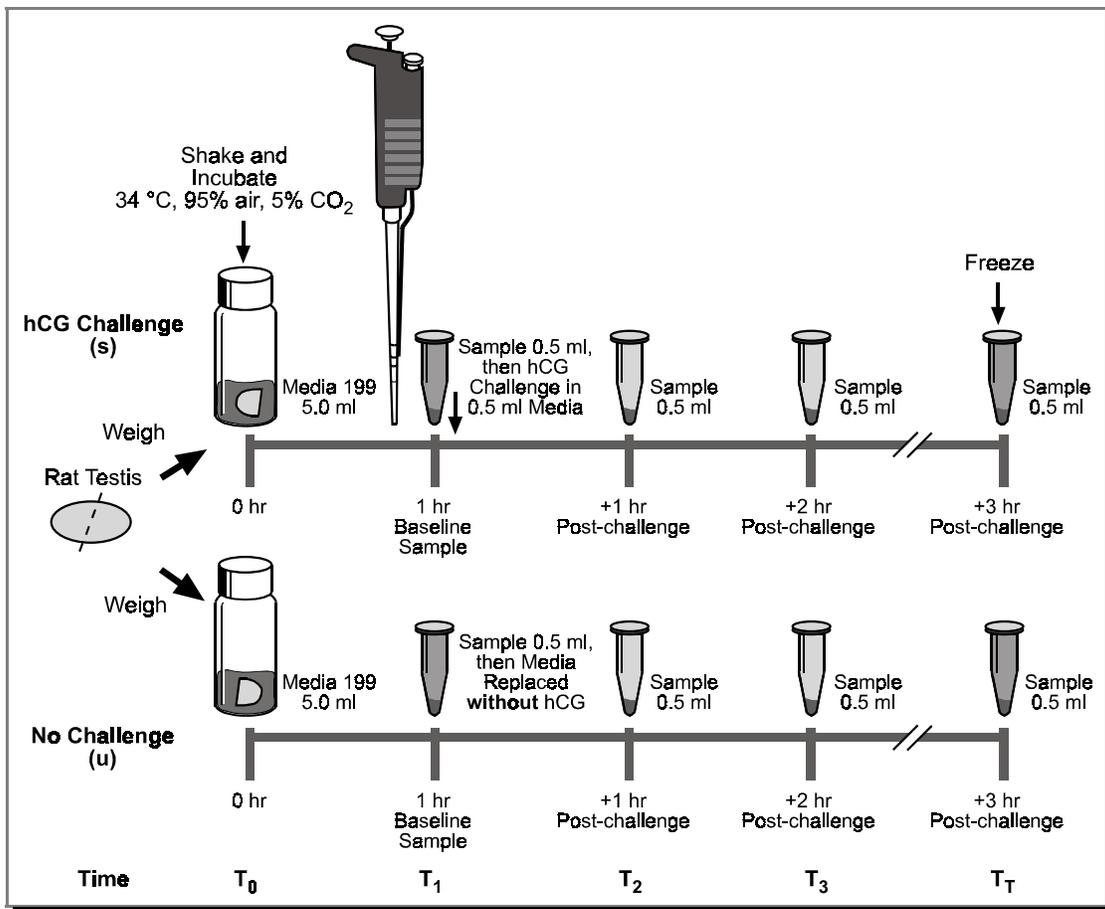


Figure 1. Technical Flow Illustration of the Testicular Steroidogenesis Assay

1 **Table 1. Experimental Design**

2

3

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>		
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, dose x	3	3
Unknown N, dose y	3	3
Unknown N, dose z	3	3

22
23

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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 34EC on a shaker in 5% CO₂/95% 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

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1 control group (within time) using either parametric ANOVA under the standard assumptions or
2 robust regression method (Royall, 1986; Huber, 1967), which does not assume homogeneity of
3 variance or normality. The homogeneity of variance assumption will be examined via Levene's
4 test (Levene, 1960), which is more robust to the underlying distribution of the data than the
5 traditional Bartlett's test. If Levene's test indicates lack of homogeneity of variance ($p < 0.05$),
6 then a log 10 conversion of the data will be made. These values will be tested for normalcy and
7 an ANOVA applied to test all treatment effects. If the assumptions of ANOVA are not met, then
8 robust regression methods will be applied. The robust regression methods use variance
9 estimators that make no assumptions regarding homogeneity of variance or normality of the data.
10 They will be used to test for overall treatment group differences, followed by individual tests for
11 exposed vs. control group comparisons (via Wald Chi-square tests), if the overall treatment
12 effect is significant. The presence of linear trends (over the time points) will be analyzed by
13 GLM procedures for homogenous data or by robust regression methods for nonhomogenous data
14 (SAS Institute Inc., 1999a,b,c,d,e; 2000). Standard ANOVA methods, as well as Levene's test,
15 are available in the GLM procedure of SAS® Release 8 (SAS Institute Inc., 1999a,b,c,d,e;
16 2000), and the robust regression methods are available in the REGRESS procedure of
17 SUDAAN® Release 7.5.3 (Shah et al., 1997).

18
19 If Levene's test does not reject the hypothesis of homogeneous variances, standard
20 ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in
21 SAS® 6.12 will be used to evaluate the overall effect of treatment and, when a significant
22 treatment effect is present, to compare each exposed group to control via Dunnett's Test
23 (Dunnett, 1955, 1964). A two-tailed test (i.e., Dunnett's test) will be used for all pairwise
24 comparisons to the vehicle control group.

25
26 A test for statistical outliers (SAS Institute, Inc., 1990b) will be performed on suspected
27 outliers. If examination of pertinent study data do not provide a plausible technologically
28 sound reason for inclusion of the data flagged as "outlier," the data will be excluded from
29 summarization and analysis and will be designated as outliers. For all statistical tests, $p \neq 0.05$
30 (one- or two-tailed) will be used as the criterion for significance.

31 32 **5.0 RETENTION OF SPECIMENS AND RECORDS**

33
34 All specimens and records will be retained in archives for two years at the performing
35 laboratory's expense. Beyond two years, continued retention will be at additional cost to the
36 Sponsor.

37 38 **6.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES**

39
40 Quality control (QC) and quality assurance (QA) procedures will follow those outlined in
41 the Quality Assurance Project Plan (QAPP) prepared for this study.

42
43 A list of all laboratory-specific SOPs will be maintained with the study records and
44 available for inspection by the Sponsor's representative.

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1
2 **7.0 REPORTING**
3

4 An executive summary will be prepared describing the number and strain of rats used in
5 the study, the doses and substances tested, and the effects with levels of statistical significance
6 for all endpoints. Electronic and hard copies of spreadsheets containing the raw data from all
7 animals will be provided for each endpoint. In addition, the spreadsheet should include
8 treatment means, standard deviation, standard error, coefficient of variation, and sample number
9 below each endpoint. Data presented should include sample identification and treatment, and
10 media progesterone, testosterone and estradiol concentrations. A data summary table containing
11 the mean, standard deviation, standard error, coefficient of variation, and sample size for each
12 treatment group should be provided for all endpoints.
13

14 **Tentative Study Dates^a** (to be added to the protocol by amendment)
15

- 16 Male Rats arrive:
- 17 Quarantine period (7 days):
- 18 Preparation of chemical solutions (test toxicants):
- 19 Sacrifice of animals:
- 20 Preparation of tissues and incubation with test toxicants:
- 21 Radioimmunoassay of media for hormones:
- 22 Statistical analysis:
- 23 Submission of nonaudited draft final report:
- 24 Submission of audited draft final report:

25
26 ^a The end dates are tentative and will depend on the dates of radioimmunoassay completion.
27

28 **8.0 STUDY RECORDS TO BE MAINTAINED**
29

- 30 Protocol and any Amendments
- 31 List of any Protocol Deviations
- 32 List of Standard Operating Procedures
- 33 Animal Requisition and Receipt Records
- 34 Quarantine Records
- 35 Temperature and Humidity Records for the Animal Room(s)
- 36 Animal Research Facility Room Log(s)
- 37 Durham City Water Analysis (analyzed monthly, reported annually)
- 38 Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results
- 39 Dosage Code Records Containing Five-Digit Rx Code, Color Code, and Concentration
- 40 Dose Formulation Receipt and Use Records
- 41 Statistical Analysis Records
- 42 Media Estradiol Analysis (E2)
- 43 Media Testosterone (T4)
- 44 Media Progesterone Analysis (P4)

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1 Correspondence
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