Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development 24-Jul-2008

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Cancels & replaces the same document of 22 July 2008

SERIES ON TESTING AND ASSESSMENT
Number 90

BACKGROUND REVIEW DOCUMENT ON THE RODENT HERSHBERGER BIOASSAY

JT03249186

ENV/JM/MONO(2008)17
Unclassified

English - Or. English

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 90

BACKGROUND REVIEW DOCUMENT ON THE RODENT HERSHBERGER BIOASSAY

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2008

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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The participating organisations are FAO, ILO, OECD, UNEP, UNIDO, UNITAR and WHO. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

FOREWORD

This background review document (BRD) provides a critical summary of the published scientific literature available as of August 2006, in support of the standardization and validation of the rodent Hershberger bioassay, undertaken as part of the OECD Test Guidelines Programme.

The rodent Hershberger bioassay is based on the principle that the accessory sex organs (ASO) are under the control of androgens to stimulate and maintain growth. If endogenous sources of this hormone are not available, the animal will require an exogenous source to initiate and/or restore ASO growth.

The objective of the OECD work on the Hershberger bioassay is to develop and validate a new Test Guideline for the detection of chemicals having the potential to act like, and consequently interfere with, endogenous male sex hormones. More specifically the rodent Hershberger bioassay is intended to identify chemicals that act like androgen agonists or antagonists. The assay, once validated for both the immature and adult castrated model, is intended to be used as a short-term assay within an overall testing strategy for the detection and assessment of potential endocrine disrupters.

This BRD was prepared by the United States and the first draft was submitted in January 2007 to a peer review panel in support of the Hershberger assay validation effort. In parallel, it was circulated for comments to the Validation Management Group for mammalian testing (VMG-mam) and to the Task Force (TF) on Endocrine Disrupters Testing and Assessment (EDTA). The draft was revised by the United States on the basis of the comments received and a second version was circulated to the VMG-mam and EDTA TF in December 2007. A final draft BRD, prepared by the Secretariat in February 2008 on the basis of the comments received, was endorsed by the EDTA TF and the Working Group of National Coordinators of the Test Guidelines Programme (WNT) at their meetings in April 2008. The WNT agreed that due to the nature of the document, it could be dealt with like validation reports, i.e. not circulated to the WNT for comments prior to its submission for endorsement.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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LIST OF ABBREVIATIONS

The following abbreviations are used in this Background Review Document:

ADME	absorption, distribution, metabolism, and elimination
AFP	α-fetoprotein
AIS	Androgen Insensitivity Syndromes
AMH	anti-Műllerian hormone
AR	androgen receptor
ASO	accessory sex organs
BRD	background review document
CALUX	chemically activated luciferase expression
CG	chorionic gonadotropin
CHO	Chinese hamster ovary cells
CNS	central nervous system
DES	diethylstibestrol
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
E2	17β-estradiol
EE	ethynyl estradiol
EACs	endocrine active compounds
ECVAM	European Center for Validation of Alternative Methods
EDCs	Endocrine disrupting chemicals
EDSP	Endocrine Disruptors Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	Endocrine Disrupters Testing and Assessment
EGF	epidermal growth factor
EPA	U.S. Environmental Protection Agency

List of Abbreviations (cont'd)

FLU	flutamide
FSH	follicle stimulating hormone
GD	gestation day
GnRH	gonadotrophin releasing hormone
GR	glucocorticoid receptor
H-P	hypothalamus-pituitary
H-P-G	hypothalamic-pituitary-gonadal axis
HRE	hormone response element
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IC_{50}	concentration of inhibitor necessary to reduce specific ligand binding by 50%
IGF-1	insulin like growth factor 1
LABC	levator ani plus bulbocavernosus complex
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LHRH-a	luteinizing hormone-releasing hormone analog
LNCaP	cell line established from a metastatic lesion of human prostatic
MIS	Műllerian inhibiting substance
mRNA	messenger ribonucleic acid
NADH	reduced form of nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NOAEL	no observed adverse effect level
NOEL	no observable effect level
OECD	Organisation for Economic Cooperation and Development

List of Abbreviations (cont'd)

PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PND	postnatal day
PO	"per os", by mouth (gavage)
PPS	preputial separation
PR	progesterone receptor
PTU	propylthiouracil
RT-PCR	real-time polymerase chain reaction
SARs	Structure activity relationships
SC	subcutaneous
SF-1	steroidogenic factor-1
SHBG	steroid hormone binding globulin
SOP	standard operating procedure
StAR	steroidogenic acute regulatory protein
T	testosterone
T3	triiodothyronine
T4	thyroxin
Tfm	mouse testicular feminization gene
TGF	transforming growth factor
TIS	Tier 1 Screening
TIS 1	tetradecanoyl phorbol acetate-inducible sequence gene
TP	testosterone propionate
TRPM-2	T-repressed prostatic message-2
TSH	thyroid stimulating hormone
VP	ventral prostate
VIN	vinclozolin
VO	vaginal opening

1.0 EXECUTIVE SUMMARY

The purpose of this detailed, background review document is to provide a critical summary of the published scientific literature available as of August 2006, in support of the standardization and validation of the rodent Hershberger assay (Hershberger *et al.,* 1953). The validation process of a test method includes:

- Standardization of the study design, protocol, and standard operating procedures (SOPs).
- Testing the export of the protocol to obtain reproducible results among qualified laboratories.
- Testing the intra- and interlaboratory variability of the endpoints over time.
- Regulatory acceptance.

The protocols under examination involve intact, stimulated weanling (sexually immature), castrated prepubertal or adult castrated male rats exposed to the test chemical (and/or a reference androgen) for a number of consecutive days (typically ten), and measurement of epididymides (if they are not removed at castration) and the accessory sex organ (ASO) weights (and also testes if the subjects are intact) at necropsy immediately following the last dose. The intended purpose of the Hershberger assay is to identify chemicals that act as androgen agonists (androgenic) or androgen antagonists (anti-androgenic) *in vivo*. It is considered a specific mechanistic screening assay, sensitive to those chemicals that interact with the androgen receptor (AR) or modulate circulating androgen levels. The assay, once validated, is intended for use as a short-term, *in vivo* assay in an overall testing strategy for the potential identification of certain endocrine modes of action (*i.e.,* androgens, anti-androgens, and 5α-reductase inhibitors) (Chapter 2.0, Introduction). Depending on the outcome of the Hershberger assay, substances may then require additional assays/tests to evaluate their potential for causing adverse reproductive and/or developmental effects.

The basis of the Hershberger assay is the absolute requirement for testosterone (T; made in the testis) and/or dihydrotestosterone (DHT; converted from T by 5α-reductase in the testis and other end target organs) for the rapid growth and maturation of the ASO during puberty in intact males and for their maintenance in post puberty. Castration with the loss of endogenous T leads to their rapid regression and involution, and for their rapid regrowth in the castrate when administered an exogenous androgen (typically T propionate; TP). The ASO of interest are predominantly the epididymides (if they are not removed at castration), the seminal vesicles with coagulating glands, the prostate (the ventral lobe or ventral plus dorsolateral lobes), and the levator ani plus bulbocavernosus complex (LABC) muscle, and Cowper's (bulbourethral) glands, as well as the other ASO less frequently used (*e.g.,* preputial glands). The mode of action for T, DHT, and other

androgens is initiated by the entrance of the androgen into the target cell and binding to the AR (*i.e.*, androgens are the ligands for the receptor). The binding of the natural ligand to the AR creates a conformational change so that the altered AR complex binds to the specific DNA-binding domain, initiates androgen-mediated transcriptional activation of a cascade of genes to produce mRNA, and translates the messages into proteins directing mass molecular, biochemical, and physiological processes to induce growth of the ASO (Chapter 3.0, Biological Basis of the Assay/Androgen Mode of Action). In contrast, an androgen antagonist also binds to the AR but either induces no conformational change or induces a change that precludes the AR-ligand complex from binding to the DNA. This sequence is the reason why an intact adult male cannot be used in the Hershberger assay, since the presence of endogenous T (and DHT) for maintenance of the ASO and other male secondary and tertiary sexual characteristics (including sexual behaviors and reproduction) and a fully developed, functional, intact hypothalamic-pituitary-gonadal axis makes the intact male insensitive to exogenous androgens. The development of the male reproductive tract and other androgen-responsive tissues and their interactions with androgens (T and DHT) are presented in Chapter 4.0, Male Reproductive Tract and Other Androgen Responsive Tissues.

The Hershberger assay was developed initially to identify the drivers (hormones) for male sexual development, and it has been used in pharmaceutical development of androgen agonists and antagonists for many years (Chapter 5.0, History of Assay).

There are three versions of the Hershberger assay in rats in use or under discussion: the prepubertal intact (weanling) male, the castrated adult male, and the peripubertal castrated male (Chapter 6.0).

- For the prepubertal intact male version or the weanling male as it is termed in the Organisation for Economic Cooperation and Development **(**OECD) validation program, after weaning on postnatal day (PND) 21, the males are exposed to the test chemical and vehicle during the postwean peripubertal period prior to acquisition of puberty (approximately PND 41-42 in rats and PND 26-28 in mice). The endpoints of interest are age at acquisition of puberty (preputial separation; PPS), weights of the testes and epididymides (since these animals are not castrated), and weights of the sex accessory organs. The timing and duration of treatment is therefore circumscribed to a maximum of approximately 14 days in rats (from PND 21-22 to PND 35 after which PPS begins to occur). These animals are intact with respect to the gonadal tissues and the hypothalamic-pituitary-gonadal axis. Therefore, the weanling assay is somewhat less mechanistically specific than the castrated male version, but the intact reproductive system enables identification of further effects induced by interactions with the intact hypothalamic-pituitary-gonadal axis and presents animal welfare benefits.
- For the castrated adult male version, the male rats are castrated after puberty with a postsurgical recovery period (so the ASO involute/regress) or no recovery period (so the androgen-dependent ASO have not yet regressed). The test chemical (or reference androgen) is administered for typically 3-5 days by per os (po) or subcutaneous (sc) injection, and the animals are necropsied 24 hours after the last dose and the ASO weighed.

- For the peripubertal castrated male version, Gray *et al.* (2005) recommended castration of sexually immature rats on PND 42 or later (so the rats can acquire PPS with/without exogenous androgens), a 12-day recovery period, initiation of treatment on PND 53-54 for ten consecutive days, and necropsy (the day after the last dose on PND 64-65). The endpoints are weights of the ASO. For this version, the tissues of the peripubertal castrate have not reached their adult size and regress more quickly to a low baseline weight. This provides a greater range of dynamic response, contributing to this version's enhanced sensitivity to exogenous androgens. The weight of the glans penis (after PPS) is another endpoint, and there may be qualitative as well as quantitative differences in the pre-/peripubertal animal versus the adult. Since this is a castrated animal model, it is specific to androgen-dependent, AR-mediated mechanisms (Chapter 3.0).

The details, procedures, and variables for each version of the Hershberger assay and the synopsis of the protocol selected to be validated by the OECD (the peripubertal castrated male rat), is described in Chapter 6.0, and briefly described below. Variables include species/strain, age at castration, recovery period (presence and duration), start and duration of treatment, route of administration, reference androgens and endpoints (such as tissue dissection, pre- and post-fixation weighing, statistical analyses), as well as animal husbandry differences such as caging, bedding, lighting, feed, housing, and drinking water.

It is important that a recommended and widely used assay be specific, robust, and reproducible. In addition, it should use the minimum number of animals, have known strengths, and identify limitations. There are a vast number of studies that have evaluated chemicals for androgenic/anti-androgenic activity in castrated rats, beginning in the 1930s that were improved and standardized by Hershberger *et al.* in 1953. The Hershberger assay has been proposed by both the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, 1998), Weybridge (1997), and OECD (1998) to be validated for use in a comprehensive screen to detect potential endocrine disruptors. The assay has also been used in the pharmaceutical industry to screen for these properties in drug candidates in development for therapeutic purposes. Chapter 7.0, Examples of Chemicals Tested in the Hershberger Assay*,* provides a compendium of chemicals, with information on protocol variables and results in terms of weights of the ASO. When tested in a relatively standardized Hershberger protocol, the vast majority of chemicals tested have produced consistent results across laboratories; with few inconsistencies (*e.g.,* the fungicide fenitrothione is an anti-androgen *in vitro* but was tested both positive [Tamura *et al.,* 2001] and negative [Sohoni *et al.,* 2001] in the *in vivo* Hershberger assay).

The Hershberger assay appears to be robust, specific, reproducible within and across capable laboratories over time. The overall testing strategy may include in silico assessment of the structure of unknown substance as a possible ligand for the AR and *in vitro* screens for AR binding affinity and transcriptional activation or inhibition, or other *in vitro* assays for androgen-related effects. However, it is important to note that *in vitro* assays do not incorporate such components as ADME (absorption, distribution, metabolism, and elimination), or response of target organs/cells/organelles, etc., which are present in *in vivo* assays. As a result, false positives (defined in this document as a substance incorrectly identified as an androgen agonist/antagonist *in vitro* not supported

by *in vivo* results), as well as false negatives (defined in this document as a substance incorrectly identified as not being an androgen agonist/antagonist *in vitro* with positive *in vivo* results) can result.

The toxico-pharmacodynamics and toxico-pharmokinetics of androgen metabolism support the necessity for an *in vivo* assay with animals in the overall assessment of possible androgen agonists and antagonists. The essential assumption (as with the estrogenic/anti-estrogenic compounds, Uterotrophic Assay Background Review Document ENV/JM/MONO, 2003) is that the active "factor" is the concentration in the serum (or plasma) of the free (not bound) bioavailable ligand in equilibrium with the target organ intracellular ligand concentrations. This portion of an administered substance is then the fraction available to bind the AR ligand. A number of considerations affect the serum/plasma concentration of the ligand and its *in vivo* activity in the Hershberger assay. These considerations include intestinal and liver metabolism, leading to active or inactive metabolites; intestinal and liver conjugation reactions; biliary, renal, fecal, and other routes of elimination; specific and nonspecific binding to serum proteins; sequestration in other body compartments; and the specific receptor concentrations in target tissues.

Also note that the Hershberger assay, involving castrated males (pre- or postpubertal), is relatively specific and limited to AR-dependent actions (T and/or DHT and those exogenous agents which also bind to the AR).

An important question is whether the Hershberger assay results are predictive of adverse effects, which is especially true of weak androgen agonists/antagonists. There is also the recognition that there may be hazard (the intrinsic capacity of the test chemical to do harm), but there may not be risk (the capacity of the test chemical to cause adverse effects at environmentally relevant doses at relevant routes of administration during sensitive life stages).

A positive response in the Hershberger assay suggests the need for additional testing for adverse reproductive and developmental effects, and that performance of more definitive tests administering the test material chronically by gavage over a wide dietary dose range, including human exposure doses (*i.e.*, a two-generation reproductive toxicity study under OECD No. 416 [2001] or EPA OPPTS 870.3800 [U.S. EPA, 1998a] testing guidelines) could result in androgen-mediated, adverse results.

Since the Hershberger assay is relatively specific and limited to AR-dependent actions, it is expected that chemicals that give a negative result in the Hershberger will not present a risk from these effects, but the Hershberger needs to be complimented by other assays, e.g., inhibition of steroidogenesis to obtain a complete picture of the potential of a chemical to affect the androgen hormone system.

The data summarized in this document provide broad support for the validation and regulatory use of the Hershberger assay as a robust, specific, mechanism-based, *in vivo* screen to detect possible androgen agonists and antagonists. In addition,

- The evidence clearly supports the binding of ligand (endogenous or exogenous) to the AR as the initial step in the transactivation of androgen-dependent genes and the subsequent cascade of molecular, biochemical, and physiological events that

results in increased growth of the ASO. Increased growth of these organs is measured by their weight.

- The extensive history of the Hershberger assay supports the ability of the assay to evaluate the androgenic/anti-androgenic potential of substances, even weak androgen agonists/antagonists which act as ligands and interact with the AR.
- Of the three major versions of the Hershberger assay, the castrated peripubertal male version tends to be the most sensitive of the three (see section 2.2.2 for strengths and limitations of the different models).
- The major procedural variables for the Hershberger assay are known, and their impact(s), have been established.
- Many laboratories have the technical skill, equipment, facilities, and experience to conduct the Hershberger assay.
- The overall specificity, robustness, and reproducibility of the Hershberger assay appear to be adequate and appropriate to different classes of chemicals. False positive and negative results are relatively rare. Criteria for accepting data (*e.g.,* acceptable control ranges of weights for ASO) and guidance on interpretation are available and useful.
- Toxico-pharmacodynamics and toxico-pharmacokinetic factors in the castrated or intact animal can modify the activity of a test substance. This activity supports the need for *in vivo* testing in animals and a tiered hierarchical framework (*e.g.,* the Weybridge Report, Proceedings of the European Workshop on the impact of endocrine disrupters on human health and wildlife, 2-4 December 1996, Weybridge, UK; European Commission DGXII, April 16, 1997; Crisp *et al.,* 1997 [U.S. EPA]; OECD, 1998; U.S. EPA [1998b] EDSTAC Report; the Aronsborg Report, Proceedings of the European Workshop on Endocrine Disrupters, 18-20 June 2001; the European Commission GD, 2001; Report of Proceedings, Copenhagen, Denmark) with ultimately relevant routes, doses, and timing of administration.
- The Hershberger assay provides a clear trigger for subsequent testing for adverse effects.
- Structure activity relationships (SARs) and *in vitro* assays appear able to identify (prescreen) substances with a potential androgenic mode of action as candidates for the Hershberger assay, thereby minimizing the use of animals and resources.
- The results for the *in vivo* Hershberger assay are suitable to identify those endocrine-active substances that act through an AR mechanism/mode of action to be appropriate and suitable for further testing for androgenic (agonistic) or anti-androgenic (antagonistic) reproductive and/or developmental effects (Chapter 8.0, Discussion and Conclusions).

2.0 INTRODUCTION

2.1 Organisation for Economic Cooperation and Development (OECD) Validation Program

In 1997, in response to growing health concerns over the potential of chemicals to adversely affect the endocrine system, the OECD reviewed its Health Effects Testing Guidelines and concluded that the existing guidelines were insufficient to identify chemicals acting through certain endocrine mechanisms (oestrogen, androgen, and thyroid), and might not be adequate to fully characterize the hazards posed by such chemicals. Therefore, a *Special Activity on the Testing and Assessment of Endocrine Disrupters* was initiated as part of the OECD Health Effects Testing Guidelines Program. The purpose of this activity was to revise existing guidelines and to develop new OECD Test Guidelines in order to, first, screen chemicals to identify substances that could interact with the endocrine system, and second, to ensure that the tests could characterize the hazards of potential disruptors. An OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) was established to provide a focal point within the OECD to distinguish and recommend priorities for the development and validation of new and improved methods to identify and assess substances acting through endocrine mechanisms (OECD, 1998).

The rodent Hershberger bioassay was proposed for use as a screen in 1998 (see below) and was given high priority for validation by several expert panels and workshops, involving both European and U.S. scientists, as a mechanistic screen for substances that would act through an androgenic or anti-androgenic mode of action (Gray *et al.*, 1997; EC/EEA/OECD/WHO, 1997; SETAC-Europe, 1997; U.S. EPA, 1998a). A series of European Commission conferences and workshops, EDSTAC, and other panels recognized that although the Hershberger bioassay has been in use since the 1930s for pharmaceutical discovery and evaluation of androgens (and anti-androgens), the bioassay would have to be validated for its use as a screen for weak androgens and anti-androgens.

Validation is a scientific process designed to characterize the operational characteristics and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose. OECD Guidance Document 34 provides the principles of test validation and practical guidance for validation that are followed by OECD. These principles were introduced in the report from a workshop on validation in Solna (OECD, 1996) and are consistent with the approaches used in Europe by the European Center for Validation of Alternative Methods (ECVAM, 1995) and the U.S. Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM, 1997).

The stages of the validation process include:

- T*est development*, an applied research function which culminates in an initial protocol. As part of this phase, a detailed background review document (BRD) is prepared to explain the purpose of the assay, the context in which it will be used, and the scientific basis of the assay's protocol, endpoints, and

relevance. The BRD reviews the scientific literature for candidate protocols and evaluates each with respect to a number of considerations, such as whether the candidate protocols meet the assay's intended purpose, the relative costs, and other practical considerations. The BRD also identifies the stage of development of each protocol, questions related to its conduct and suitability, and information necessary to answer the questions, and, when possible, recommends an initial protocol for the initiation of prevalidation.

- *Prevalidation* stage involves studies to refine, optimize, and initially assess protocols for transferability and performance. Several different types of studies are conducted during the assay's prevalidation phase, depending upon the status of the method and the nature of the questions that the protocol raises. The initial assessment of transferability is generally a trial in a second laboratory to determine that another laboratory besides the lead laboratory can follow the protocol and execute the study.
- *Inter-laboratory validation* studies are then conducted in multiple independent laboratories, after protocol optimization during prevalidation. The results of inter-laboratory validation studies are used to determine inter-laboratory variability and to set and/or cross-check performance criteria. Inter-laboratory validation is followed by:
    - *Peer review*, an independent scientific review by qualified experts, followed by:
    - *Regulatory acceptance;* the consensus is that this test method is acceptable for adoption for regulatory use by regulatory authorities.

The purpose of this background review paper is to provide a summary of the literature in support of the standardization and validation of the Hershberger assay for use in the detection and evaluation of endocrine-active compounds.

2.2 Hershberger Assay

2.2.1 Brief description

Historically, the Hershberger assay (Hershberger *et al.*, 1953) was based on a myotrophic test for the assay of protein anabolic activities of androgens that measures the levator ani muscle in castrated male rats receiving different androgens (Eisenberg and Gordan, 1950; Gordan *et al.*, 1957). In the initial myotrophic test (by Eisenberg and Gordan, 1950; Gordan *et al.,* 1957), PND 21 rat weanlings were castrated and retained for 23 days postcastration until the test chemicals were administered beginning on PND 44. In the Hershberger version (Hershberger *et al.,* 1953), the male rats (Holtzman-Rolfsmeyer) were castrated on PND 21 and administered the substances by daily ip injection for seven days, beginning on the day of castration. On the eighth day (postcastration), 22-26 consecutive hours after the last injection, the animals were sacrificed. The levator ani muscle, ventral prostate, and seminal vesicles (free of the coagulating glands) were dissected from each male and weighed. Dry weights of the levator ani muscle were also recorded after desiccation at 72°C. The results (organ

weights at various doses of each of many test materials) provided information on the relative potency of each test material for androgenic activity and/or myotrophic activity. The response of the levator ani muscle was termed the myotrophic response, and the response of the sex accessory glands was termed the androgenic response. Hershberger *et al.* (1953) reported that TP was the most potent for both myotrophic and androgenic responses, but that over the wide range of compounds tested, there was a "distinct lack of parallelism" between myotrophic and androgenic activities. Preliminary screening indicated that 19-norT and other 19-nor analogs of androgens were effective anabolic agents (*i.e.*, causing a myotrophic response) and relatively weak androgenic agents. Androsterone exhibited strong androgenic and weak myotrophic activity, and T exhibited strong androgenic and strong myotrophic activity. The subsequent identification of 5α-reductase activity potentiating T (by converting T to DHT, a more potent ligand of AR) in some tissues may explain the separable androgenic and myotrophic responses initially observed. Subsequent studies confirmed the usefulness (in terms of diagnostic capabilities, ease of use, cost, and time involved) of the assay (Deanesly and Parkes, 1936; Dorfman, 1962b; Dorfman and Dorfman, 1963; Dorfman and Kincl, 1963). A more detailed history of the assay is given in Chapter 5.

2.2.2 Versions of the Hershberger Assay

There are three principal versions of the Hershberger assay in rats currently in use. These include the castrated adult male, the peripubertal castrated male, and the prepubertal intact (weanling) male.

Castrated adult male

The initial Hershberger assay (Hershberger *et al.*, 1953), as it has evolved, employs male rats castrated at the age of 21 days. Seven to 14 days postcastration (so that T-dependent organs have involuted and regressed) or immediately after castration (so that T-dependent organs have not yet involuted or regressed), the rats are exposed to test chemicals or to vehicle control, usually by gavage, injection, or less commonly, dosed feed, daily for three to five days. Twenty-four hours after the last dose, the males are terminated and necropsied, and the sex accessory organs are weighed. If the sex accessory organs in the treatment groups are heavier than those in the vehicle control group, the test material is considered to be positive and is designated androgen-like. This version of the assay has clearly defined strengths and limitations. Its strengths are:

- With removal of the testes, the hypothalamic-pituitary-gonadal-end organ axis is disrupted, so the assay does not detect agents that act at the level of the hypothalamus, pituitary, or at nonreceptor-mediated levels. The ASO are dependent on T (or DHT) for their growth and maintenance, mediated by recognition of an androgen, and binding of the recognized molecule to the AR. Therefore, the positive response to a test material as evidenced by the increased weight of the sex accessory organs identifies the test material as not just androgen-like but as an androgen receptor agonist. Thus, the test is specific and identifies the single mechanism evaluated (AR receptor binding) by T or DHT (after 5α-reductase conversion of T to DHT), although there are possible confounders (see below).

- An anti-androgen (*i.e.*, an androgen antagonist) can also be detected if the castrated male is presented with exogenous T (usually TP), with and without the test material. If the test material is an antiandrogen, it competes with the exogenous T for binding to the AR but does not activate the down-stream genes to cause growth of the sex accessory glands. Thus, ~~I~~the diagnostic sign of an antiandrogen is that the sex accessory glands weigh less in the presence of T and test material than they do in the presence of T alone. If the male is castrated, the start of administration of the test material can be delayed and/or the duration of administration can be prolonged, allowing for flexibility (since there is no endogenous source of T).
- If there is a recovery period, so organ involution occurs (in the vehicle castrate vehicle control group), and the groups administered the test chemical exhibit increased growth and organ weights, then the test chemical is an androgen-agonist. The relative potency can be determined by the increase in organ weights in the treatment test chemical group, relative to the increase versus the organ weights in the reference androgen group. Another comparison is between the organ weights in the noncastrated controls versus the organ weights in the castrated animals administered test chemical.
- If there is no recovery period, the endpoint of interest is whether or not the ASO can maintain their precastration weight (compared to intact controls) in the presence of the test chemical. If so, the test chemical is an androgen agonist and the degree of maintenance (versus the group treated with the reference androgen) will determine the potency.
- If there is no recovery, and the castrate group treated with the test chemical and the reference androgen has lower ASO weights than in the castrate group treated with reference androgen alone, and lower organ weights than in the intact control group, then the test chemical is an androgen antagonist. The potency is based on the comparison of the ASO weights in the test chemical group versus the test chemical plus reference androgen group, and versus the intact control group.
- This assay has been shown to be a robust and specific assay. Based on the major protocol variables independently evaluated (Ashby and Lefevre, 2000a), and the OECD validation of the assay (Owens *et al.*, 2006; OECD, 2006, 2007a, 2007b), the use of different rat strains, use of the mouse, use of different diets, bedding, caging, light cycles, and animal room temperature and relative humidity does not affect the outcome as long as all the animals on a particular study are the same species and strain and are exposed to the same environmental conditions.

Limitations to the castrated adult male version of the assay are as follows:

- The assay is limited to detection of androgen agonists or antagonists (but see below). Due to castration, this version cannot detect agents that act upstream of the testis, such as at the level of central regulation, in the hypothalamus or pituitary. Also, as a consequence, it also does not detect agents that do not act

through AR-mediated mechanisms, such as effects on androgen synthesis (*e.g.*, phthalates), transport, metabolism, elimination, etc., although in the anti-androgen detection mode, metabolic modulators could be detected.

- The castrated adult male assay employs, by necessity, a postpubertal adult male. Given the evidence that younger animals may be more sensitive, possibly quantitatively (to test substances of lower potency) and/or qualitatively (with different effects) to EACs, the assay may not be the optimum choice to detect weak agents, agents at low (environmentally relevant) doses, and/or agents administered by different (environmentally relevant) routes of administration.

The Hershberger assay has been shown to be more sensitive and specific to AR-mediated alterations in the castrated adult male (or the other versions) than the assessment of endocrine activity in the intact adult male rat, which does not consistently detect the anti-androgenic activity of severe weak anti-androgenic pesticides (*e.g.*, 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene [*p,p'*-DDE]) and linuron (Cook *et al*., 1993; O'Connor *et al*., 1999, 2002). The above toxicants are easily detected in the Hershberger assay (Lambright *et al*., 2000; Parks *et al*., 2000; Yamasaki *et al*., 2003a) and in the weanling assay (Ashby *et al*., 2004). The assay is also capable of discriminating between T- versus DHT-dependent changes. Finasteride, a 5α-reductase inhibitor (which converts T to DHT), dramatically reduces the weight of DHT-dependent structures, such as the male ASO in the presence of T, but has little or no effect on the LABC muscle, which has low levels of 5α-reductase and is T dependent (Gray *et al*., 2004).

The procedures for this version include castration post puberty with a recovery period, so T-dependent (and DHT-dependent; see below) organs have involuted/regressed or no recovery period (so the male hormone-dependent organs have not yet regressed). Administration of the test chemical or reference androgen is by gavage or sc injection, typically for three to five days. Twenty-four hours after the last dose, the animals are terminated and necropsied and the ASO weighed.

Peripubertal castrated male

This version of the Hershberger assay employs sexually immature rats castrated at peripuberty by removal of the testes and epididymides on PND 42 (Gray *et al*., 2005). Gray *et al*. (2005) also recommended a 12-day recovery period, with initiation of treatment on PND 53-54 for ten consecutive days and necropsy the day after the last dose on PND 64-65. The late age at castration, plus administration of the reference androgen, allows the males to initiate and complete PPS since in Gray's experience, males castrated at a younger age do not initiate and complete PPS with or without in the presence or absence of an exogenous androgen.

The strengths include:

- The enhanced sensitivity of the peripubertal stage of sexual development to exogenous androgens, since both the AR and appropriate steroidogenic enzymes are present at this stage;

- The glans penis and other ASO display high sensitivity and small weights at this stage, which should minimize variation in responses across animals within groups (Gray *et al.,* 2005); the weight of the glans penis can also help distinguish between T- and DHT-affecting test chemicals.
- Since the males are castrated (*i.e.*, no endogenous source of T or DHT), the initiation and/or duration of dosing can be delayed and/or extended giving the assay more flexibility. Ten consecutive days of dosing are recommended by Gray *et al.* (2005).
- Since the animal is castrated, the hypothalamic-pituitary-gonadal axis is interrupted and the response of the ASO is absolutely dependent on the recognition of the test chemical by the AR, subsequent binding to the AR, and initiation of the downstream cascade of transcriptional activation of relevant genes, resulting in accessory sex gland growth (if the test chemical is an androgen agonist) or blockage of the initiation of the downstream transcription activation of relevant genes, resulting in no growth of the sex accessory glands or lesser growth in the presence of both the test chemical and the reference androgen (if the test chemical is an androgen antagonist).
- The use of younger animals (versus adults) may enable identification of the same effects at lower doses (*i.e.*, a quantitative difference) or additional or different effects (*i.e.*, a qualitative effect), reflective of the animals' stage of development. The internationally recognized standard for the Hershberger assay in peripubertal castrated male rats was developed and standardized within the OECD framework (Gray *et al.,* 2005; Owens *et al.,* 2006). This protocol included weighing the five T-dependent sex accessory glands separately (rather than as one unit) and weighing paired organs separately or as a pair as follows:
    - Seminal vesicles plus coagulating glands and their fluids
    - Ventral prostate
    - Bulbourethral (or Cowper's) glands
    - LABC
    - Glans penis

Limitations include:

- Test materials that act by an androgen-receptor mechanism will be detected. They have also exhibited the capability to address 5α-reductase inhibition in target organs. However, a test material that acts at the level of the hypothalamus, pituitary, and/or testis will not be detected.
- In most laboratory rat strains, such as the Sprague-Dawley, Long Evans, or Wistar, peripuberty occurs within the age range of five to seven weeks. In the CD® Sprague-Dawley rat at RTI, the historical control

grand mean of study means is 41.9 days of age for PPS in intact animals. This issue deserves additional consideration since the use of younger animals may capture additional effects not observed using the timing of castration and subsequent treatment(s) proposed by Gray *et al.* (2005) and employed by the OECD validation initiative (Owens *et al.*, 2006). Gray *et al.* (2005) strongly recommended castration on PND 42 to allow PPS to occur prior to castration. In their experience, PPS commonly did not occur or was not completed with rats castrated at younger ages and provided exogenous androgen.

The incomplete PPS makes the dissection of the glans penis problematic. After PPS is completed, the dissection of the glans penis is greatly improved. Please note that the onset of puberty and PPS may vary by strain and animal supplier (they clearly differ among rodent species *Rattus* versus *Mus*). Therefore, the timing of castration on PND 42 should be viewed with caution and PPS in intact animals should be evaluated prior to the Hershberger assay. There should be discussion and consensus on the acceptability of a study if the animals have not completed PPS (as has occurred in some cases employing anti-androgens and in one laboratory in vehicle control animals during the validation study [W. Owens, Procter and Gamble, personal communication as reviewer of the document]).

The Hershberger assay, as originally presented (Hershberger *et al*., 1953) used male rats castrated at weaning on PND 21, with treatment beginning on the day of castration (*i.e.*, no recovery period). Other researchers have also used animals castrated as weanlings (*e.g.*, Wakeling *et al*., 1981; Snyder *et al*., 1989) with variable lengths of the recovery period before beginning treatment.

Additional parameters include:

- Preputial glands (only after PPS has occurred)
- With the addition of other parameters, the Hershberger assay can be used to identify other targets or mechanisms:
- Liver weight and possible histopathology. This is to determine whether the test material is truly anti-androgenic, or whether the effects on the ASO are due to increased metabolism of TP (positive control material used in association with test material to detect androgen antagonistic or anti-androgenic responses) by the liver (resulting in increased liver weight and hepatocellular hypertrophy).
- Adrenal weight and possible histopathology. This is to determine whether there are effects of the test material on the adrenal.
- Kidney weight and possible histopathology. This is to determine whether there are effects of the test material on kidney structure or function.
- Obtain thyroid weight and possible histopathology. This is to determine whether there are effects of the test material on thyroid.
- Analysis of serum or plasma for T, DHT, follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH),

adrenocorticotrophic hormone, triiodothyronine (T3), and tetraiodothyronine (thyroxin; T4). A single blood collection at terminal necropsy will only provide the hormone status at that point in time. Hormone production and release are typically cyclical and change over time (*e.g.*, if T3/T4 levels drop, pituitary TSH levels increase to stimulate T3/T4 production once T3/T4 are at normal levels, TSH levels drop, etc.). It is suggested that blood samples be collected after castration and immediately prior to test material administration, and at terminal necropsy for all three versions of the protocol.

- Ashby *et al.* (2001) have suggested replacement of surgical castration (in Versions 1 and 3) by GnRH inhibition, the gonadotrophin-releasing hormone from the hypothalamus that initiates the pituitary-gonad-end organ cascade. A second suggestion is to use Leydig cell ablation. These cells are located in the testicular interstitium and synthesize T and DHT. The Sertoli cells (within the seminiferous tubules) should not/must not be affected. They produce insl3, which controls the formation and function of the gubernacular cords in males to result in testis descent in late gestation to the inguinal ring of the lower abdomen and in testis descent into the scrotal sacs in late lactation, and inhibin (a glycoprotein) which provides negative feedback on release of FSH from the anterior pituitary. FSH, in turn, binds to receptors on Sertoli cells which release factors for spermatogenesis. Sertoli and Leydig cells coordinate using FSH and T (both cell types have receptors for both hormones). These and other procedural variables will be further discussed in Chapter 6.0.

Prepubertal intact (weanling) male

A third study design for the evaluation of androgenic or anti-androgenic activity, described by Ashby and Lefevre (2000b), employs prepubertal, intact males. Immediately after weaning (PND 21 in rats and mice), the males are exposed to the test material or to the vehicle control during the postweaning prepubertal period. The endpoints of interest are the weights of reproductive (including testis and sex accessory organs) and possibly other organ systems (*e.g.*, liver). The timing and duration of the exposure period are critical and somewhat limited since natural puberty in the CD® rat begins soon after weaning, with control mean male CD® rat age at acquisition of PPS approximately 41-42 days (with no PPS-positive animals at 35 days of age), and the CD-1 mouse mean male age at acquisition approximately 26-28 days. Since the young males are not castrated, the hypothalamic-pituitary-gonadal axis is intact. It is considered as a valuable screen that will identify possible mechanisms at various organizational levels. The present performance and interpretation of this version of the assay provides recognition of its strengths and limitations. The OECD is currently evaluating this assay, and the OECD Validation Management Group reports that to date, this assay has proven equivalent to the peripubertal castrate model. The assay's strengths are as follows:

- Since the hypothalamic-pituitary-gonadal-end organ axis is intact, this assay can detect agents that act centrally as well as peripherally, act at the neuroendocrine (CNS-endocrine) interface, act via synthesis, distribution/ transport, metabolism, elimination, which act at the level of carrier proteins, etc., as well as those that act through AR agonism or antagonism.

- This assay can detect androgenic as well as anti-androgenic test materials depending on whether puberty is accelerated or delayed, and by whether the weights of the ASO increase or decrease relative to the vehicle control group values.
- This is a valuable screen. In practise, the assay responds identically to the castrate assay in detecting effects of androgens and anti-androgens. However, unlike the original Hershberger assay design, it evaluates a large number of endpoints, and several mechanisms/modes of action, and levels (*e.g.,* hypothalamus, pituitary, gonads, target organs, other organs, metabolites, etc.) in one assay.

The limitations are:

- This is a test that evaluates multiple endpoints, mechanisms, and levels, but it cannot, by itself, identify which mode(s) or mechanism(s) of action is (are) impacted.
- There is a relatively brief window of opportunity to evaluate effects on the developing reproductive system before natural puberty begins and confounds any effects from the test material. The animal must be weaned, so there is no confounding from the maternal organism (*e.g.*, nursing, behavior) or siblings, so the earliest start date is well defined. The tracking and recording of the exact ages in days at the start and end of dosing and at necropsy are imperative because of the rapid, natural changes in endpoints during this time of rapid growth and specific hormonal changes. The brief prepubertal window of opportunity is a concern, although Yamada *et al.* (2001) have reported satisfactory performance of the young adult male at PND 70, as have Kelce and Wilson (1997) for rat males out to PND 125 and Ashby and Lefevre (2000a,b) for rat males out to PND 126-127.
- Because the intact, young male is more sensitive than the castrated adult male to some agents, and because this assay involves developmental processes, the endpoints (age at acquisition of puberty, organ weights, etc.) can be affected by exogenous EACs in the bedding, feed, or water. For example, phytoestrogens in feed can cause delays in male acquisition of puberty and thus act like an anti-androgen and confound effects from the test material. Various phthalate esters interfere with T biosynthesis and could, as contaminants, also act as an anti-androgen and confound any effects from the test material. Characterization and analyses of the feed, water, and bedding, and/or use of the same environmental conditions for all groups can diminish the confounding of test compound-related effects. Perhaps the only real risks are the possibility that the presence of environmental confounders will produce a maximal response in the endpoints of interest, so no further response is possible and a false negative result will occur, or the possibility that endocrine effects from the contaminants in feed, water, bedding, etc., will be interpreted as a positive result from the test chemical (*i.e.*, a false positive).

2.2.3 Selection of the models for validation

The castrated peripubertal male and intact stimulated weanling assays are the versions OECD has chosen to validate and are basically equivalent to the weanling castrate model (Chapter 6.0). They can detect androgen agonists, androgen antagonists, 5α-reductase inhibitors, metabolic modulators (Ashby and Lefevre, 2000a,b). They are expensive and time consuming (they are *in vivo* animal assays, with treatment starting after weaning and ending before or at natural puberty [approximately ten days] for the intact model, and animals castrated on PND 42, a 12-day delay to get full involution of T/DHT-dependent tissues before onset of treatment for the castrate model, and a ten-day treatment period for all three versions to get full response), but they are straightforward.[1] Chapter 6.0 will provide a detailed description of the castrated prepubertal male Hershberger assay used by OECD in its standardization and validation program.

[1] The U.S. EPA EDSTAC proposed that screening assays be assessed against five attributes (EDSTAC Final Report, Volume I, Chapter 5, 1998). They should: (1) be inexpensive, quick, and easy to perform; (2) be validated and standardized as soon as possible (ICCVAM, 1997; Zeiger and Stokes, 1998), defining characteristics such as sensitivity and specificity against a "gold" standard once it is identified; (3) be more "sensitive" than they are "specific," meaning that they should have as their primary objective the minimization of false negative (or Type II) errors, while permitting an as-of-yet undetermined but acceptable level of false positive (or Type I) errors; (4) screening assays *in toto* should capture multiple endpoints and reflect as many modes of endocrine action as possible; and (5) screening assays *in toto* should be broadly predictive across species, gender, and age.

3.0 BIOLOGICAL BASIS OF THE ASSAY/ANDROGEN MODE OF ACTION

This chapter examines the biological role of the androgen hormones, beginning with steroid sex hormone biosynthesis, including the synthesis of T, its conversion to DHT which potentiates androgen action, and the conversion of T to E2 in the female ovary, male brain, etc. The role of the AR and the AR gene that controls the synthesis of the AR receptor is explored. Finally, the evidence presented in this chapter conclusively demonstrates the critical role of androgens in male sexual differentiation by discussing two aberrant phenotypes in both animal models and human that occur when the AR gene or the 5α-reductase gene is altered (so the protein product is altered), resulting in a spectrum of external and internal manifestations of androgen insufficiency.

3.1 Steroid Hormone Synthesis and Transport

3.1.1 Testosterone (T)

The reproductive system steroid hormones are produced primarily in the gonads, with some steroidogenic enzymative steps also found in peripheral tissues. In the male, the primary steroidogenic pathway is in the testes in the Leydig cells found in the interstitial spaces outside of the seminiferous tubules (and to a much lesser extent in the adrenal glands). In the Leydig cell, the steroidogenic pathway begins in the cytoplasm, with intermediate steps in the mitochondria, and then in the cytoplasmic smooth endoplasmic reticulum (Chen *et al.*, 1996). The Leydig cell T production is under feedback control from the HP portion of the HPG axis via circulating levels of LH produced by the anterior pituitary from stimulatory release of LHRH from the hypothalamus.

Control and synthetic pathway

For this chapter, the steroidogenic pathway to T will be considered as those processes occurring in the testes after stimulation of the hypothalamic-pituitary-testis axis. The pathway begins in the Leydig cell with: (1) intracellular signal transduction, (2) continues with cholesterol uptake into and production in the cytoplasm and its transport to the mitochondrial inner membrane, and (3) ends with a series of enzymatic conversions from cholesterol to T in the smooth endoplasmic reticulum of the testicular Leydig cell (and T to DHT or T to E2).

Signal transduction

LH from the anterior pituitary enters the Leydig cell by binding to the membrane-bound LH receptor. The LH receptor is coupled with a G-protein and, when stimulated (by LH binding), interacts with adenylate cyclase to form cyclic adenosine 3',5'-cyclic monophosphate (cAMP). Increased cAMP (termed the second messenger) stimulates protein kinase A, which initiates cholesterol biosynthesis and synthesis of the cholesterol transport protein (Cooke, 1996; Stocco, 1999). In order for maximal stimulation of steroidogenesis, intracellular calcium (Ca++) levels must increase after LH binding (Janszen *et al.*, 1976). The increase in intracellular Ca++ is due to release of Ca++ from intracellular storage depots and/or importation of extracellular Ca++ through cell

membrane-bound calcium channels. Calmodulin (a calcium binding protein) is also involved (Hall *et al.*, 1981). This series of events also enhances cholesterol transport into the mitochondrion. Chloride (Cl-) is also involved in steroidogenic signal transduction in the Leydig cell mitochondrion (Choi and Cooke, 1990). Chloride channels are present in the Leydig cell membrane, and chloride conductance is stimulated by LH and cAMP.

LH stimulation increases the release of arachidonic acid in the Leydig cell (Naor, 1991; Cooke, 1996). Arachidonic acid appears to act as an intracellular mediator with direct inhibition and indirect stimulation on steroidogenesis. Steroid hormone production is inhibited when arachidonic acid activates protein kinase C. It is enhanced by the metabolites of arachidonic acid (*e.g.*, leukotrienes) stimulating cholesterol transport into the mitochondria.

Other intracellular substances also affect steroidogenesis, including free radicals (*i.e.*, superoxide anion and hydroxyl-free radical), hydrogen peroxide, nitric oxide, molecular oxygen, and hydrogen peroxide (H_2O_2), which affects cholesterol transport and inhibits 3ß-hydroxysteroid dehydrogenase, an enzyme that converts pregnenolone to progesterone (see below and **Figure 1**; Clark *et al.*, 1993) and nitrous oxide (Davidoff *et al.*, 1995).

Cholesterol enzymatic pathway to T

Cholesterol from the serum is the common precursor to the synthesis of all gonadal steroid hormones. Cholesterol is transported to the Leydig cell by serum protein carriers (*e.g.*, high- or low-density lipoprotein). Once inside the cell, cholesterol is immediately utilized or stored in lipid droplets as cholesterol esters. A second minor source of cholesterol is *de novo* synthesis after hormone stimulation of the Leydig (or ovarian follicle) cells, beginning with acetate, which is converted to malonate, then squalene, and then lantosterol which is converted to cholesterol. LH-stimulated mobilization of stored or newly synthesized cholesterol occurs with cholesterol transported from the Leydig cell cytoplasm into the mitochondria and from the outer to the inner mitochondrial membrane with a transport protein. LH stimulation of steroidogenic cells activates rapid synthesis of the cholesterol transport protein (requires *de novo* protein synthesis and has a short t½ [half-life]). This essential protein mediates the rate-limiting step of steroid hormone production and is designated as the StAR (Steroid Acute Regulatory) protein. The StAR protein is synthesized in the cytoplasm as an inactive precursor molecule. It is transported into the mitochondrion where it is cleaved to its active form. In the mitochondrion, StAR protein transports cholesterol to the inner mitochondrial membrane where the series of enzymatic conversions of cholesterol to T take place. Steroidogenesis is therefore regulated by StAR protein production; the StAR gene is regulated by steroidogenic factor-1 (SF-1). SF-1 regulates the basal and hormone stimulated expression of the StAR gene. The effect of SF-1 is modulated by cAMP (so the signal transduction phase is linked to the control of the carrier protein). Other regulators of the StAR gene include estrogen, growth hormone, IGF-1, and calcium (which also up-regulates the StAR gene).

The StAR protein transports cholesterol from the outer to the inner mitochondrial membrane where the first cytochrome P450 enzymatic conversion takes place (see **Figure 2**).

Figure 1. Enzymatic Conversions of Cholesterol and Intermediate/End-Product Hormones

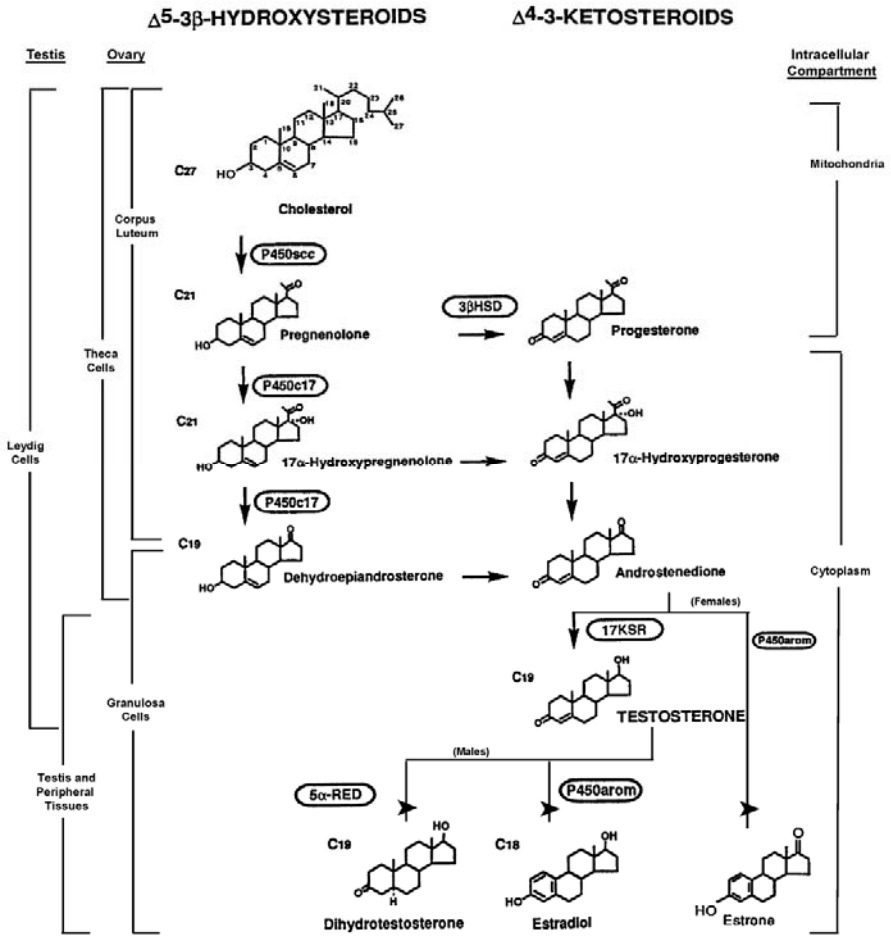
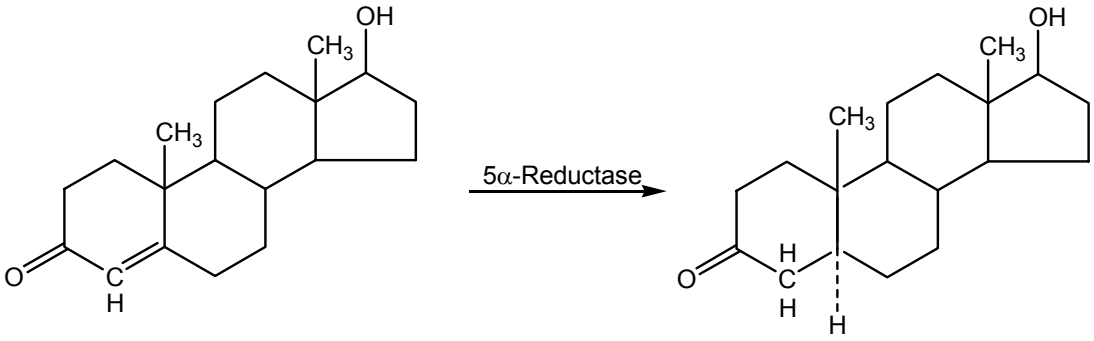


Figure 2. Transformation (Reduction) of T to DHT by 5α-Reductase



Testosterone
(17β–hydroxy-4-androsten-3-one)

5α-Dihydrotestosterone
(17β-hydroxy-5α-androstan-3-one)

Enzymatic conversions

The first enzyme reaction is the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc). This is also considered a rate-limiting step in the production of gonadal steroid hormones. This reaction on the inner mitochondrial membrane involves three oxidation reactions, each requiring molecular oxygen and NADPH. The reactions add two hydroxyl groups to cholesterol (one each at C_{22} and C_{20}), followed by cleavage between the added hydroxyl groups. Cholesterol (a 27-carbon steroid) is thereby cleaved of its 6-carbon group (the "side chain"), resulting in pregnenolone, a 21-carbon steroid (Kagawa and Waterman, 1995).

The second enzymatic reaction results in the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4-isomerase (3β-HSD). This reaction also occurs on the inner mitochondrial membrane. 3β-HSD catalyzes dehydrogenation and isomerization to convert a Δ^5-3β-hydroxy steroid (pregnenolone) to a Δ^4-3-ketosteroid (progesterone), the active form of steroid hormone. Pregnenolone may also be converted to progesterone by 3β-HSD in the cytosol. Therefore, the steroidogenic pathway bifurcates into a Δ^5-hydroxysteroid pathway, starting with pregnenolone and a Δ^4-3-ketosteroid pathway, starting with progesterone. Even though the same enzymes participate in both pathways, using different substrates along parallel pathways, both pathways ultimately converge to form androstenedione. 3β-HSD converts the Δ^5-3β-hydroxysteroid pathway substrates, 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) into their respective Δ^4-ketosteroids, 17α-hydroxyprogesterone, and androstenedione, respectively (see **Figure 1**).

The third enzymatic reaction involves cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17). This enzyme catalyzes two chemical reactions, hydroxylation and cleavage (to convert a 21-carbon steroid to a 19-carbon molecule), requiring molecular oxygen and NADPH. The products after the initial hydroxylation step are considered intermediates. Therefore, in the Δ^5-hydroxysteroid pathway, P450c17 initially converts pregnenolone to 17α-hydroxypregnenolone and then to DHEA. DHEA is then converted to androstenedione by 3β-HSD. In the Δ^4-ketosteroid pathway, P450c17 converts progesterone to 17α-hydroxypregnenolone which is then converted to androstenedione. The lyase activity of P450c17 differs among species for the intermediate substrates. For example, in humans P450c17 converts 17α-hydroxypregnenolone to DHEA (Δ^5-hydroxy steroid pathway) but not 17α-hydroxyprogesterone to androstenedione (Δ^4-ketosteroid pathway), while in rats, P450c17 converts the intermediates of both the Δ^5-hydroxy steroid and Δ^4-ketosteroid pathways equally.

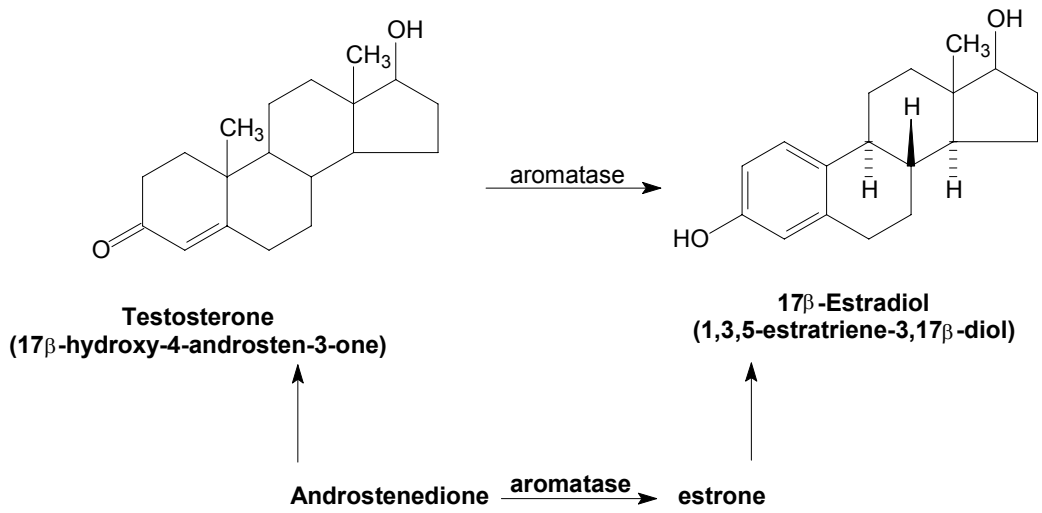
The fourth enzymatic reaction involves the conversion of androstenedione to T by 17-ketosteroid reductase (17KSR), also designated as 17β-hydroxysteroid dehydrogenase (17β-HSD). In the female, in the follicle cells, androstenedione is then converted to estrone by aromatase. The conversion of androstenedione to T is reversible, dependent on product concentrations. 17β-HSD (17KSR) can catalyze both the reduction (forward)

or the oxidation (reverse) reactions. NADH/NAD+ are co-factors in this interconversion. In the male, T is converted to DHT by 5α-reductase (see section below).

The fifth step in the steroidogenic pathway proceeds along one of two paths in males: the production of DHT or estrogen. 5α-reductase converts T to the more potent DHT (**Figure 2)** which binds more avidly to the A. The enzyme is found in cellular membranes, nuclear membranes, and endoplasmic reticulum. DHT is produced primarily in peripheral (target) organs, but it is also found in the testis and plays a dominant role in specific regions of the male reproductive system development (urogenital sinus and external genitalia) *in utero*. See sections 2 and 3 in this chapter for details on the synthesis, functions, and locations of DHT.

The other path that operates in both males and females is the conversion of T into E2 (**Figure 3**) and androstene into estrone (in females) by the aromatase enzyme. Aromatase is present in many peripheral tissues and in male and female gonadal tissue. Although aromatase does exist in the male (*e.g.,* in the brain), it is found only at very low levels in the Leydig cells, so that male Leydig cell steroidogenesis ends with T and does not proceed to E2. It is an enzyme complex (two cytochrome P450 enzymes: a reductase and an aromatase) bound to the endoplasmic reticulum. This complex catalyzes two hydroxylation steps and the aromatization of Ring A of the steroid nucleus, which results in the loss of the C-19 carbon atom, producing a C-18 molecule characteristic of estrogens. The reaction requires molecular oxygen and NADPH.

Figure 3. Conversion of Testosterone to 17β-Estradiol



Production and activity of these steroidogenic enzymes are under hormonal control. First, the gonadotrophin control: P450 scc and P450c17 enzymes, are regulated by pituitary LH. In males, pituitary FSH stimulates release of a Sertoli cell factor that increases the effect of LH on 3β-HSD activity to increase T production. In females, FSH increases the activity of aromatase to increase estrone production. FSH binds to receptors on Sertoli cells that release and metabolize factors required for spermatogenesis. FSH increases the number of LH receptors in the testis, which in turn increases T induction and testis growth. Sertoli cells have receptors for FSH and T for coordination between the Sertoli and Leydig cell population in the testis. Next, Sertoli control: Sertoli cells also produce a glycoprotein, inhibin, which provides negative feedback on the release of FSH from the anterior pituitary. Finally, the gonadal hormones themselves: gonadal hormones also provide negative feedback to the steroidogenic enzymes. T inhibits P450c17 activity by acting on the second messenger cAMP pathway. T also suppresses 3β-HSD through inhibitory effects on cAMP-mediated 3β-HSD mRNA.

The endproducts in steroidogenesis are considered T, DHT, E2, and estrone.

Locations

Sites of steroidogenesis. Reproductive system steroid hormones are produced primarily in the gonads (testes and ovaries), although some of the steroidogenic reactions are also found in peripheral tissues (*e.g.*, the adrenal glands, placenta). Other active androgenic hormones are produced in the testes and in peripheral tissues. Other peripheral tissues are also involved in T's role as a prohormone. For example, T is converted into E2 in the liver, testis, and brain (in the male hypothalamus, to determine male-specific behaviors) and is converted to DHT in the testis, liver, brain, prostate, and external genitalia. T is converted to androsterone or etiocholanolone in the liver; it is glucuronidated in the liver for excretion and converted to androstenedione in the testis and liver (Federman, 1981).

Aromatase, which converts T into E2 in males and females, is found not only in male and female gonads but also in many different peripheral tissues.

Sites of androgenic/anti-androgenic activity. Steroid hormones are carried in the circulatory system by steroid hormone binding globulin (SHBG), α-fetoprotein, albumin, etc., in perinatal rats and humans. Sites (*e.g.*, cells) with androgen-dependent activity have ARs that recognize and bind T and translocate it to the nucleus where the receptor with bound T interacts with genes to turn on or turn off specific gene expression (see later in this chapter). The cells of the embryonic anlagen (precursors) of the male reproductive tract (*e.g.*, Wolffian ducts) and the embryonic external genital folds have receptors for T and/or DHT that appear at the appropriate times to initiate hormone-dependent gene activation and structural and functional developmental changes.

At puberty in males, T and its receptors are ubiquitous in the testes, ASO, muscles, hair follicles on the face, back, underarms, pubic area, etc., skin (adolescent acne), larynx (male voice changes), growth zones in the long bones (male growth spurt), sweat glands, external genitalia, adrenal glands (glucocorticoid pathway), etc. Interference at the AR will impact all subsequent androgen-dependent activities (see later in this chapter). Interference with steroidogenesis by genetic, endogenous, or exogenous (environmental) chemical exposure will interfere with T biosynthesis and, depending on the timing of the interference, affect male internal and external sexual development, onset of puberty, etc. Effects at the level of the CNS hypothalamus and pituitary can also impact T biosynthesis.

Masculinization is an androgen-driven process, beginning under the influence of hCG, which stimulates the Leydig cells of the fetal testes to produce T (Moore *et al*., 2003). T is then converted to the more active DHT by the enzyme 5-α reductase type II. For DHT to effect masculinizing action on the developing genitalia, it must bind to ARs located in the genital tissues (G. Yamada *et al*., 2003). Cellular signaling through the ARs must be intact (Cunha and Baskin, 2004).

3.1.2 Dihydrotestosterone (DHT)

DHT, locally produced from T by the enzyme Δ^4-steroid 5α-reductase (**Figure 2**), stimulates normal differentiation and development of the urogenital tubercle, urogenital sinus, and urogenital swellings *in utero* into the urethra, prostate, and external genitalia. The growth of the perineum (anogenital distance) and apoptosis and regression of the ventral nipple anlagen in males *in utero* are also DHT-dependent processes.

Synthesis of DHT

5α-Reductase. In the male, T is converted to DHT by 5α-reductase, which is found in cellular membranes, nuclear membranes, and endoplasmic reticulum. DHT is produced primarily in peripheral tissues (at target organs), but it is also found in the testis. The activity of 5α-reductase in the Leydig cells and testes varies with age; the highest activity occurs perinatally. Genetic differences in 5α-reductase occur. In a defect for 5α-reductase activity, abnormal (reduced) masculinization is localized *in utero* to the urogenital sinus and external genitalia (since their differentiation and growth are mediated by DHT, not T). T and E2 blood levels are normal, because aromatase directly converts T to E2 (DHT is not involved). This deficiency in the male is characterized at

birth by a blind vaginal pouch, testes, and upper tract ASO (under T control), no enlarged breasts, no internal female genitalia, and masculinization at puberty when a surge in T results in differentiation and growth of external genitalia (see Androgen Insensitivity Syndromes (AIS), later in this chapter).

Locations and functions. Using a 5α-reductase inhibitor, Imperato-McGinley *et al.* (1985) developed a male pseudohermaphrodite rat which was subsequently used to examine development of various sex-specific structures and functions, such as nipple retention in males (Imperato-McGinley *et al.*, 1986), testis descent (Spencer *et al.*, 1991a; Turner *et al.*, 2002), and prostate and genital differentiation (Imperato-McGinley *et al.*, 1992). Other workers have used finasteride, a specific 5α-reductase inhibitor, to distinguish between T-mediated and DHT-mediated effects (Blohm *et al.*, 1986; Bowman *et al.*, 2003), and determined critical developmental periods for effects of low or no DHT on male rat genitalia (Clark *et al.*, 1990, 1993), on the rat gubernaculum (George, 1989), on androgen physiology in the immature rat (George *et al.*, 1989), and on embryogenesis of the rat prostate (George and Peterson, 1988). Early on, it was noted that there were differences in the amount and level of conversion of T to DHT in the prostate (now known to be DHT dependent), versus the epididymis (now known to be T dependent) (Gloyna and Wilson, 1969). The role of T and DHT in sexual ducts and the genital tubercle of rabbit fetuses was assessed during sexual organogenesis, with and without fetal decapitation (to assess the role of the hypothalamus and pituitary in *in utero* sexual development (Veyssiere *et al.*, 1982). Additional work on T versus DHT effects and targets has been done in the rat fetus (Berman *et al.*, 1995), and in both rat and rabbit fetuses (Wilson and Lasnitzki, 1971).

3.2 Androgen Receptor (AR)

There are three major classes of receptors to which hormones bind: (1) receptors found on the surface of cells (to which peptide hormones bind); (2) receptors found in the cytoplasm of cells (to which the steroid hormones bind); and (3) receptors found in the nuclei of cells (to which the thyroid hormones bind). Moreover, there are two major mechanisms of hormone action: (1) activation of plasma membrane receptors by hormone binding to transport the hormones into the cell (used by catecholamines, peptides, and protein hormones); and (2) activation of intracellular receptors to transport the hormones into the nucleus for DNA binding (used by steroid and thyroid hormones).

A vast array of receptor proteins and genes are associated with cells that may contain ~10,000 protein receptors for a single steroid hormone. As many as ~50-100 genes within a cell may be controlled by the binding of a single type of hormone to the various cellular receptors. Some genes are also affected by more than one receptor hormone complex.

3.2.1 AR gene and structure

The gene for the AR, recognizing both T and DHT, is localized on the X chromosome, with the human genes homologous to the mouse testicular feminization (Tfm) gene (Migeon *et al.*, 1981), in a highly conserved region of the X chromosome, found in monotremes, marsupials, and eutherian mammals (Spencer *et al.*, 1991b), and localized to Xq11-12 on the human X (Brown *et al.*, 1989). The absence of T, or of

appropriate receptors that recognize T or DHT, can result in full or partial AIS in humans and many other mammals (Quigley *et al.*, 1995, 2004). The newborn genetic male (XY) with AIS presents as an external female with mid-abdominal testes (triggered by the SRY gene [male determining] on the Y chromosome) and no ASO (T is made in the testis, but it is not recognized by the altered ARs in the target structures, so male ASO are not induced). Partial AIS males present with a range of external genital phenotypes that vary from near-normal female to normal or near-normal male, with or without gynecomastia (feminization of male mammary gland development), and relatively "mild" signs of under virilization (Gottlieb *et al.*, 1998). Somatic mutations in the AR have been found in advanced prostate cancer (Culig *et al.*, 1993); the somatic mutations in the AR on the X chromosome are found in cells of specific tissues (*i.e.*, the prostate), but are not in all tissues from a germline mutation (in the X chromosomes of either the sperm or ovum). The tissue distribution of the AR message or protein is presented in **Table 1**.

The AR is a member of a group of four closely related steroid receptors (the "GR-like receptors"), including the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), and the progesterone receptor (PR). This group is related by sequence homology and by the ability to activate target gene transcription by the same hormone response element (HRE). It comprises a subfamily of a larger family of nuclear transcription factors that includes the ER, thyroid hormone receptors (TRα and TRβ), vitamin D receptor, retinoic acid receptor, and a number of other related receptors. The AR, as a hormone-receptor complex, interacts directly with its target genes to regulate their transcription (as do the other members of this receptor family). Failure of the receptor to activate its target genes in the presence of hormones results in target organ resistance to the hormone.

Table 1. Tissue Distribution of AR[a]

What	Method of Detection	Where Localized	Species
AR mRNA	Northern blot analysis	Testis, prostate	Human
		Genital skin fibroblasts	Human
		Prostate and human breast cancer cell lines	Human
		Liver	Human
		Kidney, brain, epididymis	Rat
		Anterior pituitary and other neural tissues	Rat
		Lacrimal gland	Rat
		Smooth muscle cells of penis	Rat
		Kidney and liver	Mouse
		Larynx of male	Xenopus
AR protein	Immunoblot (Western blot) analysis	Genital skin fibroblasts	Human
		Penis	Rat
AR protein	Sucrose density gradient analysis	Gubernaculum	Rat
		Urogenital sinus	Rat
AR protein	Immunohistochemistry	Nuclei of glandular epithelial cells of prostate	Human and rat
		Epididymis	Rat
		Ductus deferens	Rat

		Ventral prostate	Rat
		Seminal vesicles	Rat
		Hypothalamic nuclei	Rat
		Anterior and posterior pituitary	Rat
		Epithelial cells of lacrimal gland	Rat
		Somatostatin-producting neurons of hypothalamus	Rat
		Cultured foreskin fibroblasts	Normal and AIS men
		Testis	Rat, mouse, guinea pig
		Prepubertal testis	Human
AR protein	Immunohistochemistry	Ovary	Human, monkey, rat
		Testis: Sertoli cells, peritubular myoid cells, interstitial cells, and elongated spermatids	Rat
		Ovary: granulosa, theca, and luteal cells	Rat
AR	Immunohistochemistry of paraffin-embedded tissues	In nuclei of sweat glands, hair follicles, cardiac muscle, vascular and gastrointestinal smooth muscle cells, thyroid follicular cells, renal cortical cells	Human

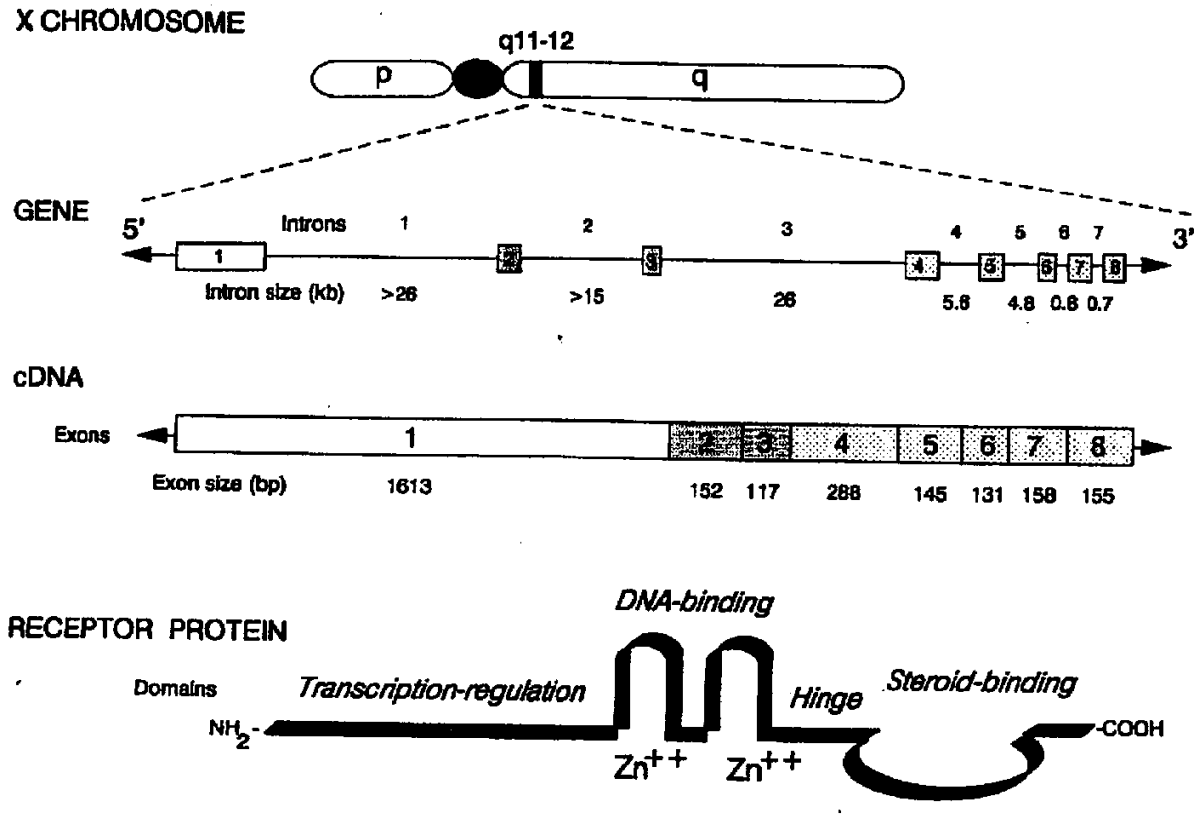
[a] Modified from text in Quigley *et al.*, 1995

The AR gene and its encoded protein are structurally and functionally similar to those of the other steroid receptors. The AR gene is a single copy X-chromosomal gene that spans 75-90 kilobases (Kb) of genomic DNA in the pericentromeric region of the long arm of the chromosome at Xq11-12. Its protein coding region (~ 2757 base pairs [bp] open reading frame) comprises eight exons (designated 1 through 8 or A through H), separated by introns from 0.7 to more than 26 Kb. At the 5' end of the gene is exon 1, containing a CAG triplet repeat region of average length of 21±2 repeats, with a range of 11-31 in a normal mixed-sex population, and a range of 14-35 in a group of unselected males. This repeat region is highly polymorphic; there are 20 different allele sizes, with 90% of females heterozygous for the size of this repeat at this locus. There are also racial differences, with the most frequent allele size (mode) equal to 18 in the African American population and 21 in whites.

The principal site of transcription initiation (designated TIS 1) in the AR gene is an adenosine residue located approximately 1100 bp from the 5' end of the translation-initiating methionine codon. A second transcription start site (TIS II) is 11 nucleotides downstream from TIS I). TIS I is used in all tissues and cell lines to date; the role of TIS II is not yet known (Quigley *et al.*, 1995). The AR promoter is like that for the PR gene promoter in that it does not contain a typical TATA or CAAT box, but does contain GC-rich elements, including a binding site for the common mammalian transcription factor SPI (Tilley *et al.*, 1990), a homopurine sequence (Baarends *et al.*, 1990), and a cAMP response element (Lindzey *et al.*, 1993), as well as binding sites for a number of other transcription factors. The minimal region of the human AR gene promoter necessary for activity includes nucleotides -74 to +84 surrounding the TIS 1 (Takane and McPhaul, 1994), but Song *et al.* (1993) have identified a more distal 5'-promoter/enhancer region of the rat AR gene at nucleotides 96-940, upstream of the transcription start site, which is critical for the activity of the rat AR gene promoter, and binds another (yet unidentified) transcription factor. A bipartite promoter element is also present, which binds two protein factors and may confer age sensitivity of hepatic AR expression (Supakar *et al.*, 1993). Another region in the rat AR gene promoter at -754 to -551 nucleotides has been identified as a repressor element by binding nuclear factor KB, which in this context functions as a negative regulator of AR gene transcription (Supakar *et al.*, 1994). A number of half-palindromic potential HRE-binding sites for AR, GR, and PR have been found within the rat AR promoter region (Song *et al.*, 1993), and one half-site for ER has also been reported (Song *et al.*, 1993). Such sequences may be important for steroid hormone regulation of AR mRNA expression. The structure of the AR gene is presented in **Figure 4**.

Regulation of the AR is age-, time-, and cell- or tissue-type dependent, and occurs at both the transcriptional (DNA to RNA) and translational (RNA to protein) levels. In addition, AR protein and mRNA have contrasting responses to androgen(s), even within the same cell type. At the protein level, androgens stabilize the receptor in transfected cells in culture by reducing protein degradation. This may account for the higher receptor levels in male versus female fetal rats and in rats exposed perinatally to high androgen levels. In contrast, the overall effects of androgens at the mRNA level are down regulation.

Figure 4. Structure of the AR Gene



Modified from Quigley *et al.*, 1995; see text for explanations

Androgen withdrawal by chemical or physical castration results in an increase in rat AR mRNA, which is reversed by androgen replacement. Down regulation of other steroid receptor mRNAs results from a decrease in transcription rate and/or decreased half-life of the receptor mRNA. By analogy with the mechanism of GR gene repression, it is likely that in the AR, sequences within the DNA-binding domain are required for receptor-mediated gene repression and mediate AR autoregulation. The Tfm (testicular feminization) rat (caused by a single amino acid change in the steroid binding domain of the AR) exhibits disrupted AR function, but not by the normal androgen-dependent down regulation of AR mRNA as in the wild-type animal. Hormones other than androgens have also been found to regulate AR in various cell or tissue types (*e.g.*, FSH up regulates AR mRNA and protein in Sertoli cells through cAMP, and epidermal growth factor [EGF] down regulates AR mRNA in LNCaP prostate cancer cells).

Mechanism of action

Androgens are transported in the blood supply, largely bound to carrier proteins (*e.g.*, SHBG, α-fetoprotein [AFP], albumin). When they reach target tissues, they dissociate from the carrier proteins, diffuse into target cells, and bind to the intracellular AR protein.

Androgen binding induces a conformational change in the AR that facilitates receptor dimerization, translocation to the nucleus, interaction with target DNA, and then regulation of target gene transcription. A variety of androgens and other steroids bind to AR. ARs have the highest affinity for DHT; less for T (primarily because T dissociates from the AR more rapidly than DHT; Wilson and French, 1976).

Androgen binding induces a change in the AR that converts the inactive receptor into its active DNA-binding state. As with other steroid receptors, hormone binding results in the removal of certain receptor-associated proteins, such as the 90 DKa (kilodaltons) heatshock protein. Removal of these proteins unmasks functional domains and initiates the conformational changes necessary for nuclear import, dimerization, and DNA binding. Receptor activation is ligand specific; functional activity of the receptor correlates with the binding activity of the ligand. Androgen binding stabilizes the receptor, which results in higher levels of receptor and higher levels of binding receptor phosphorylation, a post-translational process common to steroid receptors and other transcriptionally active proteins. It is enhanced in the presence of androgens (because of the higher AR protein levels; see above). However, the AR is phosphorylated in both the presence and absence of hormone, and both the phosphorylated and unphosphorylated forms of the AR bind androgens with high affinity; the precise role of (reason for) receptor phosphorylation is not yet clear.

Binding of androgen to the AR is necessary for DNA binding and subsequent transcriptional activity. In the absence of androgens, the steroid binding domain acts as a repressor of AR transactivation regions, likely due to an inhibitory conformation adopted by the unliganded AR protein (Quigley *et al.*, 1995).

The AR is believed to be cytoplasmic when there is no androgen bound. Once bound by androgens, the AR is clearly nuclear. Intranuclear, hormone-activated AR binds as a homodimer to the hormone response elements (HREs) of target genes and their flanking DNA. The binding of the receptor to its HRE regulates the rate of transcription of target genes through interactions with other components of the transcription complex near the transcription start site of the gene. *In vitro*, AR binds to a simple HRE consensus sequence (a 15 pb partial palindrome 5'-AGNACAnnn-TGTNCT-3'), which is also bound by GR and PR. This overlap in activity among AR, PR, and GR at certain HREs may allow for synergistic actions of androgens and glucocorticoids. In addition to acting through the simple consensus HRE, the AR also acts through selective androgen-specific complex androgen response elements (AREs). These AREs are comprised of a number of interacting elements, including the HRE itself, and recognition sequences for other transcriptional control factors. Enhancement of AR and GR binding to HREs occurs in the presence of an accessory protein identified as insulin degrading protein. This interaction may represent some type of relationship between insulin and androgen signaling.

3.2.2 AR protein and structure

The AR gene encodes a receptor protein of 110-114 KDa molecular weight, comprising 910-919 amino acids. It is a single polypeptide (like other steroid receptors) with discrete functional domains: a transcription-regulating, amino-terminal domain (corresponding to the 5' end of the gene), a DNA-binding domain, a hinge region, and a

steroid-binding domain ending at the carboxy-terminal end of the protein (corresponding to the 3' end of the gene) (see **Figure 4**).

The amino-terminal domain

This large domain encoded by exon 1 comprises more than half of the AR protein (amino acid residues 1-537). This domain is the least homologous in sequence and the most variable in size among members of the steroid receptor family. It contains a transactivation (transcription activation) domain between amino acid residues 141 and 338. There may also be other subregions for regulation of target gene transcription. This domain may also be involved in the establishment and maintenance of the three-dimensional structure of the AR molecule through interactions with other regions of the protein. This domain (in humans, rats, and mice [so far]) also contains a number of homopolymeric amino acid stretches not present in other members of its subfamily of steroid receptors. The initial amino acid stretch (at the amino terminus of the protein) is a polyglutamine stretch (approximate amino acid residues 58-79 encoded by the polymorphic CAG triplet repeat region in the gene) of average length of 21±2 glutamine residues. A shorter stretch of nine proline residues at amino acid residues 372-379 is further downstream and there is another polymorphic stretch of approximately 24 glycine residues at amino acid residues 449-472 closer to the DNA binding domain (see **Figure 4**). The polyglutamine and polyproline stretches are likely important in transcriptional regulation by protein-protein interactions with other transcription factors (Gerber *et al.*, 1994), or to maintain the three-dimensional conformation of the protein.

The DNA-binding domain

This central cysteine-rich region of the AR (amino acid residues 538-627), encoded by exons 2 and 3, contains the DNA-binding domain (amino acid residues 559-624). In GR and ER, this region is arranged as a pair of looped structures folded to form a single structural unit made up of two zinc-binding motifs (see **Figure 4**). Four cysteine residues, present in all steroid receptors, coordinately bind a zinc cation in each of the two motifs ("zinc fingers"). The first zinc finger (residues 559-579) is encoded by exon 2, and the second zinc finger (residues 595-619) is encoded by exon 3. The amino acid sequence of this domain is the most highly conserved region among the members of the steroid receptor family, with approximately 80% homology (amino acid identity) with those of MR, PR, and GR (Freedman, 1992). The DNA binding domain determines the specificity of AR (and of other steroid receptor(s)) interaction with DNA. Three amino acids at the base of the first zinc finger (glycine 577, serine 578, and valine 581) are identical with those in GR, PR, and MR and interact with transcriptional enhancer nucleotide sequences (HREs) present in or near target genes, usually in the 5'-flanking region, and appear critical for recognition. The second highly basic zinc finger stabilizes DNA-receptor interactions by contact with the DNA phosphate backbone and mediates receptor dimerization. At the carboxy-terminal end of this AR domain is an arginine-lysine pair that is part of the nuclear targeting sequence (see *The hinge region* below).

The hinge region

The hinge region is located between the DNA-binding domain (*vide supra*) and the steroid binding domain (*vide infra*). It has low sequence homology between AR and other steroid receptors. This hinge region is encoded by the 5'-portion of exon 4 and

contains the major part of the AR nuclear targeting signal, comprised of a cluster of basic residues at positions 629-633: arg (arginine), lys (lysine), leu (leucine), lys, lys. This region mediates the transfer of AR from the cytoplasm to its site of action in the nucleus.

The steroid-binding domain

The carboxy-terminal third of the AR, encoded by the 3'-portion of exon 4 and exons 5 through 8, is the steroid-binding domain at residues 670-919. Sequence homology in this region is approximately 50% among the AR, GR, MR, and PR. This region has four functions: (1) specific, high affinity binding of androgens (a principal function); (2) the binding site for inhibitory proteins such as the 90 DKa heatshock protein; (3) role in other receptor functions, including dimerization; and (4) a transactivation region (to support transcriptional activation) within this domain.

3.3 Mechanisms of Action of Androgens and Role in Fetal Masculinization

Androgens and estrogens are essential for the initial development of the reproductive system *in utero,* for the maturation of the reproductive system and ASO, and secondary and tertiary sex characteristics at puberty, and for the maintenance of the reproductive system and ASO structures and functions until death (in males) or menopause (in females). Androgens and estrogens are also needed for feedback regulation of the hypothalamus-pituitary-gonadal axis and spermatogenesis in males (and oogenesis in females).

T has two fundamental and different roles at two different life stages:

- The essential and irreversible role of T in the formation of the male reproductive tract (internal and external) *in utero*; and
- The role of T in the pubertal development of the male into a reproductively competent individual (with secondary and tertiary male characteristics) and sustaining those male structures and functions through adulthood; this role is reversible (with the removal of T

T is responsible for testis development and descent, maturation of the epididymides, vas efferens, vas deferens, seminal vesicles and coagulating glands, LABC muscle, and PPS at puberty in rodents.

DHT is responsible for development of the male urethra and prostate, formation of the penis and scrotum, and male secondary sex characteristics such as scrotal growth, development of scrotal rugae and pigmentation, and penis growth and development. There are obvious consequences in structures and functions from a reduction or increase in T production; the type of change and extent and location of the change are all dependent on the timing and direction (increase/decrease) of the alteration in hormone levels.

In pioneer experiments on rabbit fetuses, Jost (1953) demonstrated that even with surgical removal of the gonads in male and female rabbit fetuses *in utero*, female organogenesis (*i.e.*, Müllerian duct stimulation to form oviducts and uterus, and Wolffian duct inhibition to prevent development of male internal reproductive structures) still occurred in all fetuses regardless if they were XX or XY, in the absence of T, DHT, or

E2. Jost (1953) inferred that male sexual differentiation is imposed upon the natural tendency of the fetus toward femaleness. Normal male sexual differentiation requires the production and secretion of two factors by the testis; T (and DHT) to stimulate development of male structures, and Müllerian inhibiting substance (MIS) to prevent formation of female structures. In human male fetuses, at the time of sexual differentiation *in utero*, DHT formation occurs in the urogenital sinus, urogenital tubercle, and urogenital swellings. However, DHT formation does not occur in the Wolffian anlagen until after differentiation has occurred, which suggests that DHT and T have specific roles in male reproductive development (Siiteri and Wilson, 1974).

Locations

In the mammal (rat, mouse, rabbit, and human), fetal androgen production during gestation is required for normal male sexual differentiation. T is necessary for proper development of the testes (which is the site of T synthesis in the Leydig cells), as well as the stabilization and differentiation of the Wolffian ducts into the epididymides, vasa efferentia, deferentia, and seminal vesicles

3.3.1 Primary sex determination (Y chromosome)

Primary sex determination is chromosomal; the male is XY and the female is XX. The Y chromosome carries a SRY gene that encodes a testis-determining factor. If the conceptus is XY, in the presence of the testis-determining factor, the indifferent gonads will form testes in which T will be produced, and the prenatal infant will form male internal and external sexual organs. If the conceptus is XX, in the absence of the testis-determining factor, the indifferent gonad will form an ovary in which E2 will ultimately be produced, and the prenatal infant will form female internal and external sexual organs or structures. Internal and external female structures will form even in the absence of gonads (gonads removed when they are still indifferent or due to a genetic lesion).

Jost and coworkers at the Laboratoire de Physiologie Comparée, Faculté des Sciences, Paris, France in the late 1940s to late 1950s, and Friedmund Neumann, Allen Goldman and coworkers at Schering AG, Berlin, West Germany in the late 1960s and 1970s, demonstrated almost every aspect of the anti-androgenic phenotype in rats (*e.g.,* Goldman and Neumann, 1969; Neumann and Goldman, 1970; Neumann *et al.,* 1970; Goldman and Baker, 1971; Goldman *et al.,* 1973; Goldman and Klingele, 1974; Goldman *et al.,* 1976) and demonstrated the hormonal controls in the somatic sexual differentiation of the mammalian male fetus (*e.g.,* Jost *et al.,* 1970 and below) and the relative independent sexual differentiation of the female fetus.

Jost (1947, 1953, 1961, 1965, 1971) performed a series of elegant experiments on rabbit fetuses *in utero* in which they were exteriorized from the uterus, surgically altered on GD 19, 20, or 21, and allowed to completed development within the maternal abdominal cavity until GD 28 or through parturition to puberty. When the fetal females were "castrated" (i.e., ovaries removed) on GD 21 and examined on GD 28, they acquired normal female internal and external structures, with the Műllerian derivatives "somewhat" reduced in size relative to the control litter mates when the females were castrated on GD 23. There were no differences between female castrates and controls for internal or external reproductive structures (excluding the ovaries). Jost (1947, 1953) concluded that ovariectomy does not suppress female reproductive organogenesis. For

male fetuses, the consequences depend on when the castration was done (*i.e.,* before [GD 19] or at the beginning [GD 20] of somatic sexual development versus castration during [evaluate at one-day intervals between GD 19 and 24] somatic sexual development and examination on GD 28).

Early castration in male fetuses resulted in complete regression of Wolffian ducts, no Wolffian structures, and no prostate present. Castration on GD 20 resulted in two rudimentary prostatic buds. The urogenital sinus and external genitalia were female, since the Műllerian ducts persisted and differentiated into the uterovaginal tract. Unilateral ovariectomy before GD 21 in rabbit males results in normal male urogenital sinus, its derivatives, and the external genitalia (Jost, 1953). Results of rabbit male fetuses, castrated between GD 19-24 and examined on GD 28, are presented in **Table 2**. Overall "seminal" conclusions include:

- Female reproductive organogenesis (internal and external structures) proceeds in the absence of ovaries in female fetal rabbits and in the absence of testes in male fetal rabbits (**Table 2**).
- Female reproductive structures are the default state.
- Male reproductive organogenesis requires a factor from the embryonic testis. If the primordia are removed before embryonic/fetal differentiation, a phenotypic female results. If they are removed during embryonic/fetal gonadal differentiation, transition stages are observed, and if they are removed at the end of gonadal differentiation, a phenotypic male results (**Table 2**).
- Unilateral castration in the male rabbit fetus results in a male phenotype with unilateral (the side of castration) "peculiarities," including reduction or loss of the Wolffian duct and persistence of small sections or of one complete uterine horn (Jost, 1953).
- A secretion from the developing testis is required for male reproductive organogenesis *in utero*.
- Central control from the hypothalamus and/or pituitary is unnecessary (and presumably not present) in the fetus *in utero* for normal reproductive organogenesis.

Table 2. Status of Genital Tract of Castrated Male Rabbit Fetuses Examined on GD 28[a]

Date of Castration		Status of Műllerian Duct	Status of Wolffian Duct	Status of Prostate	External Genitalia
Stage	Days (No. Cases)				
I	19 (2)	Persistent	Absent	Absent	Female
II	20 (3) – 21 (8)	Persistent	"Caudal rests"[b]	2 Buds[c]	Female
III	22 (4) – 23 (2)[d]	Uterine and vaginal sections	"Caudal rests"	+ or ++	Hypospadic

IV	23 (4)[c]	Absent	Deferent duct; absent/small seminal vesicle present	+++	Male
V	24 (3)	Absent	Normal	+++	Male

[a] Modified from Jost, 1953

[b] "Caudal rests" are fragments of embryonic tissue that have been retained within the adult organism, also called "embryonic" or "fetal" rests (Dorand's Illustrated Medical Dictionary, 27th Edition, 1988, p. 1452, W.B. Sauders Co., Philadelphia, PA).

[b] To completely block prostatic development, the fetus must be castrated prior to the appearance of any bud (Stage I).

[c] Castration before Stage IV results in abnormal external genitalia, while castration at or after Stage IV results in normal male genitalia (although at gonadectomy, these structures were not yet differentiated).

A factor from the female fetus is not necessary for development of female reproductive structures *in utero*, although the development of these structures in the intact (or ovariectomized) female is much slower than that in the male fetus (Jost, 1953). The female conceptus does receive E2 prenatally from her mother and/or from the placenta since she cannot synthesize E2 in her ovaries *in utero*, or in her adrenal glands, because a number of P450 isoforms are missing (Greco and Payne, 1994). The T and DHT, made by the male conceptus (with no fetal central, placental, or maternal input), act on the development of his internal and external genitalia (Gilbert, 1997).

3.3.2 Secondary sex determination (phenotype)

Secondary sex determination involves the sex-specific structures (phenotype), excluding the gonads. A male mammal also has a penis, epididymides, seminal vesicles and coagulating glands, a prostate gland, bulbourethral (Cowper's) glands, preputial glands, as well as sex-specific LABC muscle. A female mammal also has oviducts, uterus, cervix, vagina, and mammary glands. Other sexually dimorphic features may include sex-specific body size, vocal cartilage, musculature, and body and facial hair. These secondary sex characteristics are determined, initiated, and maintained by hormone secretion from the gonads (but see above for structural females developing without gonads).

The scheme of mammalian sex determination is shown in **Figure 5**. If the Y chromosome is absent, the gonadal primordia will develop into ovaries, and E2 from the mother (and placenta) will cause the Műllerian duct from the mesonephric kidney duct remnant (used as part of the renal system in water-dwelling animals) to form the upper portion of the female reproductive tract (oviducts, uterus, cervix, and upper end of vagina). The Wolffian duct, in the absence of T, regresses in females, beginning after the regression of the Műllerian duct in males (Jost, 1953). If the Y chromosome is present, the testes form from the indifferent gonads and produce two major hormones, anti-Műllerian hormone (AMH), also known as Műllerian inhibiting substance (MIS), which causes the Műllerian duct to regress in males, beginning at 30 mm in the human (Jost, 1953). The second hormone, T (and DHT), masculinizes the fetus, stimulating the

formation of the internal and external genitalia, including the penis and scrotum, and inhibiting the development of breast primordia (Gilbert, 1997).

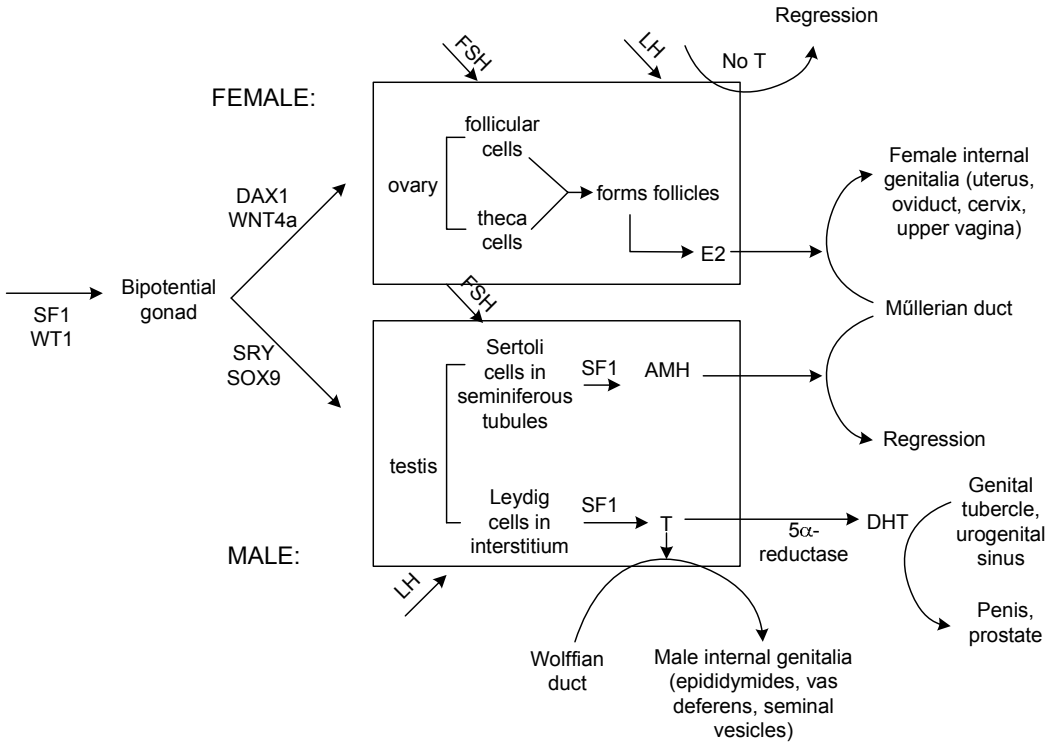
Embryologically, the genital ridge is bipotential; it can differentiate into an ovary or a testis, depending on the hormonal milieu. In humans, the genital ridge (or gonadal rudiment) forms in the intermediate mesoderm in the dorsal region on both sides of the developing central nervous system (CNS) during gestational weeks 4-7. During this indifferent stage, the epithelium of the genital ridge proliferates into the loose connective mesenchymal tissue. These cells form sex cords that will surround the germ cells that migrate into the gonad (from the yolk sac external to the body during week 6). During this period in XX and XY gonads, the sex cords remain connected to the surface epithelium.

If the fetus is XY, the sex cords continue to proliferate through the eighth week in humans, extending deeply into the connective tissue. These cords fuse with each other to form a network of internal (medullary) sex cords and, at its distal end, the rete testis. The testis cords then lose contact with the surface epithelium and become separated from it by a thick extracellular matrix, the tunica albuginea. These cords remain solid during fetal life and childhood. At puberty, the cords hollow out to form seminiferous tubules, and the germ cells begin sperm production, supported by Sertoli cells (which also make AMH and are differentiated from the cells of the early testis cords), and triggered by T produced in the interstitial cells of Leydig (which are differentiated from mesenchymal cells). The sperm are transported from the lumina of the seminiferous tubules in the testes through the rete testis, which joins the efferent ducts or vasa efferentia. The vasa efferentia, remnants of the mesonephric kidney linked the testis to the Wolffian duct, which was the collecting tube of the mesonephric kidney. The Wolffian duct differentiates into the epididymides and downstream vas deferens. The sperm from the testes and the initial seminal fluid, produced in the sex accessory glands, move through the vas efferens to the caput (head), corpus (body), and cauda (tail) of the epididymides where they are stored (transit time from head to tail in rats is seven to ten days). The sperm acquire motility and fertilizing ability in the epididymides, and are ejaculated from the caudae epididymides into the vas deferens, through the prostate gland into the urethra (where they are immersed in the secretions of the various sex accessory glands; prostate, seminal vesicles, coagulating glands, Cowper's gland, etc.) and the penis. From puberty to death in male mammals, T maintains the structures and functions of the sex accessory glands (DHT is a more potent androgen, binds more strongly to the androgen receptor than T, and plays a dominant role in *in utero* sexual development. T is responsible for development and maintenance of the testes, epididymides, vas efferens, vas deferens, seminal vesicles (plus coagulating glands), LABC muscle, testis descent, puberty, and most male secondary and tertiary sex characteristics. DHT is responsible for the development of the male urethra and prostate, the formation of the penis and scrotum from embryonic genital folds, and scrotal and penis growth and development.

If the fetus is XX, the germ cells also migrate into the indifferent gonad but reside near the outer surface of the gonad. The initial intragonadal sex cords regress and a new set of sex cords forms clusters around each germ cell. The germ cells will become ova, the clusters of sex cords will form the granulosa cells, and the mesenchymal cells of the ovary will form the thecal cells. These granulosa and theca cells will combine to form a

follicle around each ovum and synthesize E2. The Műllerian duct remains intact and forms the oviducts, uterus, cervix, and upper vagina, and the Wolffian duct, in the absence of T, degenerates (Gilbert, 1997).

Figure 5. Mammalian Sex Determination



Key:

SRY = SRY gene (on Y chromosome - begins male-specific cascade)

SOX9 = SOX9 gene (autosomal) appears essential for testis formation, is expressed in the genital ridges in mouse male embryos, expressed slightly after Sry expression

AMH = Műllerian inhibiting substance (MIS); anti-Műllerian hormone (AMH); made in Sertoli cells, causes Műllerian duct to regress in males

E2 = 17ß-estradiol; initially from mother (and/or placenta), then made in ovary; potent endogenous female sex hormone

DHT = 5α-dihydrotestosterone; made from T by 5α-reductase enzyme, predominantly in the testis (also in other end organs), binds more avidly to AR than T

FSH = follicle-stimulating hormone from anterior pituitary

LH = luteinizing hormone from anterior pituitary

T = testosterone; endogenous male sex hormone

SF1 = gene for steroidogenic factor 1, activates downstream genes in steroidogenesis pathway

WT1 = a transcription factor first expressed in the intermediate metanephrogenic mesenchyme prior to kidney formation and then in the developing kidney, gonad, and mesothelium

DAX1 = gene on the X chromosome, competes with SRY. If two DAX1 genes are present (in XX females), SRY is repressed; it is expressed in the indifferent genital ridges in the mouse embryo

WNT4a = gene considered critical for ovary formation, expressed in the indifferent genital ridge in mice. Its expression is undetectable in XY gonads (males) and is present in XX gonads (females).

Modified from S.F. Gilbert, 1997 (p. 775, Figure 20.2)

The genes involved in the differentiation of sex-specific gonads (see **Figure 5**) are:

- The SRY gene on the Y chromosome, which is the testis-determining gene located to a region near the tip of the small arm of Y. The SRY gene acts in the genital ridge immediately before and during testis differentiation (expressed in the somatic cells of the indifferent gonads), and expression is lost after the testis is formed. SRY is necessary, but not sufficient, for development of the mammalian testis. A conformational change in the SRY gene DNA is required (to allow distantly bound proteins to interact). Since SRY encodes a transcription factor, the search began for activation or suppression of "downstream" genes expressed in the genital ridge.
- The first downstream gene, likely activated by SRY-encoded protein transcription factors, is SOX9, which is essential in humans for testis formation and is expressed in the genital ridge just after SRY.
- In addition, SF1 (steroidogenic factor 1) is a transcription factor which is activated by SRY and, in turn, activates several genes involved in steroidogenesis in the Leydig cells. SF1 also plays a role in development of the adrenal glands and the gonads (the gonads develop but then degenerate in its absence) and is involved directly with testis development, activating both the Sertoli AMH and Leydig T synthetic pathways.
- The DAX1 gene on the X chromosome competes with SRY, and in XX embryos (females) appears to be the ovary-determining gene. SRY is repressed in the presence of DAX1. DAX1 is expressed in the genital ridges.
- WNT4a is also critical for ovary formation, expressed in the indifferent genital ridges, and is expressed in XX (female) gonads but not in XY (male) gonads.

Puberty in the male, triggered by a surge in T (preceded by a surge in pituitary LH), initiates the completion of spermatogenesis and development of tertiary sex characteristics (muscle mass, hair distribution, voice changes, behaviors, etc.). The production of T continues to maintain the testicular spermatogenesis and the structures and functions of the secondary and tertiary sex characteristics.

Removal of the testis when it is an indifferent gonad *in utero* results in a female phenotype (Jost, 1953). Removal of the testis after puberty (castration) results in regression of the sex accessory glands and loss of the male phenotype (including upper body strength, male pattern baldness, body hair, etc.). Administration of exogenous T *in utero* (in the absence of endogenous T) restores the development of the phenotype, and administration of exogenous T after castration post puberty restores the secondary and tertiary structures and functions (Gilbert, 1997). This sequence of castration, regression, administration of exogenous hormone, and regrowth of the sex accessory structures forms the basis for development of assays to assess active androgens (and anti-androgens).

Differentiation of the testis from the indifferent gonadal ridge begins at approximately six weeks of gestation in humans of a 39- to 40-week pregnancy, and on GD 14 in rodents with a 19- (mouse) to 22- (rat) day pregnancy. This differentiation is directed by

testis-determining factor, a DNA-binding protein transcription factor encoded by a gene (SRY) on the short arm of the Y chromosome, the sex determining region of the Y chromosome, probably in concert with other factors encoded by autosomal or X-chromosomal genes. Testis differentiation is not an androgen-dependent process; however, androgens mediated by the AR do play an indispensable role in the induction of male sex differentiation and development of the male phenotype.

After development of the testis (sex determination), the events of male sex differentiation follow two paths: one inhibiting and one stimulating. The function of the inhibitory path is to cause regression of the Műllerian ducts and therefore to prevent the development of female internal genitalia (oviducts, uterus, and upper vagina) in the male. This process occurs between gestational weeks 6 and 8 in humans and is mediated by the anti-Műllerian hormone, also known as the MIS, a glycoprotein member of the transforming growth factor β family, which is secreted by Sertoli cells in the embryofetal testis.

The stimulating events of male sex differentiation require high levels of androgen and a functional AR. Androgens are required to stabilize the Wolffian duct system to prevent its involution/regression and to induce the differentiation of the Wolffian ducts into the epididymides, vasa deferentia, and seminal vesicles during gestational weeks 9 through 13, in humans. This sequence of duct differentiation is induced by the action of T itself, probably by a paracrine effect due to its high local concentrations in the vicinity of the Wolffian ducts, which is in close proximity to the testes (source of T) (Quigley *et al.*, 1995). T secretion by fetal Leydig cells begins at eight weeks gestation and peaks between 11 and 18 weeks of gestation in humans. Maternal chorionic gonadotropin (CG) (Quigley *et al.*, 1995) is considered to be the major controller of T secretion during this period. Some researchers (*e.g.*, Word *et al.*, 1989) have reported that T synthesis and adenylate cyclase activity in the early human fetal testis appear to be independent of human CG control, so other factors may be involved.

The potent metabolite of T (DHT) is not involved in the process of developing internal male structures, since the enzyme 5-α reductase 2, required for the conversion of T to DHT (**Figure 2**), is not expressed in these tissues until approximately 13 weeks gestation in humans, at which time the process of internal masculinization is complete. Development of the prostate and prostatic urethra from the urogenital sinus, and masculinization of the external genital primordia, the genital tubercle, urethral folds, and labiosacral swellings into the penis, penile urethra, and scrotum also occur between 9 and 13 weeks gestation, and require the more potent fetal androgen DHT; 5-α reductase 2 is also expressed in these tissues at this time.

A functional AR is absolutely required to mediate the actions of both T and DHT in inducing the expression of androgen-dependent genes necessary for internal and external male genitalia. Disturbance of the production or action of androgens during this critical period of male differentiation interrupts the ordered sequence of events and results in failure of complete masculinization.

3.4 Androgen Insufficiency

3.4.1 AR mutations

The most recent AR mutational database (Gottlieb *et al.*, 1998) contains 309 entries, representing over 200 different AR mutations from over 360 patients with AIS, over 35 patients with advanced or metastatic prostate cancer, and one case of laryngeal cancer. The mutation types include insertions, substitutions, deletions, duplications, and splice-junctional deletions/substitutions. The deletions, to date, have included up to five base pairs (bp) or deletion of the entire gene. These changes can occur in the N-terminal region (amino acids numbered 1-538), the DNA binding domain (amino acids numbered 539-627), introns, exons, termination codons, etc. See **Table 3** for an overview of the types and incidences of genetic changes detected to date in the human AR. Mutations in DNA codons for single amino acids,, involving insertions or deletions, will cause frameshift mutations, resulting in the garbling of all subsequent three-base amino acid codes in the mRNA and consequent abnormal amino acid sequences in the specified protein, and the possibility of creation or loss of a stop codon. These changes can result in altered binding specificity, oligospermia, etc. Splice-junction site deletions or substitutions also result in complete or partial AIS.

Two trinucleotide repeat polymorphisms in the AR have also been identified. They encode a series of variable length glutamine (CAG) and glycine (GGC) repeats in exon 1. The CAG repeat has implications for other diseases. Its expansion causes the motor neuron disease (Spinobulbar muscular atrophy), and similar CAG expansions in a variety of unrelated genes cause a number of other neurodegenerative diseases (Gottlieb *et al.*, 1998). Gottlieb *et al.* (1998) also noted that there is a shift in the distribution of CAG-repeat sizes in the hAR gene of breast cancer tissue, that CAG-repeat sizes may act as molecular markers for prostate cancer risk, and that codon-usage variants and GGN-tract sizes may be markers for (associated with) particular diseases.

Table 3. AR Mutations in the Database[a]

	Phenotype	Mutation Type	
		Germline	Somatic
Structural Defects			
Complete gene deletions	Complete AIS[b]	3	
Partial gene deletions	Complete AIS	8	
Partial gene deletions	Mild AIS	1	
Partial gene deletions	Prostate cancer		1
Partial gene deletions	Laryngeal cancer		1
1-4 bp deletions	Complete AIS	8	
1-4 bp deletions	Partial AIS	1	
Intron deletions	Partial AIS	1	
Splice-junction deletion	Complete AIS	1	
Insertions	Complete AIS	4	
Bp Duplications	Complete AIS	1	
Subtotal:		**28**	**2**
Single Base Mutations			
Amino acid substitution	Complete AIS	119	
Amino acid substitution	Partial AIS	95	
Amino acid substitution	Mild AIS	4	
Amino acid substitution	Prostate cancer		26
Multiple amino acid substitutions	Complete AIS	3	
Multiple amino acid substitutions	Prostate cancer		7
Splice-junction substitution	Complete AIS	5	
Splice-junction substitutions	Partial AIS	1	
Premature termination codons	Prostate cancer		1
Premature termination codons	Complete AIS	18	
Subtotal:		**245**	**34**
	Grand Total:	**309**	
Trinucleotide Repeat Polymorphisms in Exon 1	**See Text**		

[a] Modified from Table 2, Gottlieb *et al.*, 1998

[b] AIS = androgen insensitivity syndrome

3.4.2 5α-Reductase deficiency

In a fascinating human "accidental experiment," 24 males (46, XY) in 13 families, in the small, isolated village of Salinas (population 4300) in the Dominican Republic, were born with ambiguous external genitalia and raised as girls. At birth, they had bilateral testes, presenting as inguinal or labial masses, a labial-like scrotum, a urogenital sinus with a blind vaginal pouch, and a clitoral-like phallus. There were no Müllerian structures present (Imperato-McGinley *et al.*, 1974).

At puberty, the voices of these girl-like boys deepened, they developed a typical male phenotype with increased muscle mass, no breast development, and the phallus enlarged to form a functional penis. The change was so striking that the townspeople referred to these individuals as "guevedoces" (penis at 12 [years of age]). In addition, the scrotum became rugated and hyperpigmented, the testes descended, and there was an ejaculate. The prostate remained small (to absent), beard growth was scanty, there was no temporal recession of the hairline (*i.e.*, no male-pattern baldness), and no acne. Their psychosexual orientation was male, and testicular biopsy indicated complete spermatogenesis with normal Leydig cells. The epididymides and vasa deferentia were normal. These males (termed pseudohermaphrodites) had normal internal male reproductive structures, incomplete differentiation of the male external genitalia at birth, and partial to complete recovery at puberty. T biosynthesis and androgen activity were therefore normal; the abnormality was likely due to a "downstream" defect in metabolism of T (*i.e.*, biotransformation of T to 5α-DHT by 5α-reductase). Analyses of the T and DHT plasma concentrations, and other 5α-reduced metabolites of T, indicated that the affected males had approximately 1/40th the DHT of unaffected males, with obligate male carriers exhibiting an intermediate concentration. Pseudohermaphrodite males are considered to be homozygous recessive for the inherited disorder of steroid metabolism; females homozygous recessive for the error are normal.

Obligate carriers of both sexes are heterozygous for the autosomal (not x-linked) gene mutation, with normal phenotypes. In 12 of the 13 families, the trait could be traced back seven generations to a woman who married four different men. The isolation of the town and common ancestors (*i.e.*, consanguinity) suggest that the increase in gene frequency was a consequence of founder effect. It is not known if the heterozygotes have a selective advantage. In 1974, the location of the biochemical error was unknown. The deficiency could have been related to the synthesis, structure, or metabolism of Δ^4-steroid 5α-reductase.

This genetic effect on 5α-reductase is not unique to the Dominican Republic or to isolated inbreeding communities. In Japan, 81 male Japanese patients (age 0-14 years) with micropenis were evaluated for the SRD5A2 gene that encodes 5α-reductase-2 (Sasaki *et al.*, 2003). The authors found mutations in the SRD5A2 gene, especially at R227Q, which resulted in only 3.2% normal enzyme activity; it is relatively frequent in Asian populations (and is likely the cause of the micropenis phenotype). In contrast, a polymorphism V89L (valine to leucine substitution at the 89th codon) at exon 1 of the gene, which results in an ~30% reduction in 5α-reductase-2 activity, is considered

unlikely to be the cause of development of micropenis. Again, interference with the function of the 5α-reductase, especially during *in utero* development, results in incomplete development of the male external genitalia controlled by DHT. As seen by the males in Salinas, the T surge at puberty causes completion of most (but not all) of the initially incompletely developed reproductive structures.

3.4.3 Androgen insensitivity syndrome (AIS)

The syndromes of androgen insensitivity are the most common, identifiable causes of male pseudohermaphroditism. Affected individuals have a male (46, XY) karyotype, normally developed, but incompletely descended testes, and an external genital phenotype from fully female (normal breast development and no or full pubic hair), to partially masculinized. AIS is classified into clinical subtypes, based on the external genital phenotype (complete AIS, partial AIS, Reifenstein syndrome, infertile male syndrome), or into biochemical subgroups, based on the presence or absence of specific high-affinity androgen binding in cultured genital skin fibroblasts. Molecular analysis of the AR gene in individuals with AIS reveals that the various AIS clinical forms and their associated AR defects form a continuum resulting from AR gene mutations of variable type and severity. The two major subtypes and their clinical features are presented below.

Complete AIS

Complete AIS is a relative rare X-linked disorder. Prevalence estimates range from as low as one in 64,200 males to as high as one in 2,000 males; although, these figures may be confounded (either positively or negatively) by ascertainment bias and by some of the studies utilizing only hospitalized cases.

Individuals with complete AIS are diagnosed at various life stages, with age at detection changing as the "index of suspicion for the diagnosis has increased" (Quigley *et al.*, 1995). Individuals are diagnosed before or soon after birth, from the discrepancy between a 46, XY karyotype (male) by prenatal amniocentesis and the presence of a female phenotype by prenatal ultrasound examination or at birth. More are diagnosed with the development of inguinal hernias (often bilateral) during infancy. Phenotypically female children are also present with inguinal hernias. Phenotypically female children are diagnosed after puberty with primary amenorrhea.

Karyotypic male individuals with classic complete AIS have unambiguously female external genitalia, with a blind-ending vagina and no uterus. The assumption has been that there are no Müllerian structures. However, in up to one third of the 43 human cases evaluated histopathologically, there were Műllerian structures such as very small fallopian tubes (Rutgers and Scully, 1991). Remnants of Wolffian structures such as vestigial vas deferens or epididymides have also been found (Bale *et al.*, 1992).

Individuals with complete AIS have excellent feminization at puberty, with normal or increased breast development and clear, smooth, acne-free complexions. The feminization of breasts and body contours is from estrogen, produced by testicular and peripheral aromatization of androgens (see **Figure 3** and **Table 4**), that is unopposed by the effects of androgens (since the mutated AR cannot recognize T, and T biosynthesis is

reduced in abdominal testes). Phenotypic women with AIS are taller than average for females and are reported to have an eunuchoid build (long arms and legs).

Smith *et al.* (1994) described the first reported case of a man (from consanguineous parents), homozygous for an ER gene mutation (a premature termination codon), that caused complete estrogen resistance. At 29 years of age, he was 204 cm tall (over 6 foot 7 inches) and still growing, with open epiphyses and severe osteoporosis. Observations from the people with complete AIS, and from this man with complete estrogen resistance, suggest that androgens alone have little direct action on epiphyseal fusion and prevention of osteoporosis, and that conversion to estrogens (which are active in epiphyseal fusion and protection against osteoporosis) is required. The normal bone maturation reported in phenotypic females with complete AIS supports the interpretation that effects of sex hormones on growth are mediated by the ER. However, delayed onset of puberty in 46, XY girls with complete AIS (and some 46, XX female carriers [heterozygous]) argues for a direct role of androgens in induction of pubertal hypothalamic-pituitary-gonadal axis activity (Quigley *et al.*, 1995).

Table 4. Sites of Estradiol Synthesis

Location	Local Function
Prenatal	
Fetal liver	Possibly to provide fetus with E2 in the absence of *in utero* ovarian synthesis (Greco and Payne, 1994)
Placenta: by aromatization of prehormones (*e.g.*, androstenedione; Figure 3) produced in the fetal adrenal glands (neither fetal ovaries nor fetal adrenal glands can produce E2; missing some P450 isoforms; Greco and Payne, 1994)	Not yet known (but see above)
Postnatal	
Ovaries: granulosa and theca cells of the follicles	Necessary to maintain oocytes in the ovary (role in estrous cyclicity, feminization, etc.)
Brain: from steroid precursors (aromatized from T in male brain; Figure 3), T crosses blood-brain barrier; E2 cannot, so it is made locally from T	E2 masculinizes male brain, resulting in male-specific behaviors in rodents (not known if in humans)
Testis: interstitial cells of Leydig (aromatizes T; Figure 4)	Prevents apoptosis of male germ cells
Fat	Responsive to environmental conditions to trigger puberty for reproduction when food is plentiful (likely responsible for precocious puberty in obese, young girls)

Partial AIS

The partial or incomplete forms of AIS express a wide spectrum of clinical (genital) phenotypes. Because of the variability of the clinical manifestation and the presence of subtle or atypical forms of androgen resistance, such as the infertile male syndrome, the prevalence of partial AIS is unknown, but is considered to be at least as common as complete AIS (Quigley *et al.*, 1995). Individuals with the most severe form of partial AIS may have a complete female phenotype with mild clitoromegaly and/or slight labial fusion, while others have significant genital ambiguity at birth, resulting in delayed or inappropriate sexual assignment. In some cases, especially those with Reifenstein syndrome, there is more extensive masculinization with the affected individuals essentially male, but with a severely undermasculinized phenotype with micropenis, perineal hypospadias, and cryptorchidism. In its mildest forms, partial AIS may be expressed only by uncomplicated hypospadias, by infertility in a phenotypically normal male, or by only gynecomastia and androgen binding abnormalities in a fertile male. In

fact, affected individuals with widely different phenotypes can be found within a single family.

Since androgen induces stabilization and differentiation of the Wolffian ducts in embryonic males, Wolffian structures may also develop to a variable extent in partial AIS, depending on the degree of responsivity/resistance to androgens. Epididymides, vasa deferentia, and seminal vesicles may therefore vary from absent, to rudimentary, to fully formed.

At puberty, virilization and/or feminization may occur depending on the hormonal milieu of the affected individual. If the testes are present, the T secreted can induce a degree of virilization proportional to the degree of masculinization which occurred *in utero*. Feminization of breasts and body curves occur because of relatively high estrogen levels in the presence of androgen resistance (as in complete AIS) (Quigley *et al.*, 1995).

Quigley *et al.* (2004) reported on partial androgen insensitivity, with sex phenotype variation in two unrelated families. This was associated with missense mutations in the AR that disrupted the NH_2-terminal/carboxy-terminal interaction. Each mutation caused a single amino acid change within the region of the ligand-binding domain that performs activation function 2 (AF2). In one family, the mutation designated 1737T was in alpha helix 4, and in the other family, the mutation designated F725L was between helices 3 and 4. Neither mutation altered androgen binding, but transactivation (transcription activation) was impaired. In the family with AR 1737T, sex phenotype varied from severely defective masculization in the proband (the identified patient) to a maternal great uncle whose only manifestation of AIS was severe gynecomastia (breast development). He was fertile and passed the mutation to this two daughters (obligate heterozygote carriers). The proband with AR F725L was also incompletely masculinized, but was raised as a male, while his affected half sibling by a different father was raised as a female. It is clear that the function of an AR AF2 mutant and male sexual development can vary greatly, depending on the genetic background (Quigley *et al.*, 2004).

4.0 MALE REPRODUCTIVE TRACT AND OTHER ANDROGEN RESPONSIVE TISSUES

4.1 Introduction

In general, androgens control gene transcription in target cells containing the AR. This gene transcription promotes protein synthesis, the growth of the target tissues, and their maintenance. This is true for the function of androgens in fetal development of the male reproductive tract and then the function in pubertal development and the adult. The resulting effects in humans are classified as virilizing or anabolic. Anabolic effects include growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth and bone maturation. Virilizing effects include maturation of sex organs including, for example, the penis and formation of the scrotum in fetuses. **Figure 6** illustrates the sexually indifferent stage of the reproductive system in the early fetus and diagrammatic view of the male fetus. **Figure 7** presents a more detailed rendering of the side view of the male reproductive system.

Relatively high levels of AR have been measured in the tissues of the male urogenital tract and in the adrenal glands and gonads of both sexes. Conversely, in other tissues such as the male LABC muscles, preputial gland, scrotal skin, and vagina, there are lower levels of AR. Using an immunoblot assay technique, one study reported that levels of AR were undetectable in the uterus, kidney, spleen, liver, gut, heart, lungs, pituitary, or hypothalamus (Bentvelsen *et al*., 1996). However, receptors have been found at a detectable level in the pituitary and hypothalamus by other techniques.

In some target tissues, such as the penis, exogenous androgens (from outside the body) decrease the AR concentration in the tissue; whereas, exogenous androgens increase the AR concentration in the ventral prostate.

Castration can also have varying effects on the AR levels of tissues. Castration seems to cause a profound decrease in the AR levels in the ventral prostate, but no detectable effect on levels in the adrenal gland. However, by seven days after hypophysectomy, AR levels decrease in both the ventral prostate and adrenal gland (Bentvelsen *et al.*, 1996). Hormones, such as prolactin (PRL), augment T-mediated growth of the prostate in a permissive manner (Prins, 1987) by increasing nuclear AR levels to promote lateral prostatic growth.

Figure 6. Sexually Indifferent Foetal Reproductive System

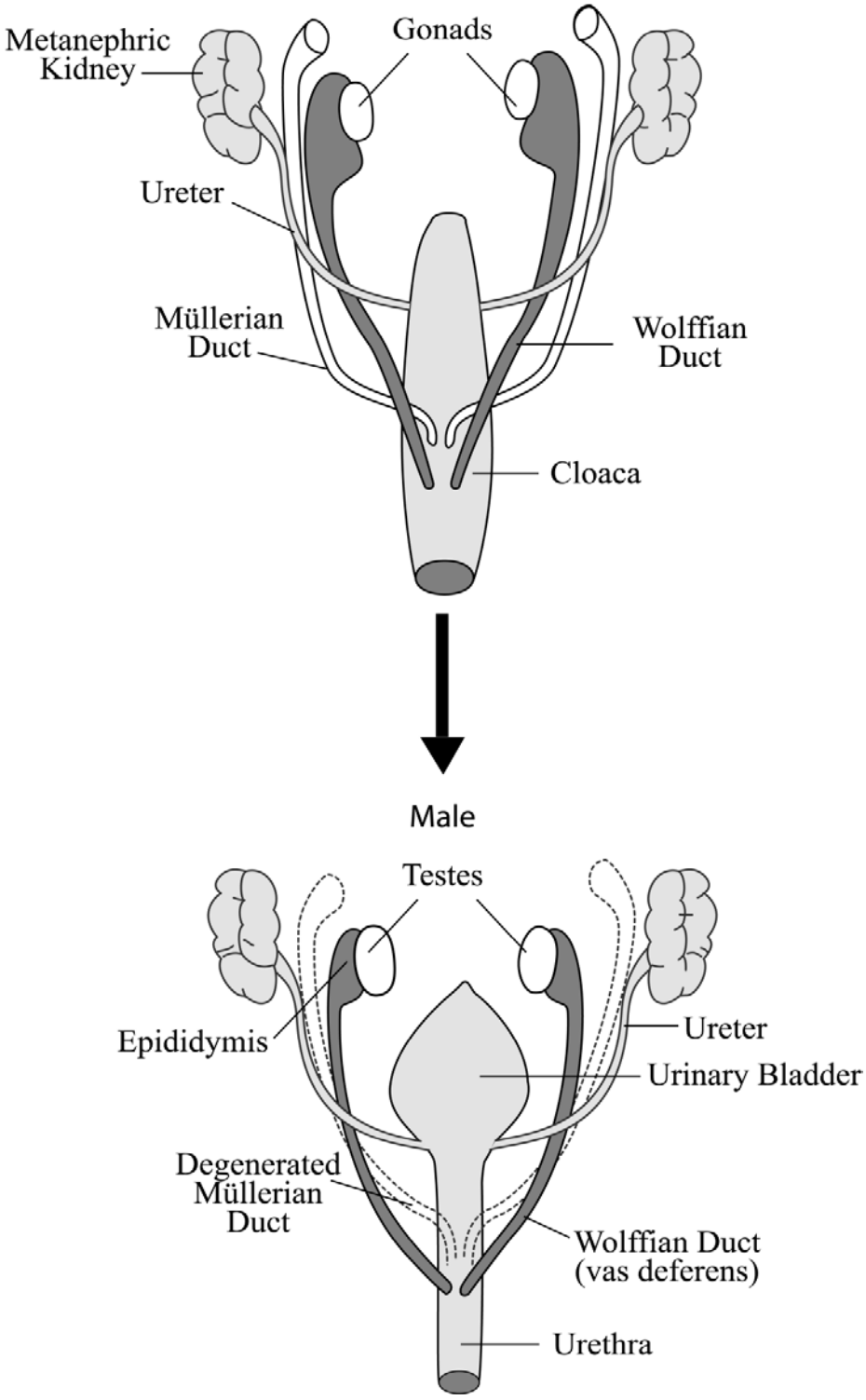
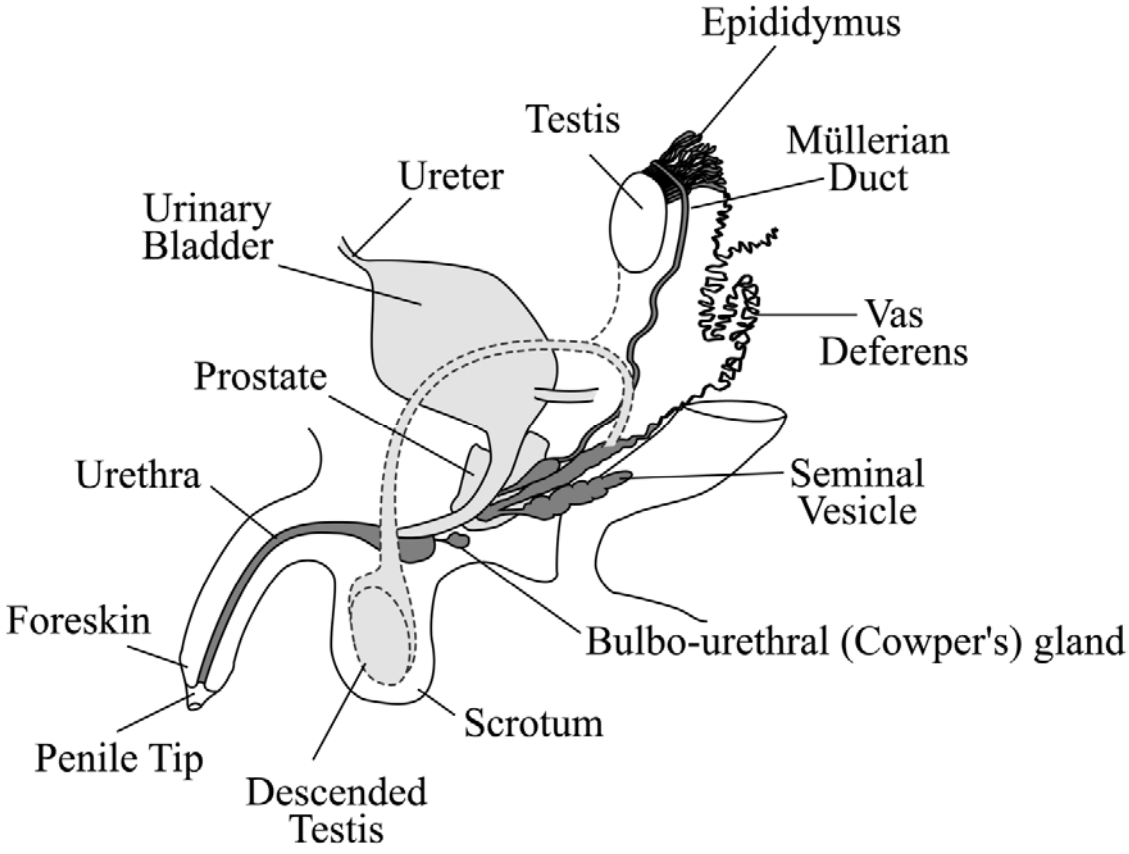


Figure 7. Lateral View of the Adult Male Reproductive System



4.2 Prostate Gland Development

The prostate is a fibromuscular exocrine gland. It is a male accessory reproductive gland which expels a complex proteolytic solution into the urethra during ejaculation. The proteolytic enzymes liquify the semen after ejaculation, and the phosphatases and salts modify the vaginal environment to enhance sperm survival, though the exact function of many of the components of prostatic secretion have yet to be determined.

At the end of the human fetal period, (*i.e.,* the seventh week of gestation), the human male and female urogenital systems are identical. The paramesonephric (Mullerian) ducts, the mesonephric (Wolffian) ducts, and the primordial ureters all lead into a rudimentary urinary bladder, which has developed from the urogenital sinus. In the male embryo, the paramesonephric ducts degenerate, leaving behind the vestiges of the uterovaginal primordium, the prostatic utricle. The ureters separate from the mesonephric ducts, which become incorporated into the urinary bladder and migrate distally to the proximal part of the urethra. The mesonephric ducts differentiate to form the ejaculatory ducts.

The first indication of the formation of the prostate is an increase in cellularity of the splanchnic mesoderm surrounding the proximal part of the urethra. During gestational week 10, the androgen responsive urogenital sinus mesenchyme induces epithelial buds in the presumptive prostatic urethra. Initially, 14 to 20 solid epithelial buds, in at least five groups, grow into the surrounding mesoderm; this is the presumptive peripheral glandular area. Shortly thereafter, a second phase of budding gives rise to the internal glandular area. The solid buds extend and branch under mesodermal control. By the 11th week, lumens form within the epithelial cords and cellular end buds form primitive acini. Mesenchymal cells differentiate into smooth muscle, fibroblasts, and blood vessels. During the 12th week, the epithelium continues to proliferate, while connective tissue septae extend into the acini and the stroma of the gland thins as the ducts and acini expand.

By 13 to 15 weeks, T concentrations have reached their peak embryonic levels. Androgen mediated epithelial mesenchyme interactions cause the simple cuboidal epithelial cells to differentiate, at first in the proximal regions of the larger ducts then progressing distally. By the end of the 15th week, the secretory cells are functional, the basal cell population has developed, and scattered neuroendocrine cells are present. Maturation of the gland continues, while embryonic T levels are high; however, as T levels fall during the third trimester, the gland enters a quiescent state.

4.2.1 Prostate at puberty

The quiescent state persists until puberty, when T levels again increase and the epithelium proliferates, giving rise to the complex folding seen in the mature gland. The prostate doubles in size during this phase of development, ARs are expressed by the epithelial cells, and the full secretory phenotype is established. By 45 to 50 years, T levels are in decline again, and the prostate undergoes a period of involution. With increasing age, atrophication of the gland may continue, though commonly, benign prostatic hypertrophy occurs in certain species such as the dog and the human.

The prostate gland is an example of sex-specific, androgen-regulated development in many species. The above describes human prostate development, though most of the genetic data has been developed using the rodent prostate as a model. Great care must be exercised when comparing late prostatic developmental events between species, though the early events during branching are sufficiently similar to be relevant. The timing of developmental events is imprecise and changes are progressive; the figures given are approximations only and may vary by +/- 10%.

In humans, the prostate gland surrounds the first 3 cm of the urethra (prostatic urethra) as it leaves the urinary bladder. The ejaculatory ducts enter dorsally and join the urethra within the gland, either side of the prostatic utricle. Anatomically, the most caudal aspect of the gland, which apposes the urinary bladder, is referred to as the base of the gland. The walls of the prostatic urethra are highly convoluted and lined with transitional epithelium. In its resting (not distended) state, the ureter has a longitudinal ridge (the urethral crest) running the length of the gland. The majority of the ductal glands secrete into longitudinal grooves (the urethral sinuses) formed on either side of the ridge. Near the junction of the ejaculatory tubes and the urethra is a short diverticulum in

the urethral crest. This is the prostatic utricle, the male vestigial remnants of the female uterus and vagina.

The prostate is covered by a thin vascularized fibrous sheath which surrounds a fibromuscular layer continuous with the smooth muscle surrounding the bladder. The fibromuscular layer extends within the organ as septae, dividing the gland into ill-defined lobules and functional areas.

4.2.2 Secretory development of the prostate

The secretory components of the gland are divided into three concentric layers. The innermost area is comprised of mucosal glands which are concentrated around and secrete into the upper region of the prostatic urethra. The middle or internal area contains submucosal glands which secrete via short ducts into the urethral sinuses. The outer or peripheral area constitutes the majority of the gland and secretes via long ducts into the urethral sinuses. The anterior isthmus is an area of the gland ventral to the urethra, relatively free of glands and rich in fibromuscular tissue.

The prostate is a compound tubuloacinar gland. Within the acini and tubules, the epithelium forms complex folds and papillae supported by a thin, highly vascularized, loose connective tissue. The secretory epithelium is mainly pseudostratified, comprising tall columnar cells and basal cells which are supported by a fibroelastic stroma containing randomly orientated smooth muscle bundles. The epithelium is highly variable, and areas of low cuboidal or squamous epithelium are also present, with transitional epithelium in the distal regions of the longer ducts. Densely packed basal nuclei are characteristic of the prostatic epithelium. The tall columnar secretory cells have an extensive basal golgi complex, apical lysosomes, and secretory granules. The epithelium contains scattered neuroendocrine cells, which partly control release and expulsion of prostatic secretions during ejaculation.

The fluid secreted by the prostate gland is rich in acid phosphatase and citric acid. It contains the proteases fibrinolysin and prostate specific antigen (PSA), the enzyme amylase, kallikreins, semenogelin, fibronectin, phospholipids, cholesterol, zinc, calcium, and many proteins of unknown function such as beta- microseminoprotein.

4.2.3 Developmental genetics of the prostate

The development of the prostate is controlled by steroid hormones that in turn induce and maintain a complex and little understood cross talk between the various cell types making up the gland. The result of this intercellular communication can be either new growth or growth quiescence, depending upon the differentiation state of the cell type being stimulated. Secretory function of the prostate is dependent upon direct stimulation of fully differentiated prostatic epithelial cells by androgens. Thus, the prostate seems to be regulated in a similar manner to other organs of the male and female genital tract, with proliferative control mediated by cell-cell interactions; whereas, differentiated function is determined by direct steroid action on the parenchymal cells.

Although the androgen-dependent nature of prostate development has been well established, it is not clear how androgens regulate prostate differentiation and growth. Identification of androgen-dependent gene pathways present during prostate development is essential in order to differentiate between normal and disease processes

of this male-specific organ. The use of high-density cDNA hybridization arrays and subtractive hybridization techniques can aid in the identification of these gene pathways. These can be further studied by analysis in transgenic mouse models. A normalized cDNA library derived from neonatal mouse prostate has been developed. This library has been screened to identify overexpressed clones in the developing male prostate compared to female reproductive tissues. Genes of interest were sequenced, their expression characterized by whole-mount *in situ* hybridization, and subjected to functional analysis using *in vitro* organ cultures, transgenic, and/or targeted mutagenesis in the mouse. Genes identified in this manner may provide useful prognostic markers for early prostate cancer.

4.3 Seminal Vesicles and Coagulating Gland Development

Using an electron microscope, the development of the seminal vesicle from the Wolffian duct and the prostate from the urogenital sinus has been studied in rat fetuses from gestational day 14 to birth. Prior to the onset of androgen secretion, the cells of the urogenital sinus and the caudal part of the Wolffian duct have a simple, undifferentiated appearance. After the onset of androgen secretion by the fetal testes at gestational day 15, "intracytoplasmic confronting cisternae" of the granular reticulum appear in both the urogenital sinus and Wolffian duct. Portions of the granular endoplasmic reticulum of the urogenital sinus become distended with a finely granular, moderately dense material. In the urogenital sinus, many hemidesmosomes are formed at the basal surface of the epithelium. Specializations of the extracellular materials are present opposite the hemidesmosomes. The formation of the seminal vesicles and the prostate begins at day 18–19 of gestation. The cells of the seminal vesicle are taller than the Wolffian duct cells from which they arise, the granular endoplasmic reticulum is moderately increased, and a patent lumen is formed. The cells of the fetal prostate do not differ greatly from those of the urogenital sinus from which they arise, except that the prostatic cells initially lack hemidesmosomes.

Development of the seminal vesicle is elicited by androgens and is dependent on epithelial-mesenchymal interactions. Androgenic signal transmission from the androgen-receptor-positive mesenchyme to the epithelium has been postulated to involve paracrine factors. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family, is produced by stromal/mesenchymal cells and acts specifically on epithelial cells. The KGF transcript was detected by reverse transcription-polymerase chain reaction in newborn mouse seminal vesicles and by Northern blot analysis of RNA from cultured neonatal seminal vesicle mesenchymal cells. Newborn seminal vesicles placed in organ culture undergo androgen-dependent growth and differentiation. Addition of a KGF-neutralizing monoclonal antibody to this system caused striking inhibition of both seminal vesicle growth and branching morphogenesis. This inhibition was due to a decline in epithelial proliferation and differentiation, as the mesenchymal layer was not affected by anti-KGF treatment. When KGF (100 ng/ml) was substituted for T in the culture medium, seminal vesicle growth was approximately 50% that observed, with an optimal dose of T (10^{-7} M). All of these findings suggest that KGF is present during a time of active seminal vesicle morphogenesis and functions as an important mediator of androgen-dependent development.

4.4 Cowper's Glands Development

Cowper's glands in humans are pea-sized glands present inferior to the prostate gland in the male reproductive system. They produce thick, clear mucus prior to ejaculation that drains into the spongy urethra. Though it is well established that the function of the Cowper's gland secretions is to neutralize traces of acidic urine in the urethra, knowledge regarding the various lesions and associated complications of this gland is scarce. Cowper's glands (also known as the bulbourethral glands) were named after the 17th century English surgeon William Cowper. Their relation to the prostate gland is, perhaps, both anatomical and metaphorical.

During the 10th week of human development, the pelvic urethra gives rise to the paired bulbourethral glands. Whereas the prostate develops from the prostatic urethra, the bulbourethral glands develop from the membranous urethra under the influence of many endocrine and paracrine signals. Moreover, Cowper's glands are most significantly under the control of DHT.

As the prostate develops, the paired bulbourethral glands sprout from the urethra just below the prostate. During the 10th week, the seminal vesicles arise from the distal mesonephric ducts in response to T, whereas the prostate and bulbourethral glands develop from the urethra in response to DHT. The vas deferens and seminal vesicles derive from the mesonephric ducts and the prostate from the cranial urogenital sinus. The bulbourethral glands develop from the intermediate urogenital sinus, which differ in the inductive capacity of its mesenchyme.

4.4.1 Cowper's gland secretory development

Sex accessory tissues include the prostate, seminal vesicles, ampullae of vas deferens, and bulbourethral glands and are believed to play an important role in the reproductive process. They are homologues of the greater vestibular glands in the female. Cowper's glands are accessory sexual organs that contribute to urethral lubrication. It has also been demonstrated that Cowper's gland secretion has a role in semen coagulation, which has been demonstrated in rodents (Beil and Hart, 1973). The two main Cowper's glands are situated within the urogenital diaphragm, with a second pair of accessory glands situated in the bulbospongiosal tissue. The main Cowper's ducts enter the ventral surface of the bulbourethra near the midline by piercing the spongiosum. The accessory ducts can enter the urethra directly or drain into the main duct. The ducts of the Cowper's gland empty into the bulbourethra. The bulbourethra extends from the inferior urogenital diaphragm to the suspensory penile ligament superiorly and penoscrotal junction inferiorly. The Cowper's gland consists of well-demarcated lobules of small, compact tubuloalveolar glands radiating from a central excretory duct lined by pseudostratified epithelium, and entrapped within fascicles of muscle. The glands have a thin connective tissue capsule composed of simple columnar epithelium. The glands histologically stain positively for mucin, smooth muscle actin (periphery of acini), and negatively for prostatic acid phosphatase (PAP), S100, carcinoembryonic antigen (CEA), and are variable for prostate-specific antigen (PSA) and CK903 (Elgamal *et al.*, 1994).

4.4.2 Functions of Cowper's glands

During sexual excitement, these glands secrete clear glycoproteins into the bulbous urethra. The male sex accessory tissues require the continued function of the testes for their development, growth, and maintenance of secretions that form the major components of the ejaculate.

Cowper's glands secrete glycoproteins during sexual stimulation, which functions as a lubricant for the semen. In response to sexual stimulation, the bulbourethral glands secrete an alkaline mucus-like fluid. This fluid neutralizes the acidity of the urine residue in the urethra, helps to neutralize the acidity of the vagina, and provides some lubrication for the tip of the penis during intercourse. Cowper's gland secretions contain no sperm. It has been shown that pre-ejaculatory fluid secreted at the tip of the urethra from Cowper's gland during sexual stimulation does not contain sperm (Zukerman *et al.*, 2003).

Cowper's glands are involved in the immune defense of the genitourinary tract and secrete many glycoproteins, including Prostate Specific Antigen (PSA) (Pedron *et al.*, 1997). Immunohistochemical studies on whole-mount cadaveric Cowper's gland and cystoprostatourethrectomy samples showed that although, PSA and PSAP are mostly produced by prostatic tissue, it was not exclusive (Elgamal *et al.*, 1994). These findings support the hypothesis of extraprostatic sources of PSA and may impact on the specificity and sensitivity of PSA serum levels after radical prostatectomy.

4.5 Glans Penis Development

The genital eminence, an external mound arising between the umbilicus and the tail, is made up of the genital tubercle and the genital swellings. The urogenital sinus opens at the base of the genital tubercle between the genital swellings. These structures form identically in male and female embryos up to week 7 of gestation.

At nine weeks of gestational age, and under the influence of T, the genital tubercle starts to lengthen. In addition, the genital swellings (also called the labio-scrotal folds) enlarge and rotate posteriorly. As they meet, they begin to fuse from posterior to anterior. As the genital tubercle becomes longer, two sets of tissue folds develop on its ventral surface on either side of a developing trough, the urethral groove. The more medial endodermal folds will fuse in the ventral midline to form the male urethra. The more lateral ectodermal folds will fuse over the developing urethra to form the penile shaft skin and the prepuce. As these two layers fuse from posterior to anterior, they leave behind a skin line: the median raphe.

By 13 weeks, the urethra is almost complete. A ring of ectoderm forms just proximal to the developing glans penis. This skin advances over the corona glandis and eventually covers the glans entirely as the prepuce or foreskin.

4.6 Penile Urethra Development

The human penis goes through a natural state of hypospadias as it develops from a primitive, undifferentiated structure into a fully differentiated penile urethra (Baskin *et al*., 2001). The urogenital system of the male embryo develops during weeks 8 to 14 following ovulation (Moore *et al*., 2003). At 8 weeks, the external genitalia of both male

and female embryos are indistinguishable. Both have a midline genital tubercle just above a urogenital membrane flanked on each side by outer genital swellings and inner urethral folds (Baskin *et al*., 2001).

The effects of T occur early and include an increase in the distance between the anus and genital structures, followed by elongation of the genital tubercle, which will become the penile shaft and glans (Baskin, 2000). The genital swellings, also called the labioscrotal folds, migrate caudally and start to fuse, forming the scrotum (Moore *et al*., 2003). A urethral groove developing on the underside of the penis becomes the urethra (Baskin, 2000). Folds of tissue, called urethral folds, that frame the lateral walls of this groove, have inner (endodermal) and outer (ectodermal) edges (Moore *et al*., 2003; Baskin, 2000).

As the urethral groove develops from the posterior to anterior surface, it is soon enclosed by fusion of the endodermal folds to form a tubular penile urethra (Cunha and Baskin, 2004; Hynes and Fraher, 2004). The fusion of the endodermal edges creates an epithelial seam that is subsequently reabsorbed (Baskin *et al*., 2001). The ectodermal edges then fuse over the urethra to fashion the penile shaft skin, leaving behind the median raphe (Baskin, 2000). The distal, or glandular urethra, develops last by one of two possible mechanisms (Cunha and Baskin, 2004). The classic theory is that the distal portion of the urethra develops as an ingrowth from the tip of the penis until it joins the proximal tubular urethra (Moore *et al*., 2003; Kurzrock *et al*., 1999). Recent evidence however suggests that the entire urethra, from base to tip, is formed by continuous extension and fusion of the endodermal urethral groove (Hynes and Fraher, 2004; Belman, 2002).

The main defect of hypospadias, the abnormally located urethral opening, is considered a failure of some stage in the orderly process of development outlined above. A normal penile urethra, with a meatus at the tip of the glans, requires proper formation of the urethral groove, urethral folds, and fusion of the folds with seam formation and seam removal. Failed seam formation during fusion of the urethral folds results in hypospadias (Baskin *et al*., 2001) and the site of failure dictates the final position of the urethral meatus (G, Yamada *et al*., 2003).

The two features that commonly accompany hypospadias, penile curvature and incomplete foreskin, also represent normal stages of embryologic development. The developing fetal penis is curved ventrally because the penis and the shaft skin grow faster on the dorsal (upper) than on the ventral aspect (Snodgrass *et al*., 2002). Concurrent with urethral development, at about 8 weeks following ovulation, the prepuce (foreskin) arises from the base of the glans, growing primarily on the dorsal surface of the penis. As the prepuce advances distally, it also grows ventrally to completely cover the glans (Baskin, 2000). If the urethral folds fail to fuse, as in hypospadias, the preputial growth is also interrupted (Baskin, 2000). Hypospadias at more distal locations (*e.g.*, distal glanular hypospadias) is associated with fusion of the urethral folds to the base of the glans, at least. Thus, in these very minor degrees of hypospadias, normal preputial development is possible (Hynes and Fraher, 2004).

4.7 Preputial Glands

Preputial glands are sebaceous glands, and their sebocytes (which have AR) are stimulated to proliferate and produce secretions by androgens. The AR gene expression increases as the sebocytes begin to differentiate (Miyake, 1994).

The preputial glands of rodents are highly modified, paired sebaceous glands lying anterior to the male prepuce between the body wall and the skin. The rodent glands are not homologous with the glandular developments of the preputial skin found in other mammalian groups, but are unique to the order Rodentia (Mallick, 1991). Early studies demonstrated that preputial glands of hypophysectomizd rats grew in response to exogenous steroids such as T (Noble and Collip, 1955) and 4-androstene-3,17-dione (Selye and Clarke, 1943). The preputial glands were highly responsive to steroids which induced androgenic effects in animals but not to steroids of the phenolic estrogen type.

4.8 Demonstration of Androgen Receptors in Tissue

ARs are present in most tissues, as demonstrated by a variety of methods. 3H-androgen binding assays were the initial approach used to determine tissue distribution of AR. By injecting (3H)-T into rats, followed by quantitation of (3H)-androgen uptake in various tissues, Gustafsson and Pousette (1975) were able to identify target organs of androgens. The development of anti-AR antibodies has complemented the autoradiographic methods of AR detection (Husmann *et al*., 1990; Tilley *et al*., 1994; Takeda *et al.*, 1990; and Chang *et al*., 1989). Using anti-AR antibodies, Takeda *et al*. (1990) evaluated AR distribution in various rat tissues. They were able to demonstrate that all male sexual organs in the rat showed a strong, positive nuclear staining for AR, whereas several other tissues, including hepatic, renal, neuronal, muscular and female reproductive organs had weak, albeit positive, nuclear staining. In fact, the only tissue which did not demonstrate staining for AR was the spleen. The use of microwave-based antigen retrieval for AR (Janssen *et al*., 1994) enhanced the immunohistochemical detection of AR in paraffin sections allowing for evaluation of archival tissue sections.

In addition to identification of AR protein, detection of AR mRNA in tissues has been accomplished by several methods. Cloning of human AR (hAR) and rat AR (rAR) cDNA (Chang *et al*., 1988; Lubahn *et al*., 1988) allowed for development of probes which were used to detect in various tissues the AR mRNA by Northern blotting or by *in situ hybridization*. In addition to confirming previous immunohistochemical staining data, the analysis of AR mRNA by Northern blot resulted in identification of two isoforms in the A/B domain of AR mRNA in the vocal organ of *Xenopus* (Fischer *et al*., 1993) and the brain of rodents (Burgess and Handa, 1993). The sensitivity of detection of AR mRNA was greatly improved by competitive RT-PCR (Young *et al*., 1994). **Table 5** summarizes their results for the relative expression (compared to prostate) of AR mRNA in various tissues of male and female Sprague-Dawley rats based on competitive RT-PCR. These results demonstrate agreement between mRNA levels and immunohistochemical staining intensity (Takeda and Chang, 1991).

AR expression is modified during fetal development, sexual development, aging, and malignant transformation. Regulation of AR levels may occur anywhere along the path from AR gene transcription to post-translational modification. A variety of factors, including androgens, have been implicated in modulating the AR protein and mRNA expression.

In the case of mouse fetal development, AR mRNA, based on *in situ* hybridization, was not found in the urogenital sinus at 13.5 days of gestation, whereas at 15.5 days of gestation, both AR mRNA and protein levels were detectable (Takeda and Chang, 1991).

If, as discussed below, one subscribes to the idea that both T and DHT mediate their effects by interacting with a single nuclear receptor protein, then how can we account for the requirement of both T and DHT for sexual development? The answer may be found by examining the ability of these androgens to interact with the AR. Several investigators have demonstrated that T has approximately three times faster association and dissociation rates than DHT on both the rat (Wilson and French, 1976) and human (Grino *et al*., 1990; Zhou *et al*., 1995) AR. In agreement with these binding kinetics, Zhou *et al.* (1995) have demonstrated that T is less effective at stabilizing AR than DHT. These observations suggest that DHT, by enhancing the stabilization of AR and its action, amplifies the T signal in those tissues which contain 5 alpha-reductase. Perhaps in these tissues, the AR expression is not sufficient for T to mediate a physiologic response, but due to the ability of DHT to enhance AR activity, a response is observed.

Table 5. Relative Abundance of Androgen Receptor mRNA in Various Organs to the Level in the Prostate Gland (prostate = 100) in rats

Organ	Male	Female
Hypothalamus	42	217
Adrenal gland	141	186
Epididymis	115	NA
Thyroid gland	68	ND
Harderian gland	58	ND
Pituitary gland	56	9
Preputial gland	44	38
Quadriceps muscle	35	ND
Levator ani muscle	30	NA
Kidney	27	7
Coagulating gland	25	NA
Seminal vesicle	25	NA
Testis	20	NA
Liver	18	9
Submaxillary gland	17	ND
Bulbocavernosus muscle	16	NA
Vagina	NA	9
Heart	8	7
Ovary	NA	4
Uterus	NA	2

The relative abundance of AR mRNA in rat tissues (Young *et al.*, 1994). Total RNA from the indicated tissues was subjected to competitive reverse transcription-polymerase chain reaction for quantitation of AR mRNA levels. The data are reported as percentage of AR mRNA relative to the prostate AR mRNA levels (averaged from 5 male rats). The data represent mean values from 3-5 rats.

ND = not determined

NA = not applicable

In contrast to the inability of T to mediate various androgen-dependent events during sexual development, the biology of hair growth offers an example of the different effects that androgens exert on the proliferation of similar populations of epithelial cells.

Specifically, T can stimulate facial hair growth but causes the regression of scalp hair in aging individuals (Randall, 1994). Hair follicles are intimately associated with the mesenchymally-derived dermal papilla, which is believed to provide an important influence on the follicular proliferation.

Mesenchymal-epithelial interactions are critical in the development of prostate tissue [reviewed in (Cunha *et al*., 1992)]. Prostate mesenchyme, when coincubated with Sertoli cells, synthesizes extracellular matrix in an androgen-independent manner; however, when exposed to an androgen, it produces a diffusible substance similar in action to P-Mod-S (Verhoeven *et al*., 1992). P-Mod-S, a protein secreted by peritubular cells, can activate a variety of responses such as inhibin production and aromatase activity in Sertoli cells. The effect of P-Mod-S on the prostate epithelia is currently unknown; however, it may also stimulate the activity similar to that observed in Sertoli cells, thus accounting for one mechanism of mesenchymal-epithelial interaction within the prostate. Gleave *et al*. (1992) demonstrated that prostate fibroblasts secrete a diffusible substance which can stimulate the growth of LNCaP prostate carcinoma cells. Androgen deprivation mediated by castration resulted in expression of tenascin in the rat prostate (Vollmer *et al*., 1994).

Despite the binding of T and DHT to a single receptor, the effect of these hormones on a single gene may be quite distinct. For example, differential regulatory effects of T and DHT on expression of several genes, including Far-17a (Seki *et al*., 1991) and the cytokines interleukin-4 (IL-4), IL-5 and g-interferon (gIFN), has been reported (Araneo *et al*., 1991). The observation that both T and DHT can differentially regulate the expression of the androgen responsive genes has led to the controversial idea that more than one AR may exist. This hypothesis is supported by the following observations [reviewed in (Sheridan, 1991)]: (1) (3H)T, when injected into rats, concentrates in the hypothalamic nuclei, and 100x unlabeled DHT does not inhibit this localization; (2) hypothalamic localization is not observed after injection of tritiated DHT; and (3) the ability of T, but not DHT, to induce neuronal proliferation or male sexual behavior in castrated male rats. These data are consistent with three different possibilities. One possibility is that two different ARs indeed exist. Alternatively, these data may be due to different metabolic interactions of the androgens on a single AR. As discussed above, T dissociates from the AR 3 times faster than DHT and is less effective in stabilizing the AR (Zhou *et al*., 1995). This difference in the dissociation rate has been directly related to the androgens' ability to stimulate transcription of an androgen responsive gene (Deslypere, 1992). A third explanation is that labeled T, being an aromatizable androgen, is converted to estradiol by brain aromatase and subsequently binds to the ER in various nuclei in the hypothalamus. The inability of excess DHT to displace bound T is because DHT is not an aromatizable androgen; it cannot be converted to estradiol and, therefore, is unable to displace the aromatized T (*i.e.,* estradiol) from its cognate receptor. These observations could account for the differential effects of these androgens mediated by one receptor.

Perhaps the strongest evidence for the presence of one AR is derived from the observation that genotypic XY mice and rats with testicular feminization syndrome (Tfm) do not have a fully functional AR (Deslypere, 1992; Yamamoto *et al*., 1983; Aarden, 1989; Yarbrough *et al*., 1990), and even though they express both T and DHT, they

develop into phenotypic females. This experiment of nature demonstrates that loss of one AR can result in loss of action of both androgens.

The presence of two androgens acting on one receptor may serve several possible functions. Cells which contain steroid 5 alpha-reductase can convert T into DHT. Thus, the overall effect of this system may be to amplify the action of T via conversion to DHT within these cells, thus providing a mechanism of local regulation.

Another method of studying the multi-hormonal nature of the developmental process has been to treat the animal with an antagonist to the developmental signal for the duration of the critical period and then discontinue treatment. Higgins *et al*. (1981) used an estrogen as a probe to identify critical periods of development of the male accessory glands and treated rats for five days with estradiol benzoate [50 µg in oil (20µl)] during either the neonatal, prepubertal, or pubertal stages or as adults. Neonatal rats treated at birth for five days show several abnormalities in the male reproductive system development. These include smaller testes, epididymides (caput affected more than the cauda, with DNA content decreased to approximately one half off the control value), seminal vesicles and ventral prostate. The seminal vesicles and ventral prostate weights were approximately one quarter that of the controls. The DNA content for both was decreased, but not proportionately to the tissue weight, indicating that the estradiol affected cell size as well as cell proliferation. When animals were allowed to go out to 120 days beyond treatment, some compensatory growth occurred in the seminal vesicles, but not in the ventral prostate, and full secretory activity was not restored in the seminal vesicles; therefore, delayed maturity may not be the only explanation.

Rats treated for five days during the prepubertal period (aged 20-25 days) had normal sized testes. Tissue weights and DNA contents for the seminal vesicles and ventral prostates were near control values. Caput and cauda epididymides were smaller with proportionally less DNA, but no different than the amounts noted in the animals treated as newborns. Thus, this developmental process seems to be insensitive to estrogen treatment during the prepubertal period. However, when rats were treated during the pubertal period, 40-45 days of age, they had slightly smaller testes and underdeveloped seminal vesicles and ventral prostate, but less marked than when treated in the neonatal period. This suggests that the estrogen had affected cell proliferation and not cell size. The effects on the epididymides were the same as in the two earlier periods.

Rats treated in the adult period of life caused a small decrease in seminal vesicle weight and a 40% decrease in ventral prostate weight.

4.9 Conclusions

Many types of substances can have an influence on the reproductive tissues of both males and females. Androgenic, estrogenic, anti-androgenic, and anti-estrogenic compounds all have been shown to have effects on the target organs that are responsive to androgens. The Hershberger assay is one attempt to detect these types of compounds, based on their relative influence on the target tissues: ventral prostate, LABC, seminal vesicles, glans penis, and Cowper's glands.

Although differences in metabolism could result in differing results between species, the basic physiological systems are similar for humans and rodents. There are no major differences between the circulatory, respiratory, endocrine, urinary, skeletal, and central nervous systems of the human and rodent male. The gall bladder is not present in the rat, but this does not appear to influence the reproductive system to produce any differences. The major difference is the timing of the events in the development of the human versus the rodent. The development of the endocrine system organs and their hormone production, release, and secretion are intrinsically different between species and even strains.

Similarities in male reproductive physiology between humans and rats (modified from Gray et al., 2004, Table 1a)

- T and DHT, made in the embryofetal testis, control the initiation of the other male reproductive structures *in utero*.
- Central nervous system (CNS) – hypothalamic secretion of gonadotrophin-releasing hormone (GnRH) controls anterior pituitary synthesis and release of FSH and LH.
- LH in the male induces and regulates interstitial cells of Leydig outside the seminiferous tubules in the testes to produce T and DHT. These androgens, in the rat, initiate spermatogenesis in the early postnatal period. *In utero* production of T is initiated by the SRY gene on the Y chromosome. FSH in the male induces the Sertoli cells in the seminiferous tubules *in utero* to initiate nourishment and support of the developing gonocytes.
- At puberty, LH triggers a surge of T, which initiates completion of the process of spermatogenesis in boys (from spermatogonia to spermatids to spermatozoa).
- Both FSH and LH regulate germ cell development after puberty. T (and DHT) is required to maintain male spermatogenesis and initiate and maintain secondary sex characteristics in both boys and male rodents.
- Some sexually dimorphic behaviours (e.g., "rough and tumble" play and mating behaviours) are imprinted by early exposure to androgens and

maintained by estrogen made locally in the brain by conversion of T to estradiol (E2) by aromatase (the sexually dimorphic nucleus of the pre-optic area [SDN-POA] in the brain is the likely location; E2 cannot cross the blood brain barrier, but T can, so E2 is made in the brain).

- Puberty in males (and females) is initiated by dramatic endocrine changes resulting from CNS-HPG (hypothalamic-pituitary-gonadal) axis. Males generally acquire puberty (external indication is acquisition of PPS, separation of the glans penis from the prepuce in rodents) later than females (acquisition of vaginal opening in rodents) for both humans and rodents. The most important androgen in the human and rat prostate is DHT, made locally by conversion of T to DHT by 5α-reductase in the prostate.

Differences in male reproductive strategies between humans and rats (modified from Gray et al., 2004, Table 1b)

- In the rat, sexual differentiation of the reproductive tract is perinatal, with CNS sexual differentiation a postnatal event, regulated predominantly by local aromatization of T to E2 (play behaviour, an exception, is androgen dependent in both rats and humans).
- In nonhuman primates (and presumably humans), more CNS events are prenatal, and androgens are more important than in rats.
- Male rat sexual behaviour can be induced by estrogens and involves multiple series of ejaculations in a single mating. Mating involves approximately ten mounts, with intromission before each ejaculation, followed by a postejaculatory interval before the onset of the next series. The male rat responds to a female displaying sexual receptivity (only during estrus after E2 and then progesterone surges). In nonhuman primates (and humans), male sex behaviour is androgen mediated and does not depend on female behavioural or physiological estrus.
- Spermatogenesis begins at approximately five days of age in the rat. The postpubertal spermatogenic cycle (from spermatogonia to spermatozoa) is approximately 70 days in the rat (and approximately 56 days duration in mice). Sperm first appear in the rat epididymides at approximately 55 days of age. In humans, spermatogenesis begins during puberty at 10-14 years of age, and the entire spermatogenic cycle is approximately 75 days in duration.
- Puberty in male rats (measured by PPS, an androgen-dependent event) occurs at approximately 42 days of age in CD® (SD) and LE rat strains.
- Spontaneous male reproductive malformations are very rare in the rat. In humans, some male reproductive malformations, such as cryptochidism (undescended testes; under INSL3 [transabdominal descent] and DHT [inguinoscrotal descent] control), and hypospadias (when the opening of the urethra occurs along the ventral aspect of the penis, not at the tip), are

considered the most frequent complications in newborn boys (~ 3% incidence).

Research for the past 15 years has started to focus on the development of the male reproductive tract at a cellular and molecular level. This approach may lead to biomarkers which enhance *in vivo* and *in vitro* assays that are under consideration for assessment of possible endocrine-active chemicals and help to reduce some of the variability between species.

Ashby (2002) emphasized the importance of the resolution of study design and data interpretation issues before human hazard assessment for exposure to EDC can be approached with confidence.

5.0 HISTORY OF THE ASSAY

5.1 History of Androgenic/Anti-androgenic Assays

The initial work on assays for testicular hormone was performed on capons (castrated male chickens), assessing comb regrowth, in the late 1920s and 1930s. Around the same time, mammalian assays (predominantly in rodents) were being developed, looking at sperm evaluations, prostate, seminal vesicles, vas deferens, and Cowper's gland size and/or cytology. The long evolution of the so-called Hershberger assay began in 1932 with reproductive organ weights (Korenchevsky, 1932), and continues to change slightly under various validation efforts (*e.g.,* Gray et al., 2004, 2005; Freyberger *et al.,* 2005; Fang *et al.,* 2003; Yamasaki *et al.,* 2003a, Yamasaki, 2004).

5.1.1 Comb growth

The comb growth of capons, after injection of the test material, was reported by many researchers (cited in Korenchevsky, 1932). Upon castration, the male comb regresses in the absence of "testicular hormone." Injection of an endocrine-active material causes the comb to grow. The extent of the growth was used as a measure of potency of the test materials (all at the same dose).

Strengths

- Only a short time (five days) is necessary for its completion.
- The results are "precisely" expressed as increases in length and height of the comb.
- The same birds can be used for several assays since the comb regresses to its initial size after injections cease (Korenchevsky, 1932).

Weaknesses

- The comb is present in both sexes of chickens, although differing in size and appearance, and comb growth can be obtained in birds of both sexes after injection of the hormone (Korenchevsky, 1932). However, it is likely that comb growth in both sexes is sensitive to androgens. Injections of yolk or ovarian extract cause similar comb growth to that produced by testicular extract (Korenchevsky, 1932), so (in modern terms) estrogenic as well as androgenic compounds can cause comb growth. In fact, Champy (1930; cited in Korenchevsky, 1932) considered the hormone-producing comb growth assay to be specific and useful for both sexes.[2]

[2] From a more modern perspective, Robaire *et al.* (1979) reported that exposure to E2 in the male rat reduced prostate, seminal vesicle, and testis weights and epididymal sperm counts, with the effects likely due to reduced serum luteinizing hormone (LH) and therefore reduced T from the inhibiting action of estrogens at the hypothalamus-pituitary (H-P) level in males. Also, in support of the effects of E2 on males, Hess *et al.* (1997) identified E2 receptors in the testis, efferent ducts, epididymides, and prostate of the male mouse. Male mice carrying a null mutation in the E2α-receptor gene were infertile (Hess *et al.*, 1997). The complexity of the feedback loops from sex steroid hormones to the H-P axis in birds created difficulties in interpretation of the results of the comb test without knowledge on the feedback from the hypothalamic-pituitary-gonadal (H-P-G) axis.

- An extract that is inactive on comb growth can be active on the growth of genital organs of castrated guinea pigs and vice versa (Korenchevsky, 1932). From the perspective of 2006, this is not necessarily a disadvantage; different organs in different species may be differentially sensitive to different androgens.
- Although low doses give satisfactory results in this assay, high doses of testicular preparations produce "exceedingly irregular results" (Gallagher and Koch, 1930; cited in Korenchevsky, 1932). For example, when 45 capons were injected with the same dose of the same testicular preparation, the results were 0% comb growth in 2.2%, 2 mm in 13.3%, 3 mm in 15.5%, 4 mm in 28.8%, 5 mm in 28.8%, 6 mm in 4.4%, and 7 mm in 6.6%. This is a useful model since the data sort into a bell-shaped curve, with an overall mean of ~3.8 mm, and individual variability in response is the rule, not the exception. However, these data probably imply that a large number of animals must be tested to obtain sufficient statistical power to detect an effect, which is a significant drawback for its use as a routine assay.

5.1.2 Mammalian assays

Mammalian assays were also being proposed or developed at this time (late 1920s, early 1930s) to identify testicular hormones. Korenchevsky (1932, with references therein) describes them as follows:

- In rats: assessment by sperm motility tests, prostate cytology, seminal vesicle cytology, vas deferens cytology, and Cowper's gland cytology;
- In mice: assessment by seminal vesicle cytology; and
- In guinea pigs: assessment by electroejaculation test.

These tests in the various species were viewed as satisfactory by researchers during this time (late 1920s to early 1930s) (*e.g.*, Korenchevsky, 1932, and others) to detect testicular hormones. However, Gallagher and Koch (1930; cited in Korenchevsky, 1932) criticized them as taking too long, lacking quantitative precision, and the need for operating on new animals for each assay. Two additional assays were proposed by Korenchevsky *et al.* (1932):

- Measurement of the size of the prostate and/or seminal vesicles in rats; this assay was discarded due to contradictory results (Korenchevsky, 1932, with references within); and
- Weighing the prostate plus seminal vesicles and the penis in castrated rats.

5.2 Evolution of the Rat Assays

5.2.1 Development and use of the male reproductive accessory sex organs (ASO)

In 1932, Korenchevsky described in detail procedures for the assay above and provided data on organ weights (not linear measurements, which were considered

unsuitable because of the difficulty in accurately measuring glands of irregular shape and the need for length and height or width to characterize the organ's size) of castrated rat males with and without injection of testicular hormone. Korenchevsky weighed the prostate with seminal vesicles, the penis, adrenal glands, thyroid gland, hypophysis (pituitary gland), and retroperitoneal fat. The selection of organs was based on the atrophy of the prostate, seminal vesicles, penis, and thyroid with castration; the hypertrophy of the adrenals and hypophysis with castration; and the increase in retroperitoneal fat in castrated male rats and their return to normal with injections of testicular hormone. He standardized the weights of the organs as relative to a rat body weight (200 g), used matched pairs of rats from the same litter (one with and one without injection), standardized the diet, and standardized the route and duration of injections (SC, for six to seven days). However, he did not standardize the age at castration or the time from castration to the start of injections, indicating only that "the pairs differed in age and in the period of time since castration," and also did not standardize the age at termination (Korenchevsky, 1932; p.421).

Korenchevsky and Dennison (1935a) evaluated the effects of the newly discovered androgen, transdehydroandrosterone (previously, only androsterone, T, and oestrone were known to be present in reproductive organs and urine), on gonadectomized male and female rats. For the males, there was no information on the age at castration or the time from castration to treatment. The duration of treatment was 7 or 21 days, with sc injections twice daily of the test material in sesame oil (at three different doses) or sesame oil alone (vehicle control). At necropsy (specified at 57-65 days or 68 and 86 days of age), body weights and the weights of the seminal vesicles, prostate, seminal vesicles plus prostate, penis, preputial glands, adrenal glands, hypophysis, thymus, liver, kidney, and heart were recorded. Korenchevsky and Dennison (1935a) also recognized that the principle and approach of this male assay could be applied to a female assay to identify estrogens (and anti-estrogens). They used each assay to inform and enhance the other as they built the understanding of the hypothalamic-pituitary-gonadal axis.

For the females, the following information was provided. Ovariectomy occurred "before sexual maturity," and injections were begun 14-34 days after ovariectomy and continued daily for 21 days. The females were terminated at 61-77 days of age. The female body weights and weights of the following organs were recorded: uterus (in diestrus), cervix, vagina, preputial glands, adrenal glands, thyroid glands, hypophysis, thymus, liver, kidney, heart, and retroperitoneal fat. This study was important for two reasons:

- Korenchevsky and Dennison (1935a) defined a "rat unit" (a specific level of quantitative response on specific organs) as resulting from 8γ of T, 20γ of androsteronedial, 170γ of androsterone, and 940γ of transdehydroandrosterone to characterize relative potency. They also refined Korenchevsky's previous work on the comparison of capon units and rat units for relative potency of various androgenic/anti-androgenic compounds (Korenchevsky *et al.*, 1932, 1935).
- This work on ovariectomized female rats, with or without injections, was the first step toward the development of the uterotrophic assay in mature

ovariectomized female rats for detection of estrogenic (and anti-estrogenic) compounds.

Korenchevsky *et al.* (1933) and Korenchevsky and Dennison (1935a,b) continued to explore the potency of various testicular hormonal preparations, the effect of differing durations from castration to treatment, and of differing durations of treatment. He and his co-authors began to specify the age at castration (*e.g.*, 22-29 days of age), and the duration between castration and treatment (*e.g.*, 28-42, 32-75 days). Korenchevsky *et al.* (1937) also examined the response of castrated and ovariectomized rats to prolonged treatment with TP which had been shown to be the most effective of the T metabolites (esterification increased the androgenic activity of T) from short-term treatment studies. TP also prolonged the duration of the effect of a single injection or two injections (five days apart), with the maximum effect observed in the 11th day after the first injection, with activity lasting two to four weeks, versus injection of T, with the maximum effect observed "a few days" after the injection and activity gone by the 11th day postinjection. They, therefore, dosed the male rats with TP or T for 23 days and terminated the males one or nine days after the last injection. TP was more effective than T, and the changes persisted (partially or completely) out to nine days after the last dose.

Korenchevsky *et al.* (1937) also dosed ovariectomized females with TP, T, estrone, or TP plus estrone. The age of the females at castration was not provided, nor was the interval from castration to start of treatment. The duration of treatment was 21 consecutive days, and the females were terminated at 56, 61, 82, or 106 days of age. At the necropsy, the typical list of organs were weighed, plus the addition of vaginal smears to detect vaginal cornification (*i.e.*, persistent estrus) and examination of the uterus for distension (at least) to enlarged and thickened horns. The authors reported that the vaginal cornification test was "unreliable and insufficient;" organ weights plus histopathology were both "satisfactory and accurate." TP caused more profound estrogenic effects in the female than estrone or T. TP plus estrone resulted in abnormally large vaginas and female preputial glands, with histological changes in the uterus and vagina "reminiscent of the changes observed during pregnancy in normal rats," and the peripheral end of the female uterus (with TP or T injections) reportedly developed into a structure "having the appearance of a clitoris" (Korenchevsky *et al.*, 1937).

Additional researchers in the 1930s continued to evaluate both the chemicals in the assay and the castrated male assay itself in the rat (early work did not specify the rat strain), varying the numbers per group, number of groups, the age at castration, the duration between castration and the start of treatment, duration of the treatment, duration from end of treatment to termination, which organs were weighed, and organ weights fresh or fixed (usually in Bouin's fixative). Organ weight data were presented as absolute weight in mg or gram, and/or in mg or g per gram body weight, per 200 g body weight, or per kg body weight (*e.g.*, David *et al.*, 1934; Callow and Deanesly, 1935; Bülbring and Burn, 1935; Dingemanse *et al.*, 1935; Deanesly and Parkes, 1936).

Deanesly and Parkes (1936) used rats castrated at 40-50 g (likely on PND 21 at weaning, based on the weight) and used "not less than one month later," with administration of one of 11 androgen-like compounds by sc injection for ten days. At termination, prostate and seminal vesicles, fixed in Bouin's, were weighed. Several compounds were administered at different doses, with dose-response curves presented.

Responses were specific for the organs weighed. Dingemanse *et al.* (1935) used rats castrated at 21-25 days of age and not used until 6-6.5 months of age. Five rats/group were dosed with urine or testicular extracts for 24 days by twice daily sc injections. At termination (age not specified), the prostate, seminal vesicles, and penis were weighed.

Greene and Burrill (1940) castrated immature rats at 32-38 days of age. Twenty rats/group were administered TP by sc injection once 24 hours after castration. At termination, 24 hours after the single dose, some organs did respond significantly at higher doses. The prostate weight increased by 30%, and seminal vesicle weight increased by 16.5%. Considerable variability in organ weight was present within groups. In a second paper, Greene and Burrill (1941) used immature rats castrated at 22-24 or 33-38 days of age. Castrated rats were administered TP by sc injection 48 hours postcastration and terminated 48 hours after injection, with seminal vesicles weighed. Organ weight variability was greater at 33-38 days of age than at 22-24 days of age. There were statistically significant differences between organs from treated versus nontreated castrates evaluated at 22-24 days of age.

Mathieson and Hays (1945) used immature Wistar male rats as well as a series of castrations at different ages, recovery times, and dosing times. The animals were treated with TP by sc injection during several time periods for two- to five-day dosing durations. At necropsy, only seminal vesicles were weighed. Their results indicated that atrophy of the seminal vesicles was a slow process once systemic growth had been stimulated after days 35-38. They concluded that rats castrated at 50 days of age were unsuitable due to slow regression of the seminal vesicles after castration and inconsistent responses to TP (a very potent androgen). They strongly recommended the juvenile castrate.

5.2.2 Development and addition of male musculature endpoints

In the late 1940s and early 1950s, Eisenberg and Gordan (1950) and others published a number of papers on the "myotrophic assay" in rats. This assay evaluated the protein anabolic activities of androgens by measuring the oxygen uptake and/or the weight of the androgen-dependant levator ani muscle in castrated rats receiving different androgens. In 1949, Eisenberg *et al.* used the Long-Evans and Slonaker-Wistar rats (four to five rats per group), castrated them at 30 days of age (prepubertal) with no apparent recovery period, and dosed them by sc injection once daily for 30 days with one of nine compounds plus pituitary growth hormone. They reported the oxygen uptake and weights of the liver and levator ani muscle (the weights of other sex accessory glands were not reported; the focus was on anabolic activity). Eisenberg and Gordan (1950) used the Long-Evans rat, castrated at approximately 30 days of age (prepubertal), with a 23-day recovery. The rats were then dosed by sc injection for seven consecutive days with one of nine compounds, including estradiol dipropionate. The authors concluded that the levator ani weight was a reasonable indicator for anabolic activity from androgenic substances, but its growth was not wholly androgen dependent. Seminal vesicles were also weighed. The seminal vesicle response was androgen dependent, although estradiol also stimulated seminal vesicle weight. As the assay evolved, the response of the levator ani muscle (wet or dry weight) was termed the myotrophic response, and the response of the sex accessory glands (initially weighed as one unit, then separately) was termed the androgenic response. Gordan *et al.* (1951) reported that

different androgens expressed different myotrophic and/or androgenic potencies. For example, methylandrostenediol was a potent protein anabolic steroid with little androgenic activity using this assay.

The LABC muscle, shown to be responsive to castration and exogenous T by changes in mass, is now one of the five androgen-dependent target organs to be weighed in the current, standardized Hershberger assay. It is especially important since the target tissues differ in their response to T (*e.g.,* LABC) versus their response to DHT converted from T (by irreversible reduction by 5α-reductase) and a more avid ligand for the AR (*e.g.,* ventral prostate and seminal vesicles). Joubert *et al.* (1994) also showed that T induced initial masculinization of the rat levator ani muscle during puberty (important for the intact weanling version of the Hershberger assay). In addition, trenbolone is a potent synthetic androgen and anabolic agent (like T) *in vitro* and *in vivo* after SC injection (and approximately 100 times less active if administered orally). This first pass metabolism (in the liver after oral administration) acts at the 17β-hydroxyl group that is not protected from metabolic activity by the presence of a 17α-methyl or 17α-ethinyl group. However, due to its structure, it cannot be activated metabolically to a structure comparable to DHT. In support of the T-like (but not DHT-like) function of trenbolone, it causes significant increases in absolute weights of the glans penis, LABC, and Cowper's glands, but not of the ventral prostate or seminal vesicles (Freyberger *et al.,* 2005). In fact, the responsivity to T versus DHT is one of the bases for the separable androgenic and myogenic responses observed in different tissues and a major reason to retain the weight of LABC as an important androgen-dependent endpoint.

5.3 The Hershberger Assay

Hershberger *et al.* (1953) improved and standardized the prepubertal castrated male assay as follows.

- Male rats were castrated on PND 21 (day of weaning).
- The castrated rats were dosed with the test material by sc injection for seven consecutive days, beginning on the day of castration (no recovery period).
- On the eighth day postcastration, 22-26 hours after the last injection, the animals were terminated.
- The levator ani muscle, ventral prostate, and seminal vesicles (free of the coagulating glands) were dissected from each male and weighed.
- Dry weights of the levator ani muscle were also recorded after desiccation at 72°C.
- The results, expressed as organ weights versus a range of doses of each of many test materials, provided information on the relative potency of each test material for androgenic activity (from ventral prostate and seminal vesicle weights) and/or for myotrophic activity (from the levator ani muscle weight).

In 1954, Eisenberg and Gordan reasoned that: (1) since the growth of the levator ani muscle of castrated, immature male rats was induced by various anabolic agents, (2)

since the amount of growth paralleled the anabolic potency of these agents, and (3) since purified pituitary growth hormone extract also stimulated growth of the levator ani, then it is likely that the muscle could be used as an index of changes in nitrogen balance induced by steroids and other growth-promoting substances. Therefore, they studied whether the levator ani muscle changes also reflected the loss of protein that accompanied growth-inhibiting conditions, such as hypothyroidism. Since small doses of thyroid hormone are known to be anabolic in hypothyroid rats and large doses are catabolic, the weight of the levator ani muscle was measured after induction of hypothyroidism, after hypothyroidism treatment with low doses of thyroid hormone, and after induction of severe hyperthyroidism. The effect of the catabolic steroid, cortisone, was also studied. Long-Evans rats were divided into seven groups:

- Normal controls
- Hypothyroid rats with intact gonads (made hypothyroid by 0.1% propylthiouracil in the drinking water during the second month of life)
- Hyperthyroid rats with intact gonads (made hyperthyroid by feeding each rat 30 mg of thyroid substance every other day from the 35th to the 60th day of life; the thyroid-dependent oxygen consumption in liver slices for these rats was two-fold the value of liver slices from normal animals)
- Castrate controls (castrated on PND 30; prepubertal)
- Castrate hypothyroid animals
- Castrate hypothyroid rats treated with 2 mg desiccated thyroid substance daily from the 53rd to the 60th day of life
- Castrate animals treated with cortisone (0.1 mg of cortisone in sesame oil) by sc injection for seven days preceding necropsy

All animals were terminated by decapitation on the 60th day of life, and the levator ani and seminal vesicles were removed and weighed. Their results were as follows:

- Relative to the normal control values, terminal body weights were reduced in the hypothyroid and hyperthyroid rats with intact gonads.
- In the hypo- and hyperthyroid animals with intact gonads, the weights of the levator ani muscle and the seminal vesicles were reduced relative to the normal control values (but see #1 result).
- In the hyperthyroid group, body weight and weight of the levator ani were both lower than those of castrate rats (the hyperthyroid group values were approximately normal for rats at 30 days of age, the age at castration).
- The seminal vesicles in the hyperthyroid group weighed 2.5 fold more than in the castrate animals.
- In the castrate hypothyroid group, body weight was statistically equivalent to that in the hypothyroid group with intact gonads but lower than that of the intact or castrate controls.

- In the castrate-hypothyroid group, the levator ani muscles weighed only slightly less than those in the castrate control rats, but only 50% of the weight of the levator ani in the hypothyroid group with intact gonads.
- Administration of thyroid substance to the castrate hypothyroid group resulted in restoration of the weight of the levator ani muscle and gain in body weight, but there was no effect on the weight of the seminal vesicles.
- Injection of cortisone for seven days prior to termination caused further atrophy of the already atrophic levator ani muscle ($p<0.01$) but did not affect the weight of the seminal vesicles.

The authors concluded that the weight of the seminal vesicles was completely dependent on the presence of androgen, but that the growth of the levator ani was not solely an androgenic phenomenon since the levator ani muscle in the castrate rat grew with treatment with thyroid hormone, with pituitary growth hormone, with nonandrogenic doses of methylandrostenediol (Gordan *et al.*, 1951), or with nonandrogenic molecules. In all of these cases, the weight of seminal vesicles remained unchanged, so the assumption was that there was no androgenic activity.

In addition, Hershberger-like protocols for the pharmaceutical industry (*e.g.*, Dorfman, 1969a,b) and a regulatory screen for steroidal androgens (Hilgar and Vollmer, 1964) have been published. More recently, Ashby and Lefevne (2000a), Yamada *et al.* (2000), and Yamasaski *et al.* (2001a) have investigated protocol variables with weak anti-androgens.

The Hershberger assay, employing peripubertal castrated male rats, is described in detail in Chapter 2.0. Its strengths are its specific nature (it can identify mechanism), its relative speed (the males are castrated at 42 days of age, treated for ten days starting 12 days postcastration from PND 53-54 to 64-65, and terminated immediately after treatment ceases, Gray *et al.*, 2005; or castrated at 45 days of age, treated for ten days, starting seven days after castration and terminated on one day [24 hours] after the last treatment; Freyberger *et al.,* 2005]), and relatively low cost (Hershberger *et al.*, 1953; Dorfman, 1962a,b; Dorfman and Dorfman, 1963; Dorfman and Kincl, 1963).

5.4 Detection of Anti-androgens

5.4.1 Definition, actions, and examples

Anti-androgens are defined as substances that act to antagonize, prevent, block, or reduce the activity of an androgen (usually endogenous). These substances can act by:

- Competitive binding to the AR, thereby displacing the endogenous androgen on the AR or preventing the endogenous androgen from binding to the AR and initiating the stimulatory cascade (these agents are termed androgen antagonists).
- Interfering with the synthesis of the endogenous androgen (at the level of cholesterol biosynthesis, the integrity of the mitochondria, steroidogenic acute regulatory [StAR] protein, and the steroidogenic enzymes).
- Interfering with the transport of the endogenous androgen via steroid binding globulin, α-fetoprotein, albumin, etc.

- Increasing the metabolic degradation of endogenous androgen by induction of metabolizing enzymes predominantly in the liver (typically accompanied by hepatocellular hypertrophy).
- Preventing or reducing the conversion of T to DHT, which more avidly binds to the AR and is considered more potent than T, especially during reproductive organogenesis perinatal stage (but note that for some ASO, such as the LABC, T is more stimulatory than DHT).

Examples of substance activity in one or more of the five modes of action include:

- Competitive binding: *p,p*'-DDE (1,1-bis-[4-chlorophenyl]-2,2-dichloroethylene) (Freyberger *et al.,* 2005), vinclozolin (after metabolic activation) (Freyberger *et al.,* 2005), linuron and procymidone (Gray *et al.,* 1999, 2006), polybrominated diphenyl ethers (PBDES) (Stoker *et al.,* 2005), spironolactone, flutamide and RU486 (Charles *et al.,* 2005), diethylstilbestrol (DES), estradiol (Kelce *et al.,* 1995; Kelce and Wilson, 1997), linuron and fenitrothion (Tamura *et al.,* 2001).
- Interfering with synthesis: a series of phthalate diesters (*e.g.,* diethylhexyl-phthalate [DEHP], dibutylphthalate [DBP], butylbenzylphthalate [BBP]) and prochloraz (Wilson *et al.,* 2004).
- Interfering with transport: The human has SHBG throughout life, the rat only has SHBG pre- and perinatally, and both genera have the other less effective transport proteins such as albumin and α-fetoprotein. There have been no compounds identified as interfering with transport by any mechanism other than spontaneous mutations or knock-outs.
- Increasing metabolic degradation: *p,p*'-DDE (Freyberger *et al.,* 2005; Freyberger *et al.* 2007), benzo(a)pyrene (Charles *et al.,* 2005) both via liver enzyme induction and hepatocyte hypertrophy.
- Preventing or reducing the conversion of T to DHT: finasteride (inhibits 5α-reductase)

5.4.2 Anti-androgens and the Hershberger assay

The Hershberger assay, using the weanling, peripubertal, or adult castrate model, will not detect agents that interfere with steroidogenesis (*e.g.,* the phthalate esters; Foster *et al.,* 2001) since the testis (predominant site of steroidogenesis) is removed, but it can detect those chemicals requiring metabolic activation for *in vivo* activity (*e.g.,* vinclozolin) or chemicals rapidly degraded from induction of liver enzymes (*e.g., p,p'*-DDT). Fryberger *et al.* (2005) examined livers histopathologically from castrated males treated with *p,p'*-DDE and detected hepatocyte hypertrophy and increased weights of the liver, kidneys, and adrenal glands. They also detected dose-related increases in hypertrophy of the thyroid follicular epithelium from exposure to *p,p*'-DDE, and concurred with Chowdury *et al.* (1984) and Yamada *et al.* (2004) that the Hershberger assay could be used to assess thyroid effects if the protocol was "amended accordingly" (Freyberger *et al.,* 2005; p. 138).

An anti-androgen is detected in the castrate Hershberger assay by co-administration of a potent androgen (typically TP at 0.4 mg/kg/day by SC injection, methyl testosterone, AR agonist R-1881 [methyltrienolone, a synthetic androgen], and the possible anti-androgen test compound. Other castrate groups only received vehicle (negative control) or only the androgen (positive control). The comparison is made for the androgen-dependent organ weights (the typical five discussed previously) for the vehicle control versus the positive control group (to define maximal response) and for the positive control group versus the group with both androgen and the possible anti-androgen test compound. If the test compound plus androgen group exhibits lower androgen-sensitive organ weights versus the positive control group, then the test material interferes with the action of the exogenous androgen and is therefore anti-androgenic. *In vitro* AR receptor binding assays have also been performed (*e.g.,* Freyberger and Ahr, 2004, on 50 chemicals; Fang *et al.,* 2003, on 202 chemicals) to detect both androgenic and anti-androgenic compounds. They were successful in detecting receptor-mediated androgen agonists and antagonist activity *in vitro* but did not detect binding from phthalate esters (mechanism does not involve AR receptor binding) or from vinclozolin (which requires metabolic activation to generate androgen-active metabolites).

The rodent Hershberger assay was first described for use as a screening agent for androgenic and myotrophic agents (Hershberger *et al.*, 1953). From that time to the present, the Hershberger bioassay has been primarily used by the pharmaceutical industry (by Hershberger and others) as a screen to identify strong androgens/anabolic agents and by modifying the screen, as described above, to use T supplemented castrated rats to identify effective anti-androgens. Neumann and his co-workers at Schering AG (Berlin) published a series of papers in the 1960s and 1970s demonstrating almost all of the reproductive endpoints affected by anti-androgens and almost every aspect of the anti-androgen phenotype (*e.g.,* androgen insensitivity; Chapter 3.0).

Pharmaceutical investigations of 5α-reductase inhibitors followed. In the 1990s, industrial and environmental chemicals were identified with androgen-like and anti-androgen-like activity (*e.g.,* Kelce *et al.,* 1994, 1995, for vinclozolin; Lambright *et al.*, 2000, Soto *et al.,* 2004, Wilson *et al.,* 2002, and Durhan *et al.,* 2005 for 17β-trenbolone acetate, an androgenic feedlot contaminant; and Durhan *et al.,* 2002 and Ellis *et al.,* 2003 for androstenedione, the androgenic component of contaminated river water downstream from a pulp and paper mill effluent. These findings in industrial and environmental chemicals were the major impetus for the international initiatives in developing and validating a robust protocol to detect these effects and which can be considered as the basis for an OECD test guideline (Freyberger *et al.,* 2005).

6.0 TEST PROTOCOL PROCEDURES AND SYNOPSIS

6.1 Procedural Variables

This section has been written from the standpoint of the castrate models for which a wider literature is available.

6.1.1 Species/Strain

The Hershberger assay was initially introduced in rats (Hershberger *et al*., 1953), and the early papers (1920s-1950s) usually did not provide strain information. Mice were also evaluated for use for androgenic (Dorfman, 1969a) and anti-androgenic (Dorfman, 1969b) activity. Since then, the rat has been the species of choice.

Yamasaki *et al*. (2001a) evaluated strain sensitivity differences in the Hershberger assay based on the presence and degree of alteration in the weights of ASO in castrated males from three rat strains; Fischer 344 (F344; inbred), Sprague-Dawley (SD; outbred), and Wistar (outbred). The rats were castrated at 49 days of age, and eight days later began the ten-day treatment (from 56 days of age through 65 days of age). The rats were terminated approximately 24 hours after the last dosing by ether anaesthesia and exsanguination. The negative control group for each strain was comprised of intact rats given vehicle, and a second vehicle control group was comprised of castrated rats given vehicle. The positive control group was comprised of castrated rats given TP. The treated group for each strain received both TP and FLU. At necropsy, the authors measured terminal body weight, ventral prostate, seminal vesicle, bulbocavernosus/ levator ani muscle, glans penis, and Cowper's glands. The rats were orally administered one dose level of FLU (a known androgen antagonist) at 3.2 mg/kg and, for the positive control group, TP by sc injection at 0.4 mg/kg.

There were no abnormalities noted in clinical signs *in vivo*, body weights, or gross findings at necropsy in any strain or group. In all three strains evaluated, there were no effects on the weights of any of the ASO in the intact vehicle control group, no differences among strains for reduction in ASO weights in the castrated vehicle control group, and no differences in the positive control group (castrated rats given TP only). The TP plus FLU group in all three strains exhibited significant reductions in the weights of the ventral prostate, seminal vesicles, bulbocavernosus/levator ani muscle, and Cowper's glands. The SD and Wistar rats in this group also exhibited a significant reduction in the weight of the glans penis. Analysis of covariance (ANCOVA) between absolute organ weights and body weights across strains indicated significant effects between the SD and F344 rats and between the Wistar and F344 rats.

Specifically, the interaction in the ventral prostate, seminal vesicles, and glans penis weights and strain by two-way ANOVA was significant between SD and F344 rats and between Wistar and F344 rats, but not between SD and Wistar rats. In short, the SD and Wistar reacted similarly, and the F344 responses were different from both the SD and Wistar strains. The authors concluded that "these findings demonstrate that F344 rats are

less suitable than SD or Wistar rats for detecting FLU-induced changes." They suggested that, at least for the Hershberger assay, outbred strains may be more sensitive, and that the difference in sensitivity may be due to the smaller size of the F344 rat, and lower body weights and concomitantly lower weights of the accessory sex glands. It is not clear whether the implication by the authors is that it is a detection problem rather than a sensitivity problem (Yamasaki *et al*., 2001a).

O'Connor *et al*. (1999) compared the CD (SD) and Long-Evans rats in detection of the environmental anti-androgen, *p,p'*-DDE, using the EPA (EDSTAC) screening battery and Hershberger assay, and found both strains to be similarly sensitive. Both strains exhibited reduced weights of the seminal vesicles and prostate, with reduction of the seminal vesicles weight statistically significant in both strains. The reduced prostate weight was statistically significant only in the Long-Evans rat. You *et al*. (1998) reported strain differences in sensitivity to DDE for the SD versus the Long-Evans hooded rats after *in utero* and lactational exposure. Ashby and Lefevre (2000a) employed the Alpk:APf SD (AP) rat from Astra Zeneca Pharmaceuticals, with comparable sensitivity.

6.1.2 Age at castration

Age at castration varied across studies and researchers as follows:

- Hershberger *et al*. (1953) castrated their rats at weaning (PND 21), long before acquisition of puberty, as did Eisenberg and Gordan (1950) and Gordan *et al*. (1951). Rats were also castrated at weaning by Wakeling *et al*. (1981) and Snyder *et al*. (1989) for use in the Hershberger assay.
- Yamada *et al*. (2001) castrated their rats at three weeks (*i.e.*, weaning; Experiment 4), six weeks (Experiments 2 and 3), and at ten weeks of age (Experiments 1, 2, and 3) after one week of quarantine (so the rats two weeks old arrived with their dams). The authors concluded that castrated rats at all three ages could detect the anti-androgenic effects of FLU and *p,p'*-DDE (Yamada *et al*., 2001).
- Gray *et al*. (2005) castrated their rats at "peripuberty" on PND 42 (removing both the testes and epididymides), with the rationale that earlier castration interfered with subsequent PPS. This timing was adopted for the OECD Hershberger assay validation.
- Ashby and Lefevre (2000a), Kang *et al*. (2004), and Charles *et al*. (2005) castrated their rats at six weeks of age (PND 42).
- Young adults (less than four months old) were also castrated for use in the Hershberger assay by Raynaud *et al*. (1980) and Shao *et al*. (1994).
- Kelce and Wilson (1997) castrated rats at 125 days old.
- Ashby and Lefevre (2000a) also used rats castrated at 126-127 days of age. Adult animals were castrated for this assay at a number of ages (*e.g.*, O'Connor *et al*., 1999; also see Chapter 5.0, History of the Assay, for the early adult Hershberger assay procedures).

For animals castrated and immediately treated, the endpoint is whether or not the ASO regressed or continued to grow normally in the presence of the test chemical (if castrated prior to puberty). For animals castrated with a delay before initiation of treatment (to allow the ASO to regress/involute), the endpoint is whether the organs of interest have grown in animals exposed to test chemical relative to the regressed ASO weights in the castrated negative control group.

As an aside, a number of researchers have suggested intact (noncastrated) prepubertal males (*e.g.*, Kelce and Wilson, 1997; Monosson *et al*., 1999; Stoker *et al*., 2000; Ashby and Lefevre, 2000b). Since these rats have an intact H-P-G axis and have not yet achieved puberty, they can detect androgens or anti-androgens by accelerated or inhibited sex organ growth, by development of androgen (T, DHT)-dependent ASO, and by accelerated or delayed puberty. This animal model is also sensitive to metabolic modulators and 5α-reductase inhibitors, as well as chemicals that act at the level of the hypothalamus and/or pituitary, and addresses animal welfare concerns.

In summary, there are strengths and weaknesses for each age at castration:

- Early castration prior to puberty allows for immediate initiation of treatment (no need for a regression period) at a time when the T and DHT tissues are ready to become (or are becoming) sensitized to androgens (endogenous or exogenous). However, Gray *et al.* (2005) noted that in his laboratory, some rats castrated prior to PND 42 failed to initiate or complete PPS, even in the presence of potent androgens, so castration on PND 42 was adopted for the OECD assay validation.
- Castration at and after PPS (~PND 42) requires a delay before initiation of treatment to allow the ASO to regress/involute. However, many researchers have reported appropriate responses to exogenous androgens in rats castrated as young adults.

6.1.3 Recovery period

The recovery period between castration and the start of treatment is also variable in the Hershberger assay. Hershberger *et al*. (1953) and other workers (Kelce *et al*., 1997) used no recovery period, instituting treatment on the day of or the day after castration. Yamada *et al*. (2000) inserted capsules containing T (as the positive control) subcutaneously through a dorsal incision immediately after castration. Subsequent administration of test chemicals began seven days later (Yamada *et al*., 2000). Seven days for recovery from the surgery and to allow involution of the ASO (in the absence of T) is relatively common (*e.g.*, Snyder *et al*., 1989; Shao *et al*., 1994). Ashby and Lefevre (2000a) used eight days of recovery ("to insure complete recovery and healing from the operation," p. 93). Charles *et al*. (2005) used ten days, and O'Connor *et al*. (1999) recommended 11 days of recovery. Gray *et al*. (2005) recommended 12 days of recovery; this duration was adopted for the OECD Hershberger assay validation. Prior to Hershberger's assay, as presented in 1953, Eisenberg and Gordan (1950) waited 23 days postcastration before beginning treatment.

In summary, the duration of the regression period, prior to initiation of treatment for males castrated at or after puberty, extends from seven days (shortest) through 12 days

(adopted for the OECD assay validation) out to 23 days (early in the assay's development). All of the studies and researchers cited indicated appropriate responses to androgen from castrated males, with at least seven days of holding after castration to allow regression of the ASO prior to initiation of treatment.

6.1.4 Start and duration of treatment

Based on the length of the recovery period (see above), the age of the rats at the start of treatment range from 21 days of age (at weaning immediately after castration) to 54-55 days of age (Gray *et al*., 2005; adopted for the OECD Hershberger assay validation). The duration of treatment has been from three days (Peets *et al*., 1973), four days (Rittmaster *et al*., 1991; Ashby and Lefevre 2000a), five days (Kelce *et al*., 1997; Yamada *et al*., 2000, 2001), seven days (Hershberger *et al*., 1953; Ashby and Lefevre, 2000a), ten days (Snyder *et al*., 1989; Gray *et al*., 2005), 11 days (Ashby and Lefevre, 2000a), to 14 days (Shao *et al*., 1994; Ashby and Lefevre, 2000a). Dorfman (1969b) suggested that a longer period of treatment is required for oral administration versus sc injection. In fact, he recommended 20 days of treatment by the oral route and ten days by the sc route (see below for routes of administration).

6.1.5 Routes of administration

The basic issue in choosing the experimental route of administration is that of relevance for human exposure and screening, as well as evaluating consequences of test chemical metabolism by the various routes. The most common routes chosen are SC injection and oral gavage. The increasing use of skin patches for human drug delivery has not yet translated into use of experimental cutaneous (dermal) application for endocrine disruptors.

The sc injection route for the test chemicals (dose volume ~ 1 ml/kg) appears to be very common (*e.g.*, Hershberger *et al*., 1953; Dorman, 1969b; Rittmaster *et al*., 1991; Ashby and Lefevre, 1999; Gray *et al*., 2005). The oral route (~ 5 ml/kg dosing volume) has also been widely used (*e.g.*, Peets *et al*., 1973; Snyder *et al*., 1989; Kelce *et al*., 1997; Yamada *et al*., 2000). As indicated above, Dorfman (1969b) suggested that a longer treatment period is required for oral versus sc injection administration (he recommended ten days by sc injection and 20 days by oral administration). Silicon capsules, placed subcutaneously, have also been used (*e.g.*, Yamada *et al*., 2000), usually for the positive control chemical (see below).

The oral route provides direct, first-pass metabolism/elimination through the liver (the hepatic portal system carries the blood from the GI tract directly to the liver). Therefore, if the parent compound is the active moiety, it will most likely be sequestered/metabolized/cleared rather rapidly by this route. If a metabolite is the active moiety, again it will be formed, likely in the liver, rapidly by the oral route of administration. The sc injection route of administration provides systemic exposure (once it gets into the circulatory system) without first-pass metabolism/elimination directly to the liver. If the parent compound is the active moiety, it will persist longer by non-oral routes of administration and only gradual access to the liver. If the active moiety is a metabolite, it will be formed more slowly (versus oral administration) and over time as the chemical reaches the liver from the systemic circulation. Sloan *et al.* (2002) evaluated gavage (per OS, PO) administration of methyl T and SC injection of TP

with or without the androgen antagonist FLU (PO) in castrated rats. Both routes worked well; methyl T (PO) was slightly less potent than TP (SC) in restoring the weights of the ASO. FLU was effective in blocking the androgenic effects of either methyl T or TP by either route. However, if the anticipated route of exposure is cutaneous (dermal), neither oral nor SC administration assesses the consequences of metabolic activity localized in the skin, such as dermal esterases, on the effects of the test chemical.

6.1.6 Reference androgens

T has been administered both as a silastic capsule implant (Kelce *et al*., 1997; Yamada *et al*., 2000) and by sc injection at doses up to 3 mg/kg/day to adult rats and total dose of 2.4 mg to weanlings (Dorfman, 1969b). TP has also been widely used at daily sc doses of 200 μg/kg (0.2 mg/kg; Wakeling *et al*., 1981; Ashby and Lefevre, 2000a), 0.25 mg/kg/day (Yamada *et al*., 2000), 0.4 mg/kg/day (Kang *et al*., 2004), and to 1 mg/kg (Shao *et al*., 1994). Other reference androgens that have been used include T enanthate (at ~ 2 mg/kg; Sunahara *et al*., 1987) and DHT (at 8 mg/kg/day; Snyder *et al*., 1989). MethylT (a potent androgenic pharmaceutical) has also been used by injection by Gray *et al*. (2004) and by oral administration (twice daily) by Sloan *et al*. (2002). The implants are inserted once and are present for the duration of the treatment period. The sc injections and oral administration are usually performed once daily for the duration of the period.

The reference androgens are used for two reasons: (1) as a standard against which the response of the ASO to the test chemical is compared; if there is growth, the test chemical is androgenic and the potency can be determined from comparison of the results in the test chemical group with the results from the reference androgen group; and (2) the reference androgen and test chemical are administered to the same animals simultaneously to measure the ability of the test chemical to compete with the reference androgen and block its androgenic action. If the weights of the ASO are lower in the group exposed to both and reference androgen and test chemical (less than maximal response) than in the group exposed to the reference androgen alone, then the test chemical is acting as an anti-androgen (antagonizing the effects of the reference androgen). The degree of inhibition can be determined from the comparison of ASO weights, in the presence of both the test chemical and the reference androgen, versus the ASO weights of castrated males in the presence of only the reference androgen.

6.1.7 Husbandry

There is concern that the endocrine system can be readily modulated by many experimental factors, including diet. Standard laboratory animal diets can contain high and variable levels of phytoestrogens which can modulate physiologic and behavioral responses similar and comparable to endogenous estrogen or exogenous estrogenic chemicals. Stokes (2004) concluded in his introduction to 11 papers on this topic that it is critical to select animal models and appropriate diets for endocrine disruptor studies that will provide optimal sensitivity and specificity to "minimize confounding experimental variables, increase the likelihood of replicable experimental results, and contribute to more reliable and relevant test systems." This section therefore examines the major husbandry variables.

The major husbandry components are:

- Feed
- Bedding
- Caging
- Light/dark cycles
- Drinking water (source modifications)
- Temperature and relative humidity
- Air changes

Feed

A number of different feeds has been used in androgenic (anti-androgenic), estrogenic (anti-estrogenic), and multi-generation toxicity studies in rats (**Table 6**). Some of the diets are certified, some are not, some are open formula, some are closed formula, some are semi-synthetic, some are specially formulated with varying levels of vegetable protein (usually soybean meal and yeast), some are soy free, some are phytoestrogen free (soy and alfalfa free with casein and corn oil as replacements). Many researchers have had the diets analyzed for genistein and daidzein (and sometimes glycitein), the main phytoestrogen components. Thigpen *et al.* (1999) analyzed (by HPLC) 12 rodent diets and six major ingredients for phytoestrogens (daidzein, genistein, formononetin, biochanin A, and coumestrol, with the last three phytoestrogens not detected). Three rodent diets recently formulated to reduce phytoestrogen content were also assayed. Soybean meal was the major source of phytoestrogens. They stated that high, variable concentrations of daidzein and genistein are present in some rodent diets, and dietary phytoestrogens have the potential to alter results of studies evaluating estrogenicity of chemicals. They also recommended that careful attention be given to diet phytoestrogen content, and their concentration(s) should be reported. They concluded that a "standardized, open-formula diet in which estrogenic substances have been reduced to levels that do not alter results of studies that are influenced by exogenous estrogens is recommended."

Table 6. Rodent Feeds

Designation	Vendor	Characteristics	References
Purina 5002 (Purina Certified Rodent Chow)	PMI Feeds (St. Louis, MO)	Daidzen: 131 ppm (114-167 ppm) Genistein: 128 ppm (113-139 ppm) Glycitein: 50 ppm (2000-2001)	Tyl *et al.,* 2002
		Daidzein: 149 ppm (99-209 ppm) Genistein: 164 ppm (108-222 ppm) Glycitein: 43 ppm (30-71 ppm) (2001-2002)	Tyl *et al.,* 2006
NIH-07 (No. 7022 CM "open formula"	Harlan-Tekland (Madison, WI)	12% soybean Daidzein: 98-127 ppm Genistein: 102-138 ppm	Tyl *et al.,* 2006
Purina Rodent Chow No. 5001 (not certified)	PMI Feeds, Inc. (St. Louis, MO)	No information	Gray *et al.,* 2000 Wilson *et al.,* 2002
Syn 8.IT	No information	Phytoestrogen free	Vinggaard *et al.,* 2002
L5 semi-synthetic diet	NRA (Jouy-en-Josas, France)	No phytoestrogens	Stroheker *et al.,* 2003
Rodent Diet D04 and D03	VAR (Epinay Sur Orge, France)	D04: 8.5% vegetable protein (soybean meal, yeast) Genistein: $3.9x10^{-3}$ % Daidzein: $2.3x10^{-3}$ % D03: 22.5% vegetable protein (soybean meal, yeast) Geinistein: $6.3x10^{-3}$ % Daidzein: $3.0x10^{-3}$ %	Stroheker *et al.,* 2003

(continued)

Table 6 (continued)

Designation	Vendor	Characteristics	References
Open Source Diets™	Research Diets, Inc.	Phytoestrogen free, purified ingredient	
Rodent chow (altromin 1324)	Altromin GmbH (Lage, France)	No information	Talsness *et al.* 2000; Schonfelder *et al.,* 2004
R&M No. 1	Special Diet Services (Witham, Essex, UK)	Not specified (used to maintain postweaning rats)	Ashby *et al.,* 2004b; Ashby and Lefevre, 1997
R&M No. 3	Special Diet Services (Witham, Essex, UK)	Not specified (used for preweaning rat pups)	Ashby and Lefevre, 1997
AIN-76 (American Institute of Nutrition)	PMI Feeds, Inc., Zeigler Bros., Harlan Tekland	Soy free	
MF and CRF-1	Oriental Yeast Co (Tokyo, Japan)	"Commercial diets"	Yamasaki *et al.,* 2003
NIH-31	Purina Mills, Inc. (St. Louis, MO)	Supplemented with soy and alfalfa Daidzein: 30.4 μg/g Genistein: 31.9 μg/g	Latendresse *et al.,* 2001
NIH-31C	Purina Mills, Inc. (St. Louis, MO)	Phytoestrogen free (protein contributed by soy meal and alfalfa replaced by casein, and soy oil replaced by corn oil)	Ainoo *et al.,* 2007
SK96	Purina Mills, Inc. (St. Louis, MO) (modification of NIH-31)	Soy and alfalfa free (replaced by casein and corn oil) Daidzein: 0.3 μg/g Genistein: 0.54 μg/g	Latendresse *et al.,* 2001

Thigpen *et al.* (2003) subsequently grouped diets into Group 1 (low phytoestrogen, 0.20 μg/g): AIN-76A, PMI SK96, HSD (Harlan Sprague Dawley) 20145, 2016 S, HSD2919, Zeigler 5412-01, and 5412-00; Group 2 (medium phytoestrogens, 01-210 μg/g): AIN-76A soy, PMI 5058, HSD 20185, NIH-31, NIH-07, NTP 2000; and Group 3 (high phytoestrogen, 270-370 μg/g): PMI 5002, 5053, 5015, HSD 7012, 8656, 8760, and 7004. Immature female CD-1 mice were weaned on PND 15 and fed the various Group 1, 2, and 3 diets from PND 15 through 30. Vaginal opening (VO) was assessed on PND 22, 24, 26, 28, and 30. The groups, based on total daidzein and genistein (low to high in μg/g) also corresponded to the acceleration in acquisition of VO, with the diets in Groups 1-3, and the associated ranges of percent of mice with VO as follows (Thigpen *et al.,* 2003):

Group	PND 22	PND 24	PND 26	PND 28	PND 30
1	0-10%	13-37%	20-47%	26-80%	86-100%
2	0-68%	13-95%	27-96%	90-100%	60-100%
3	17-60%	53-90%	60-100%	67-100%	80-100%

The Group 1-3 (low to high) diets also correspond to low to high levels of daidzein, genistein, and equol in the plasma and urine. Thigpen *et al.* (2004) provided the following conclusions:

- "Rodent diets differ significantly in estrogenic activity, due primarily to the phytoestrogen content that is directly proportional to the level of soybean meal in the diet.
- The phytoestrogen content can vary three- to six-fold between different batches of the same diet producing significant differences in the time of VO in CD-1 mice and in F344 rats.
- Diets high in phytoestrogens can have the same effects on the time of VO and uterine weight in mice as diets containing 4 or 6 ppb of added DES, and this factor may mask the ability to detect weak but significantly estrogenic substances.
- Dietary estrogens affect the timing of sexual development in rodents and can influence outcomes of reproductive, carcinogenicity, and endocrine disruptor studies.
- Dietary estrogens, including the phytoestrogens daidzein and genistein and total metabolizable energy significantly accelerate the time of VO and increase uterine weights in CD-1 mice, thus altering the results of endocrine disruptor studies.". In the uterotrophic validation program it was noted that

mice were more sensitive to EDCs than rats and immature animals were more sensitive than adults.

If dietary estrogens can alter endocrine-related endpoints, then a phytoestrogen-free diet, defined as <20 μg/g, should be selected that contains a low level of metabolizable energy and consequently will not cause any of the following effects: reduced sensitivity of the assay for conducting estrogen bioassays comparing the time of VO; affect uterotrophic assays where increases in uterine weight are measured; or influence assays with molecular endpoints to evaluate estrogenic or anti-estrogenic activity of an EDC (Thigpen *et al.,* 2004).

The focus has clearly been on estrogenic components of the feed. Researchers using various feeds in the Hershberger assessments have reported no differences in the quality or quantity of the responses (*i.e.,* which organs, increase/decrease, relative response) with different feeds, with one exception. Stroheker *et al.* (2003) exposed castrated male Wistar rats for ten days to diets differing in soybean meal content (L5, D04, or D03; see **Table 6**), with and without vinclozolin (0, 25, 50, 100 mg/kg/day, PO) and/or TP (0, 1, 0.2, 0.4, 0.5 mg/kg/day, SC injection). There was no effect of diet on the weights of the ASO (seminal vesicles, prostate, LABC muscle) nor on the dose-related, reduced ASO weights from vinclozolin. However, diet did affect the androgenic response to TP in the relative and absolute weights of the seminal vesicles and prostate, but not the LABC. In every case, D03 diet resulted in reduced ASO weights relative to the L5 diet at each TP dose. They therefore recommended the use of a standardized, open-formula diet without soy isoflavones, such as L5, in the Hershberger assay (Stroheker *et al.,* 2003).

The concern for other contaminants in feed (*e.g.,* pesticides, heavy metals, organophosphates, PCBs, aflatoxins, etc.) has abated now that feed manufacturers provide standards and analyses for these contaminants as well as for nutrients (protein, fat, fiber, etc.). Although some researchers have the opinion that as long as all groups in the study (negative control, positive control, test chemical, etc.) have the same environmental conditions, then there should be no problem, other researchers have voiced the concern that estrogenic components in the feed may result in maximum response, so no further changes can occur in the presence of test chemicals and/or that feed should be chosen with little or no phytoestrogens (Thigpen *et al.* 2004). However, Latendresse *et al.* (2001) have reported that soy/yeast-free feed apparently causes polycystic kidneys in rat pups exposed to nonylphenol; polycystic kidneys do not occur from exposure to nonylphenol in the presence of feed with phytoestrogens (Tyl *et al.,* 2006).

Different feeds may result in different patterns of weight gain. Marty *et al.* (2003) evaluated the impact of changes in growth on Hershberger pubertal male assay endpoints. Feed restriction and reductions in body weights impacted ASO and other organ weights when expressed as absolute as well as relative to terminal body weights. Therefore, these Hershberger assay ASO and other weights, both with and without T stimulation, can be influenced by feed restriction, resulting in a 10% decrease in terminal body weight. The authors concluded that objective criteria for determining a positive or negative result may be problematic due to the confounding effects of body weight on some endpoints. They also noted that a 10% decrement in body weight appears to be excessive as a requirement for high-dose toxicity in these assays since tested chemicals may be incorrectly classified

as positive (when they are not if the effects are due to general toxicity) or negative (if endocrine-dependent effects are masked by body weight changes) in the presence of biologically significant weight gain reduction. At least caution is recommended in interpreting assay results in the presence of a 10% body weight change due to the possible confounding effects of this degree of growth suppression, especially in young adult animals (Marty *et al.,* 2003).

Bedding

A variety of bedding materials has also been used (see **Table 7**), as well as the use of hanging cages with no bedding. The bedding materials range from soft pine shavings (heat treated to destroy resins which induce hepatic metabolizing enzymes) to pure alpha cellulose. There has been no major concern about different bedding materials since the Hershberger validation studies used various bedding materials with no impact on outcomes, again as long as all groups in the study were exposed to the same husbandry components.

Caging

Caging (when specified in publications) has ranged from clear plastic (polycarbonate) shoebox changes, translucent polypropylene shoebox cages (polycarbonate) is a polymer of bisphenol A (BPA), a weak environmental estrogen, and badly crazed/cracked polycarbonate cages have been shown to release BPA which apparently resulted in meiotic aneuploidy in females from susceptible mouse strains [Hunt *et al.,* 2003]) to stainless steel, wire-bottom hanging cages and stainless steel caging with steel plate floor inserts during gestation (for nest building) and lactation (for dams and their pups) (see **Table 8**). Again, in the Hershberger validation studies, caging used by the various laboratories did not affect the study outcomes.

Light/Dark Cycles

Light cycles, when they are reported, are 12 hours light and 12 hours dark (Yamasaki *et al.,* 2003; Stroheker *et al.,* 2003) or 14 hours light and ten hours dark (Stoker *et al.,* 2005), with the times of lights on and off rarely specified and likely variable. Again, this husbandry component, when specified, did not appear to affect study results. The NRC Guide (NRC, 1996) recommends light intensity of 325 lux (30 foot candles) at one meter (3.3 ft) above the floor, since this intensity does not cause phototoxic retinopathy in albino rats.

Drinking Water

Drinking water variables include the source (municipal city water, wells, etc.), modifications (filtered, reverse osmosis, acidified, etc.), delivery systems (automatic watering system versus water bottles [glass or plastic] with caps [rubber stoppers, stainless steel, plastic, screw-top], and sipper tubes [stainless steel]), etc. Again, these variables did not appear to affect study results.

Table 7. Rodent Bedding

Designation	Vendor	References

Sani-Chip® cage bedding	P.J. Murphy Forest Products, Inc., Montville, NJ)	Tyl *et al.,* 2002
Tapvai bedding	Not provided	Vinggaard *et al.,* 2002
Heat-treated (to eliminate resins which induce liver enzymes), laboratory-grade pine shavings	Northeastern Products, Warrenburg, NY	Wilson *et al.,* 2002
ALPHA-dri® (purified alpha cellulose)	Shepherd Specialty Papers (Watertown, TN)	
Ab-Sorb-Dri® cage litter (semi-purified)	Laboratory Products, Garfield, NJ	Tyl *et al.,* 1996

Table 8. Rodent Caging

Designation	Vendor	References
Clear plastic cages (20x25x47 cm)	Not specified	Wilson *et al.,* 2002
Solid-bottom translucent polypropylene cages	Laboratory Products, Rochelle Park, NJ	Tyl *et al.,* 2002
Clear polycarbonate cages (20x25x17 cm)	Not specified	Gray *et al.,* 2002
Stainless steel hanging cages (during prebreed exposure)		Tyl *et al.,* 2002; Cagen *et al.,* 1999 (rats)
Stainless steel hanging cages with steel plate floor inserts (during gestation and lactation		Cagen *et al.,* 1996 (mice)

Temperature and Relative Humidity

Temperature (in °C or °F) and relative humidity (in %) required ranges are specified in the National Research Council Guide for the Care and Use of Laboratory Animals. For rodents, the temperature range is 64-79°F (18-26°C) and the relative humidity is 30-70% (NRC, 1996). This information is typically not provided in the open published literature.

Room Air Changes

Room air changes are also specified in the NRC Guide (1996) as 10-15 fresh air changes/hour, with the recommendation to not use recycled air. Again, this information is usually not provided in the open published literature.

6.1.8 Endpoints

Early in the development of the assay, Korenchevsky (1932) chose to weigh the prostate, seminal vesicles, and penis (as well as systemic organs) as a measure of a test material's ability to compensate for the removal of the testes, since these organs exhibited the most profound regression after castration. In 1932, Korenchevsky and Dennison (1935a) weighed the seminal vesicles, prostate, and preputial glands as part of their assessments. The addition of the LABC occurred soon after as the distinction between androgenic (*i.e.,* DHT dependent) and myotrophic (*i.e.,* T dependent) responses became apparent.

Hershberger *et al*. (1953) recorded terminal body weight, ventral prostate weight, seminal vesicle weight, and levator ani weight at terminal necropsy, and calculated the ratio of increase in levator ani weight to increase ventral prostate weight (if levator ani increase was highly significant, $p<0.01$, as compared to controls). These organs were also monitored in the studies reviewed by Dorfman (1969a,b) for assessment of androgens and anti-androgens. More recent studies have also monitored the same organs (Snyder *et al*., 1989), weighed only the prostate (Raynaud *et al*., 1980; Rittmaster *et al*., 1991; Shao *et al*., 1994), or weighed the prostate plus the seminal vesicles (Wakeling *et al*., 1981; Kelce *et al*., 1997). Ashby and Lefevre (2000a) weighed the prostate, seminal vesicles (including the coagulating glands), LABC muscle complex, and Cowper's glands. Gray *et al*. (2004) weighed the ventral prostate, Cowper's glands, seminal vesicles (with coagulating glands and fluids), glans penis and LABC muscles. For the pubertal intact male rat assay, Gray *et al*. (2004) recommended weighing the same organs as above plus the testes and epididymides. Yamada *et al*. (2004) weighed the ventral prostate, dorso-lateral prostate, seminal vesicles with coagulating glands, LABC muscles, glans penis, Cowper's glands, and pituitary.

Additional data collected included mortality, clinical observations, regular body weights (predominantly to calculate dosing volume based on the most recent body weight but also as a measure of androgen-dependent increases in muscle mass and terminal body weight to allow calculation of relative ASO weights), feed consumption, and blood at termination for subsequent determination of circulating serum hormones (*e.g.*, T, LH, T3, T4, TSH, etc.). The organ weights were presented as absolute and relative to terminal body weight. The organs to be weighed were dissected free, trimmed, and weighed either

fresh or postfixation (*e.g.*, thyroid, adrenal glands, pituitary), wet (fresh or in fixative), or dry (after dessication).

Histopathology after fixation, embedment, and sectioning at ~5 μm thickness, and staining with H&E (hematoxylin and eosin) is routine for the thyroid (if evaluated; Yamada *et al*., 2004; Noda *et al*., 2005) and is sometimes employed for ASO. When peripubertal animals are used, age at PPS is also recorded (note that the rationale for castration on PND 42 by Gray *et al*. [2005] was to allow acquisition of PPS). A number of investigators have noted that the early-castrated males (even with androgenic administration) cannot acquire PPS (Ashby and Lefevre, 2000a,b; Gray *et al*., 2005).

Although the endpoints in the Hershberger assay (specifically changes in androgen-dependent ASO weights) are not necessarily considered adverse, Gray *et al*. (2004, p. 429) "have found that chemicals that are positive in the Hershberger assay often produce adverse effects during puberty and after *in utero* exposure."

6.2 Synopsis of Protocol(s)

6.2.1 Animal requirements

The first protocol, which was validated by OECD, used sexually immature castrated male rats. As mentioned in Chapter 2.0, this model involved rats castrated at peripuberty by the removal of testes and epididymides (orchidoepididymectomized). In most laboratory rat strains (such as Sprague-Dawley, Long Evans, or Wistar), peripuberty is expected to take place within five to seven weeks of age. Peripuberty is marked by separation of the prepuce from the penile shaft (glans penis). Chemicals such as TP will initiate PPS so that it is possible to weigh the glans penis. At this peripubertal stage of sexual development, the glans penis and other androgen-sensitive tissues exhibit their sensitivity to androgens, since both ARs and appropriate steroidogenic enzymes are present during this time. The advantage of using rodents of this age is that the ASO tissues display high sensitivity and relatively small weights, both of which aid in minimizing the variability in responses between individual animals.

Another protocol under validation is the intact stimulated weaning male rat assay used by Asby and Lefevre (2000). The reproductive tissue weight changes in this male have shown sensitivity to a range of biochemical modulators, estrogens and anti-androgens.

6.2.2 Principles

The Hershberger assay is based on changes in the weight of male ASO in these sexually immature peripubertal castrated or intact weanling male rats. The five ASO used for this protocol are the ventral prostate, seminal vesicles plus coagulating glands, LABC muscles, glans penis, and Cowper's (or bulbourethral) glands in the peripubertal male. The six androgen-dependent organs in the intact weanling include; the ventral prostate, seminal vesicles plus coagulating glands, LABC muscles, Cowper's glands, and testes with epididymides (since the animals are intact), but not the glans penis (since it cannot be reliably weighed until after acquisition of preputial separation at puberty).

In addition to the weight of the ASO, body weight gain should be determined to provide information on the general health of the animals. Liver weight can be used as an

endpoint to determine if a test substance is truly anti-androgenic or simply appearing that way due to inducing an increased metabolism of the reference androgen (*e.g.,* TP) by the liver, which would be indicated as increased liver size (cell hypertrophy). Necropsy of the adrenals and kidneys as optional endpoints may provide supplementary information about the effect of the test substance on other related biochemical pathways. Similarly, measurement of serum T and LH may also be determined in this context.

To test for androgen agonists, the test substance is administered to immature, castrated rats for ten consecutive days. The vehicle is the negative control, and a substance such as TP (a potent androgen) can be used as a positive control.

For androgen antagonists, the test substance is administered to immature, castrated rats for ten consecutive days, together with a reference androgen agonist such as TP. Administration of TP alone can be used as the positive control, to which treatments are compared for anti-androgenic activity. The weights of the ASO after co-administration of the test substance and reference androgen are compared to the weights of tissues from this control group.

6.2.3 Husbandry

The immature, male rats are preferably group housed (2-3/cage), with food and water *ad libitum* and a 12/12 light cycle.

6.2.4 Initial procedures

Animals should be uniquely identified and weighed upon receipt. At 42 days of age, the animals are castrated using the testing laboratory's Institutional Animal Care and Use Committee (IACUC) approved methods that eliminate pain and distress. Approximately one day prior to dosing, at PND 53 to 54, the rats should be weighed to the nearest 0.1 g, and a homogeneous population of study animals should be created by eliminating the highest and lowest outliers. All unhealthy animals (or those not gaining weight) should also be removed from the study. The animals should be assigned to dose groups using a randomized, complete block design. The body weight means and standard errors should be calculated for each treatment group to ensure they are as similar as possible.

6.2.5 Testing for androgenic activity

The administration of vehicle or test substance in vehicle by gavage should be performed daily for ten days, starting at 53-54 days of age, using a separate 16-gauge, 2-inch gavage needle for each group. The rats should be weighed daily during treatment. In the weanling rat assay, the animals are used at 21-22 days of age. The test substance should be prepared at the selected doses in a vehicle, such as corn oil at 2.5 ml/kg body weight, and administered between 7:00 a.m. and 10:00 a.m. daily. The volume administered should be adjusted daily for body weight changes. The body weight and dose volume should be recorded for each animal. Monitoring of feed consumption should also be performed.

6.2.6 Testing for anti-androgenic activity

TP in corn oil should be administered orally on a 0.2 to 0.4 mg/kg body weight/day basis, or alternatively at 0.1 mg/rat/day, to the appropriate groups by sc

injections at the same time of day on the dorsal surface, caudal to the nape of the neck, but anterior to the base of the tail, with a 25-G x 5/8-inch needle using a separate 1 cc glass tuberculin syringe for each treatment condition. The test compound should be administered by gavage at the appropriate dose in 2.5 ml corn oil per kg/body weight. Dosing should be repeated for ten days, and the body weight and dose volume should be recorded daily.

6.2.7 Necropsy

Overview

Animals should be necropsied by block design across dose groups. Approximately 24 hours after the last day of treatment, the animals should be assessed for PPS, necropsied, the data collected, collated, and analyzed, and the report prepared.

The rats at 64-65 days old are necropsied to isolate organs and tissues for the study of androgenic or anti-androgenic effects. The endpoints evaluated at necropsy are:

- Body weight
- Seminal vesicles plus coagulating gland with fluid weight
- LABC muscle weight
- Glans penis weight
- Cowper's glands (bulbourethral glands) weight
- Liver weight

Optionally, the following may be determined:

- Serum T and luteinizing hormone levels
- Paired kidney weight
- Paired adrenal weight

Procedure

- Weigh the rats.
- Anesthetize the animals.
- Collect blood by cardiac puncture if hormone levels are to be measured.
- Euthanize the anesthetized animals humanely.
- Dissect and weigh the ASO (ventral prostate, seminal vesicle with coagulating glands and fluid, Cowper's glands, LABC muscles, and glans penis), as well as other organs of interest such as the liver, kidneys, and adrenal glands if desired, to the nearest 0.1g.

- Determine if the prepuce of the penis has separated from the glans penis. If it has, retract the prepuce, remove the glans penis, and weigh to the nearest 0.1 mg. If it has not separated, note this and do not weigh the glans penis.
- Remove the abdominal skin and muscle layers to expose the viscera.
- Optionally, remove and weigh the liver, the paired kidneys, and the paired adrenal glands each to the nearest 0.1 mg.
- Expose the seminal vesicles plus coagulating glands and bladder.
- Dissect the **ventral prostate** by separating the bladder from the ventral muscle layer by cutting the connective tissue along the midline with microdissecting scissors. Displace the bladder anteriorly towards the seminal vesicles, revealing the left and right lobes of the ventral prostate covered by a layer of fat. Carefully tease away the fat layer from the right and left lobes of the ventral prostate with microdissecting forceps. Displace the right lobe of the ventral prostate from the urethra and dissect this lobe of the ventral prostate from the urethra with scissors. While holding the right lobe of the ventral prostate with forceps, displace the left lobe away from the urethra and then dissect this lobe from the urethra with microdissecting scissors and weigh in tared weigh boat to the nearest 0.1 mg.
- Displace the bladder caudally, expose the vas deferens and right and left lobes of the **seminal vesicles plus coagulating glands**, and clamp a hemostat at the base of the seminal vesicle where the vas deferens joins the urethra to prevent leakage from the seminal vesicle. Using the microdissecting scissors, dissect the seminal vesicle from the urethra. Trim the fat and adnexa and remove the hemostat. Place the seminal vesicles plus coagulating glands with fluid in a tared weigh boat and weigh to the nearest 0.1 mg.
- Expose the **LABC** and the base of the penis with penile bulbs by removing the skin and adnexa from the perianal region extending from the base of the penis to the anterior end of the anus. With forceps and microdissecting scissors, remove the fat from these tissues until the muscles can be identified. Grasp the bulbocavernosus muscle with blunt forceps and dissect the muscle from the penile bulb so that the white connective tissue and reddish corpus spongiosum are detached from the bulbocavernosus muscles on each side. Lift the bulbocavernosus muscles upward and away from the body. Cut the colon in two with the scissors, pull the LABC further upward, and pull the fat and adnexa off with forceps. Remove the LABC, trim the fat, and weigh it to the nearest 0.1 mg.
- Remove the round **Cowper's or bulbourethral glands** at the base of, and slightly dorsal to, the penile bulbs with dissecting scissors. Avoid nicking the thin capsule to void leakage. Weigh the paired glands to the nearest 0.1 mg.

6.2.8 Expected outcomes/data interpretation

The weights of the androgen-dependent tissues will increase with exposure to androgenic compounds. This response in the castrated, immature male rat will generally

be dose dependent. Anti-androgenic compounds will inhibit the TP-induced growth of the ASO in a dose-related manner.

6.3 Considerations

- Doses should be carefully selected so that the lowest dose shows no or minimal effects and the highest dose should not produce signs of toxicity. Therefore, the animals should also not show a loss of ≥ 10% body weight over the course of the assay, so that the toxicity of the compound does not become part of the evaluation (*i.e.*, confounder).
- The individuals involved in the conduct of this assay should be well trained and consistent in their dissections and observations.
- The entire assay, with all test and control groups, should be run at one time.
- This version of the Hershberger protocol, used in the OECD standardization and validation initiative (Owens *et al.*, 2006) and described by Gray *et al*. (2005), specifies castration of the "prepubertal" male rat on PND 42, with dose administration commencing on PND 53-54. In the authors' laboratories, with the CD® (SD) rat, the grand mean of the individual study control group means for age at PPS is 41.9 days, with the range of individual study means from 41.1 days to 43.6 days. This means that on average, at castration on PND 42, 50% of the prepubertal males are, in fact, postpubertal, having acquired PPS prior to castration. The data to be collected at scheduled necropsy (after the treatment period) is the percentage of males with PPS (Gray *et al.,* 2005); therefore, the authors make two recommendations for future use of this protocol:
  1. That the evaluation of males for PPS should also be made at castration (so the baseline of animals already with PPS is known); and
  2. That the males be castrated much earlier, *e.g.* the latest on PND 35 (when no control CD® SD males have acquired PPS in the authors' laboratories), or the earliest on PND 22 (the day after weaning on PND 21). Hershberger *et al.* (1953) castrated their males on PND 21 and began treating them on the same day.

A sample of a basic protocol using castrated animals is located in Appendix A.

It could be easily modified for the use of intact weanling rats.

7.0 EXAMPLES OF CHEMICALS TESTED IN THE HERSHBERGER ASSAY

Testing of chemicals for determination of androgenic properties in castrated rats began in the 1930s with the experiments of Tschopp, 1935; Ruzicka *et al.*, 1934; Korenchevsky and Dennison, 1933, 1935a; and Korenchevsky *et al.*, 1935 testing androsterone and androsterone-diol. They found that the secondary sex organs (prostate, seminal vesicles, and penis) became enlarged after the injection of androsterone and even more enlarged with androsterone-diol. Korenchevsky *et al.* (1937) tested TP in this bioassay, and it also produced increased weight in these organs. Wainman and Shipounoff (1941) also demonstrated the stimulating effect of TP upon the perineal complex and thus the appropriateness of the muscles (LABC) as an index of myotrophic activity. Variations of this early assay occurred over the next 15-20 years, with scientists trying different ages for castration, different time periods between castration and testing, various amounts of potent androgens, and determination of the weight of various organs.

In 1950, Eisenberg and Gordan further used the levator ani weight and showed that some of the increased muscle weight produced by TP over T was retention of water, and that it actually produced slightly less weight increase in the tissue over T. Still, even based on dry weight, unesterified T, TP, and methyl T were the most potent group of steroids tested for myotrophic activity. Progesterone showed a moderate response, and no significant increase in weight was seen with estradiol dipropionate, desoxycorticostrone, cis-T, or ethinyl T. A distinct lack of parallelism was seen between the degree of androgenic effect (growth of seminal vesicles) and the degree of myotrophic activity.

In 1953, Hershberger *et al*., improved the assay and made the first attempt to standardize it for prepubertal castrated males. Rats were castrated at weaning on PND 21, and sc injections were given for seven consecutive days, beginning on the day of castration. On the eighth day after castration, approximately 22-26 hours after the last injection, the animals were terminated. The levator ani muscle, ventral prostate, and seminal vesicles (without the coagulating glands) were dissected from each animal and weighed. The dry weight was also determined for the levator ani muscle after it was desiccated at 72ºC. Results from this assay, expressed as organ weights versus a range of doses of each test material, provided information on their relative potency for androgenic (from the ventral prostate and seminal vesicle weights) and/or for myotrophic activity (from the levator ani muscle weight). The Hershberger assay is also sometimes called the Hershberger anti-androgen assay.

This *in vivo* bioassay has been proposed by both EDSTAC and OECD to test chemicals that have the potential to act as androgens or anti-androgens, and it has been widely used by the pharmaceutical industry for screening drugs with these potentials designed to be used therapeutically. For assessing androgenicity, chemicals that act as

agonists are identified in the Hershberger assay if they produce statistically significant increases in the weight of the target, androgen-dependent tissues in the castrated animal. For assessing anti-androgenicity, chemicals that act as antagonists cause decreases in the stimulated target tissues weights when they are co-administered with a potent androgen such as TP. Inhibitors of androgen synthesis and anti-androgens may cause male reproductive tract malformations, and thus a screen for these chemicals is necessary to attempt to reduce the number of these occurrences.

The assay variables are currently being standardized and assay validation in multiple laboratories is being established. TP (commonly 0.4 mg TP/kg-bw/day) is generally used as the reference androgen agonist and FLU (0.1-10 mg/kg-bw/day) as the reference androgen antagonist. Corn oil is the vehicle used most often; peanut or olive oil have been found to cause a significant increase in body weight (Yamasaki *et al.,* 2001a). Issues still exist as to the comparison of this assay with the use of the weanling male rat assay (Ashby *et al.*, 2004a) and whether the castration by surgery can be eliminated by using a GnRH antagonist (Ashby *et al.*, 2001).

Results for some chemicals have been inconsistent in the assay. For instance, the fungicide fenitrothione, which acts as an anti-androgen *in vitro*, was positive in the Hershberger assay performed by Tamura *et al*. (2001), but negative in one performed by Sohoni *et al. (*2001). However, the majority of chemicals tested have given fairly consistent results across laboratories when a more standardized protocol is used.

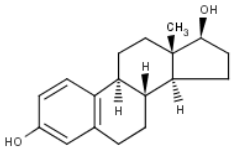
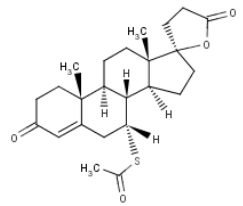
The reference androgens are used as a standard against which the response of the ASO to the test chemical is compared; if there is growth, the test chemical is androgenic and the potency can be determined from comparison of the results in the test chemical group with the results from the reference androgen group.The reference androgen and test chemical are administered to the same animals simultaneously to measure the ability of the test chemical to compete with the reference androgen and block its androgenic action. If the weights of the ASO are lower in the group exposed to both and reference androgen and test chemical (less than maximal response) than in the group exposed to the reference androgen alone, then the test chemical is acting as an anti-androgen (antagonizing the effects of the reference androgen). The degree of inhibition can be determined from the comparison of ASO weights, in the presence of both the test chemical and the reference androgen, versus the ASO weights of castrated males in the presence of only the reference androgen.

Table 9 lists the results of some chemicals that have been tested in the Hershberger assay and the variables that were used when they were available from the publications. **Table 10** lists the chemicals tested in the Hershberger assay by mode of action. **Table 11** lists chemicals that appear to bind to the AR but have not been tested in the Hershberger assay.

The Hershberger assay is valuable as an *in vivo* androgenic screen for AR antagonists, AR agonists, and 5α-reductase inhibitors that prevent T to DHT conversion. This bioassay furnishes the natural targets for androgens, a relatively rapid growth response yielding quantifiable weight changes, and no requirement for specialized equipment unless hormone determinations are added as an enhancement.

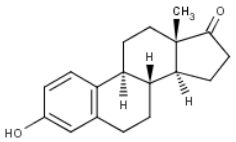
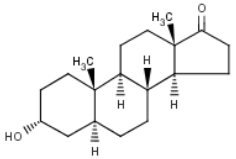
These attributes of the assay become important in various fields such as risk assessment, toxicology/teratology, and drug development. For instance, the aim of prostate cancer therapy is to block the androgens. The ideal drug would have potent anti-androgenic activity and yet be devoid of androgenic, glucocorticoid, progestational, estrogenic or any other hormonal or antihormonal action. Thus, appropriate screening for the various classes of androgenic and anti-androgenic compounds becomes vital in these roles.

Table 9. Examples of Chemicals Tested in the Hershberger Assay

Chemical	CAS No.	Citation	Strain Age at Castration # Per Dose Group	Doses Given (mg/kg/day) Days Given TP Dose	Route for Test Chemical Vehicle Used	Body Wt. Gain (g)	Accessory Sex Organ Weight Change				
							Ventral Prostate	Seminal Vesicle	LABC	Glans Penis	Cowper's Gland
STEROID											
17 β-Estradiol 	50-28-2	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.1,0.4,2.0 PND 56 for 10 days 0.2TP sc	Gavage olive oil	↓ 2.0	-	-	↓ 2.0	-	↑ 0.4
						↓ 2.0 +TP	-	-	-	↓ 2.0 +TP	-
Spirolactone 	52-01-7	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	8,40,200 PND 56 for 10 days 0.2 TP sc	Gavage olive oil	-	-	-	-	-	-
						-	↓ All +TP	↓ 40,200 +TP	↓ 40, 200 +TP	↓ 200 +TP	↓ 200 +TP

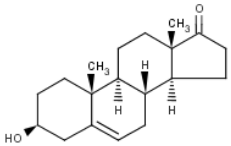
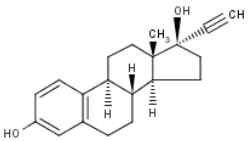
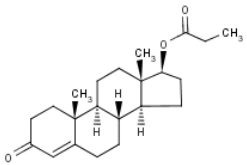
(continued)

Table 9 (continued)

Estrone 	53-16-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.5,2,10 (6) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 2,10(6)	↑ 10(6)	↑ 10(6)	-	-	-
						↓ 10(6) +TP	-	↑ 0.5,2, 10(6) +TP	-	↑ 2,10(6) +TP	-
Androsterone 	53-41-8	Korenchevsky et al., 1935	NA PND 21-26 4-10 rats per group	1993,3986,7971,11957 rat units 30-38 days post castration	Sc injection 2-3x per day oil or water	-	↑	↑		↑	
Androsterone-diol		Korenchevsky et al., 1935	NA PND 21-26 2-9 rats per group	97,194,389,777,1555,3109 rat units 30-38 days post castration	Sc injection 2-3x per day oil or water	-	↑	↑		↑	
androstrone		Korenchev-sky and Dennison, 1935	NA PND 20-28 4-7 per group	67,200,450,600,900,13501800 rat U 28-42 days after castration(PND 53-66)	Injection 2x per day for 7 days oil	-	↑ All doses	↑ All doses			

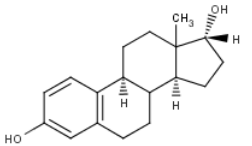
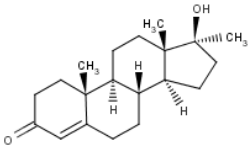
(continued)

Table 9 (continued)

Trans-Dehydroandrosterone 	53-43-0	Korenchev-sky and Dennison, 1936	NA NA	0.8, 1,2.4 1,2,3 necropsied at PND 68/86 injected for 7 or 21 days	Sc sesame oil		↑	↑		↑	
Ethynyl estradiol 	57-63-6	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,50,200 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 50,200 ↓ 200 +TP	↑ 50 -	↑ 50 ↑ 50,200 +TP	-	↑ 50,200 ↑ 200 +TP	- -
Testosterone propionate (TP) 	57-85-2	Kang, *et al.*, 2004	Sprague-Dawley PND 42 6 rats/group	0.1,0.2,0.4,0.8,1.6 PND 50 for 10 days	Sc corn oil	-	↑ 0.2,0.4,0.8,1.6	↑ All doses	↑ 0.2,0.4, 0.8, 1.6	↑ 0.2,0.4, 0.8, 1.6	↑ 0.2,0.4,0.8, 1.6

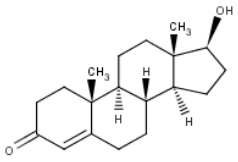
(continued)

Table 9 (continued)

TP	57-85-2	Edgren, 1963	Charles River PND 26 6 rats per group	03,1 PND 28 for 7 days	sc corn oil	↑ 1	↑ 0.3,1	↑ 0.3,1	↑ 0.3,1	NA	NA
17 Alpha estradiol 	57-91-0	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.5,2,10 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	- ↓ 2,10 +TP	- -	- -	- -	- -	- -
Methyltestosterone 	58-18-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.5,5,50 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	- -	↑ 0.5,5,50 ↑ 50 +TP	↑ 0.5,5,50 ↑ 50 +TP	↑ 50 ↑ 50 +TP	↑ 5,50 ↑ 50 +TP	↑ 0.5,5,50 ↑ 50 +TP
methyltestosterone	58-18-4	Yamada *et al.*, 2003	Crj:CD(SD)IGS PND 42 6 rats/group	100 PND 56 for 10 days	Gavage corn oil	-	↑	↑	↑	↑	↑

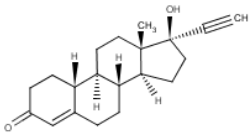
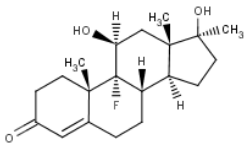
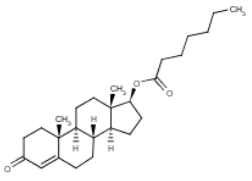
(continued)

Table 9 (continued)

methyltestosterone	58-18-4	Kennel *et al*., 2004	Sprague-Dawley PND 46 6 rats per group	0.5,2,10,40 PND 53-57 for 10 days	Gavage MC 500	-	↑ 10,40	↑ 10,40	↑ 10,40	↑ 40	↑ 40
Methyltestosterone	58-18-4	Edgren, 1963	Charles River PND 26 10 rats per group	0.625 PND 28 for 7 days	sc corn oil	↑	↑	↑	↑	NA	NA
Δ^1-Methyltestosterone		Edgren, 1963	Charles River PND 26 6 rats per group	3.0 PND 28 for 7 days	Sc corn oil	-	↑	↑	↑	NA	NA
Testosterone (T) 	58-22-0	Edgren, 1963	Charles River PND 26 5 rats per group	PND 28 for 7 days	sc cornoil					NA	NA

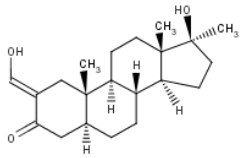
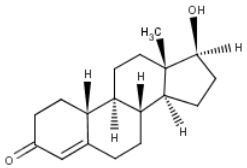
(continued)

Table 9 (continued)

Norethindrone 	68-22-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.5,2,10 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 10	-	-	-	-	-
						↓ 10 +TP	-	-	-	↑ 10 +TP	-
Fluoxymesterone 	76-43-7	Dorfman, 1962b	Charles River PND 25-28	4,8,16,32,64 from day of castration for 10-30 days	Gavage aqueous	NA	↑	↑	↑	NA	NA
Testosterone enanthate 	315-37-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	-	↑ 50,200, 600	↑ 50,200, 600	↑ 50,200, 600	↑ 50,200, 600	↑ 50,200, 600
						-	↑ 50,200, 600 +TP	↑ 50, 200,60 +TP	↑ 200, 600 +TP	↑ 600 +TP	↑ 200,600 +TP

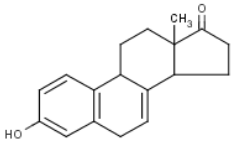
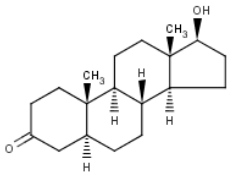
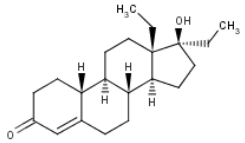
(continued)

Table 9 (continued)

2-Hydroxymethylene-17-α-methyl-4,5α-dihydroTestosterone 	434-07-1	Edgren, 1963	Charles River PND 26 10 rats per group	3.0 PND 28 for 7 days	Sc corn oil	-	↑	↑	↑	NA	NA
19-Nortstosterone 	434-22-0	Edgren, 1963	Charles River PND 26 10 rats per group	1.5 PND 28 for 7 days	Sc corn oil		↑	↑	↑	NA	NA
19-NorTestosterone. B-phenylpropionate		Edgren, 1963	Charles River PND 26 6 rats per group	0.5 PND 28 for 7 days	Sc corn oil	-/↑	↑	↑	↑	NA	NA

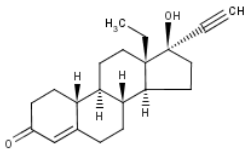
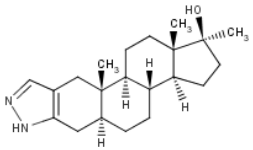
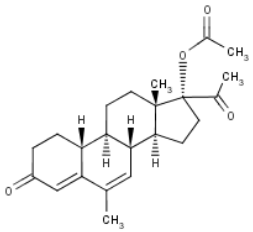
(continued)

Table 9 (continued)

Equilin 	474-86-2	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.5,2,10 (6) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 0.5,2,10 (6)	↑ 2,10(6)	↑ 2,10(6)	-	↑ 2,10(6)	-
						↓ 0.5, 2,10(6) +TP	-	↑ 0.5,2,10 (6) +TP	-	↑ 10(6) +TP	↑ 0.5,2,10(6) +TP
5α-Dihydrotestosterone 	521-18-6	Yamasaki *et al.*, 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	8,40,200 PND 56 for 10 days 0.2 TP sc	Gavage olive oil	-	↑ 40,200	↑ 200	↑ 200	↑ 200	↑ 200
						-	-	-	-	-	-
Wy 3475 	797-58-0	Edgren, 1963	Charles River PND 26 6 rats per group	0.3 PND 28 for 7 days	Sc corn oil	↑ 0.3	-		↑	NA	NA

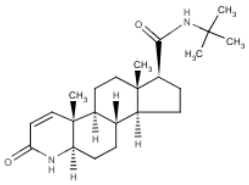
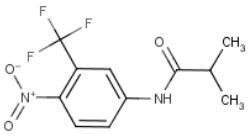
(continued)

Table 9 (continued)

Norgestrel 	797-63-7	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,30,100 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 100 ↓ 100 +TP	↑ 30,100 ↑ 100 +TP	↑ 30,100 ↑ 100 +TP	↑ 30,100 ↑ 100 +TP	↑ 100 ↑ 100 +TP	
Stanazol 	10418-03-8	Edgren, 1963	Charles River PND 26 10 rats per group	2.0 PND 28 for 7 days	Sc corn oil	↑				NA	NA
Nomegestrol acetate 	58652-20-3	Duc *et al*., 1995	Sprague-Dawley PND 21	2.5 – 20 PND 21 0.5 mL rat/day sc	Gavage olive oil		↓ 2.5,5,10 +T	↓ 2.5,5,10 +T			

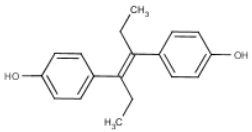
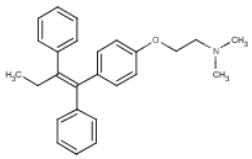
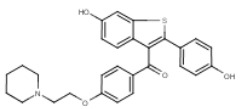
(continued)

Table 9 (continued)

Finasteride 	98319-26-7	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	0.2,1,5,25 PND 50 for 10 days 0.4 TP	Gavage stripped corn oil	- -	- ↓ All doses +TP	- ↓ All doses +TP	- ↓ 1,5,25 +TP	- ↓ All doses +TP	- ↓ 1,5,25 +TP
Finasteride	98319-26-7	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	0.008,0.04,5 PND 50 for 10 days 0.4 TP	Gavage stripped corn oil	- -	 ↓ All doses +TP	 ↓ All doses +TP	 ↓ 5 +TP	 ↓ All doses +TP	 ↓ 0.04,5 +TP
ANILIDE											
Flutamide (Positive Control) 	13311-84-7	Kang, *et al*., 2004	Sprague-Dawley PND 42 6 rats/group	1,5,10,20/ PND 50 for 10 days 0.4 TP	Gavage corn oil	- -	 ↓ All doses +TP	 ↓ All doses +TP	 ↓ All doses +TP	 ↓ 5,10,20 +TP	 ↓ 5,10,20 +TP
Flutamide (positive control)	13311-84-7	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	3 PND 50 for 10 days 0.4 TP	Gavage stripped corn oil	-	↓	↓	↓	↓	↓

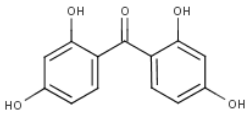
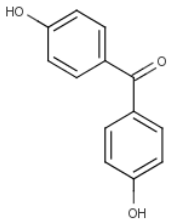
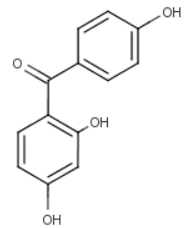
(continued)

Table 9 (continued)

STILBENE											
Diethylstilbestrol 	56-53-1	Yamasaki *et al*., 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.002,0.01,0.05 PND 56 for 10 days 0.2TP sc	Gavage olive oil	↓ 0.05	-	↑ 0.05	-	-	-
						↓ 0.05 +TP	↓ 0.05 +TP	-	-	-	-
Tamoxifen 	10540-29-1	Yamasaki *et al*., 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	0.004, 0.02,0.1 PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 0.1	-	-	-	-	-
						↓ 0.1 +TP	-	-	-	-	-
BENZOTIOPHENE											
Raloxifene 	84449-90-1	Nubauer et al., 1993	Immature males castrated 3 days before dosing 7 rats per group	0.05,0.5,5.0 for 7 days 0.2 TP	Corn oil	-					
							↓ All doses +TP				

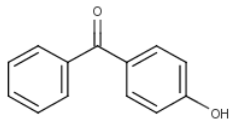
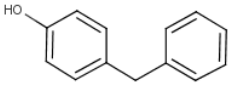
(continued)

Table 9 (continued)

BENZOPHENONE											
2,2',4,4'-Tetrahydroxybenzo-phenone 	131-55-5	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42/6 rats group	50,200,600 (400) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 200,600 (400)	-	-	-	-	-
						-	-	-	-	-	-
4,4'-Dihydroxybenzo-phenone 	611-99-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2TP sc	Gavage Olive oil	-	↑ 50	-	↓ 200	-	-
						↓ 600 +TP	↓ 200 +TP	-	↓ 20 +TP	↑ 600 +TP	-
2,4,4'-Trihydroxybenzo-phene 	1470-79-7	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 600	-	-	-	-	-
						-	-	-	-	-	-

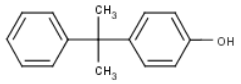
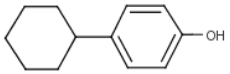
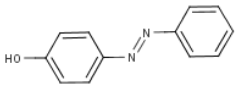
(continued)

Table 9 (continued)

2,4,4'-triOH-BP	1470-79-7	Suzuki et al., 2005	F344 PND 28 6 rats per group	100,300 PND For 10 days 0.5 TP sc	Sc DMSO	NA	-	-	NA	NA	NA
						NA	↑ 300 +TP	↑ 300 +TP	NA	NA	NA
4-Hydroxybenzo-phenone 	1137-42-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	-	↓ 600	-	-	-	-
						-	-	-	-	-	-
PHENOL											
4-(Phenylmethyl) Phenol 	101-53-1	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 (400) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 600 (400)	-	-	-	↑ 50,200	-
						-	-	↑ 50 +TP	-	-	-

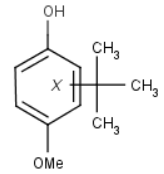
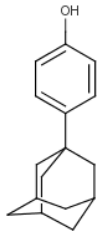
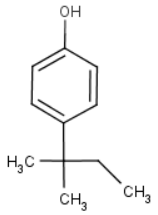
(continued)

Table 9 (continued)

p-cumyl phenol 	599-64-4	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 600	-	-	-	-	-
						↓ 600 +TP	-	-	-	-	-
4-Cyclohexylphenol 	1131-60-8	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 (400) Reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 600 (400)	-	-	-	-	-
						-	-	-	-	-	-
4-Hydroxy azobenzene 	1689-82-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,30,100 PND 56 for 10 days 0.2TP sc	Gavage olive oil	-	-	-	↓ 30	-	-
						-	-	-	-	↑ 10 +TP	-

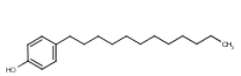
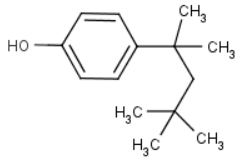
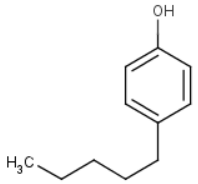
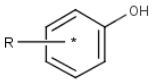
(continued)

Table 9 (continued)

Butylated hydroxyanisole (BHA) 	25013-16-5	Kang *et al.*, 2005	Crj:CD (SD) PND 42 8 rats per group	50,100,250, 500 PND 51 for 10 days 0.4 TP sc	Gavage corn oil	↓ 250,500	-	-	↑ 50	-	-
						-	↑ 250 +TP	↑ 250 +TP	↑ 250 +TP	↑ 250 +TP	↑ 250 +TP
4-(1-Adamantyl)phenol 	29799-07-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,50,200 PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 200	-	↑	-	-	
						↓ 200 +TP	-	200 +TP	-	-	↑ 200 +TP
ALKYLPHENOL											
p-(Tert-pentyl)phenol 	80-46-6	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 (400) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage olive oil	-	-	-	-	-	-
						-	-	↑ 50 +TP	-	-	-

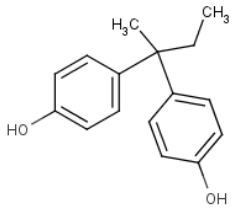
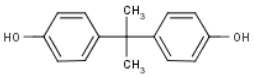
(continued)

Table 9 (continued)

p-Dodecyl-phenol 	104-43-8	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,30,100 PND 56 for 10 days 0.2TP sc	Gavage olive oil	-	-	-	↓ 100	-	-
						-	-	-	-	-	-
4-Tert-octylphenol 	140-66-9	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 (400) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 200	-	↑ 50	-	-	-
						-	-	-	-	-	↑ 200 +TP
4-n-Amylphenol 	14938-35-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 (400) Dose reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 600 (400)	-	-	-	-	-
						↓ 600 (400) +TP	-	-	↓ 600 (400) +TP	-	-
Nonylphenol 	25154-52-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,50,200 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 200	-	-	-	-	-
						-	-	↓ 200 +TP	-	-	-

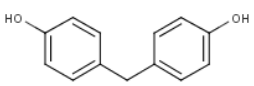
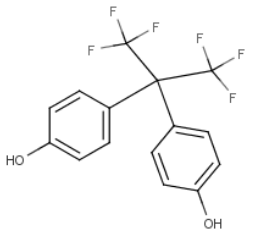
(continued)

Table 9 (continued)

BISPHENOL											
Bisphenol B 	77-40-7	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 600	-	-	↑ 200, 600	-	-
						↓ 600 +TP	↑ 200,600 +TP	↑ 200,600 +TP	↑ 600 +TP	↑ 600 +TP	↑ 600 +TP
Bisphenol A 	80-05-7	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,600 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	-	-	-	-	↑ 600	-
						-	-	-	-	-	-
Bisphenol A	80-05-7	Nishino, et.al, 2006	Wistar/ NA 13 rats per group	3,50,200,500 NA for 7 days	Gavage propylene glycol	↓ 200,500 ; 500 +FL	-↓ Rel wt. 200,500	-↓ Rel.wt. 200,500	NA	NA	NA
Bisphenol A	80-05-7	Kim *et al*., 2002	Sprague-Dawley Crl:CD/PND 35 or 42/ NA	10,100,1000 or 50,100,250, 500/PND 42or 49 for 7 days 0.4 TP sc	Gavage Corn oil	-	-	-	-	NA	NA
						-	-	-	-	NA	NA

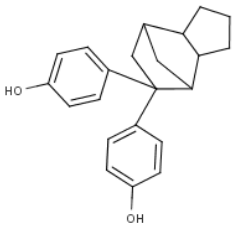
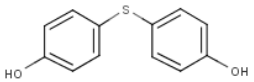
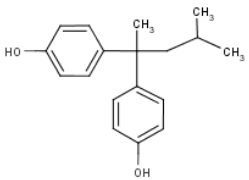
(continued)

Table 9 (continued)

Bisphenol F 	620-92-8	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,1000 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 1000;	-	-	-	-	-
						↓ 200, 1000 +TP	-	-	-	-	↑ 200 +TP
4,4'-(Hexafluoroisopropylidene)diphenol 	1478-61-1	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200, 600 (400) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 200,600 (400)	-	-	-	-	-
						↓ 200,600 (400) +TP	-	↑ 50, 600 (400) +TP	-	↑ 50, 600 (400) +TP	↑ 600 (400) +TP

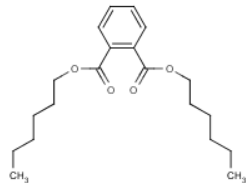
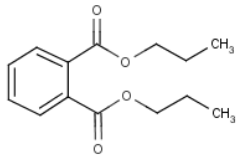
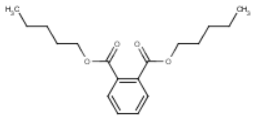
(continued)

Table 9 (continued)

4,4'- (Octahydro-4,7-methano-5H-ubden-5-ylidene)bisphenol 	1943-97-1	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	2,10,50 PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	-	-	-	-		-
						↓ 10,50 +TP	-	-	-	↑ 10,50 +TP	-
4.4'-Thiobis-phenol 	2664-63-3	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,50,200 PND 56 for 10 days 0.2 TP sc	Gavage olive oil	↓ 200	-	-	-	-	-
						-	↑ 10,200 +TP	↑ 10,200 +TP	-	-	-
2,2-bis(4-Hydroxyoxyphenyl)-4-methyl-n-pentane 	6807-17-6	Yamasaki *et al.*, 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	10,50, 200 (100) reduced due to toxicity PND 56 for 10 days 0.2 TP sc	Gavage Olive oil	↓ 50,200 (100)	-	-	-	-	-
						200 (100) +TP	↑ 10,200 (100) +TP	-	-	-	-

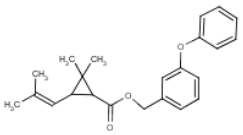
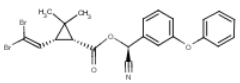
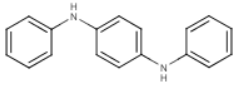
(continued)

Table 9 (continued)

PHTALATE											
Phthalic acid di-*n*-hexyl ester 	84-75-3	Yamasaki *et al*., 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	40,200,1000 PND 56 for 10 day 0.2TP sc	Gavage olive oil	-	-	-	-	-	-
						-	-	-	-	-	-
Phthalic acid di-*n*-propyl ester 	131-16-8	Yamasaki *et al*., 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	40,200,1000 PND 56 for 10 days 0.2TP sc	Gavage olive oil	-	-	-	-	-	
						-	-	-	-	-	↑ 200 +TP
Phthalic acid di-*n*-amyl ester 	131-18-0	Yamasaki *et al*., 2004	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	40,200,1000 PND 56 for 10 days 0.2TP sc	Gavage olive oil	-	-	-	-	-	-
						-	↓ 1000 +TP	↓ 100 +TP	↓ 200, 1000 +TP	-	↓ 40, 200 +TP

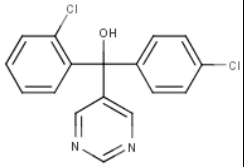
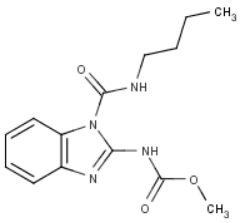
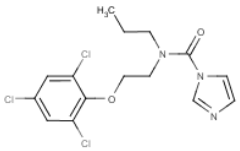
(continued)

Table 9 (continued)

PYRETHIN											
d-phenothrin 	26002-80-2	Yamada *et al*., 2003	Crj:CD(SD)IGS PND 42 6 rats/group	100,300,1000 PND 56 for 10 days 0.2 TP sc	Gavage corn oil	- -	- -	- -	↑ 100 	- -	- -
Deltamethrin 	52918-63-5	Andrade *et al*., 2002	Wistar 8 or 9 per group	2.0,4.0 for 7 days	Gavage distilled water was used for the test chemical, canola oil for others	NA NA	- -	- -	NA NA	NA NA	NA NA
ORGANONITROGEN COMPOUND											
Diphenyl-p-phenylenediamine 	74-31-7	Yamasaki *et al*., 2003b	Brl Han:WIST Jcl (GALAS) PND 42 6 rats/group	50,200,800 PND 56 for 10 days 0.2 TP sc	Gavage olive oil	- -	- ↑ 50,800 +TP	↑ 800 -	- -	↑ 50 -	- -

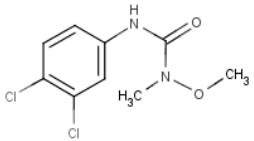
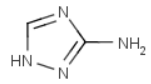
(continued)

Table 9 (continued)

Fenarimol 	60168-88-9	Vinggaard, *et al*., 2005	Wistar PND 28 6 rats per group	200 PND 42 for 7 days 0.5 TP	Gavage peanut oil	-	-	-	-	-	-
						-	-	↓ 200 +TP	↓ 200 +TP	-	-
CARBAMATE											
Benomyl 	17804-35-2	Yamada *et al*., 2005	Crj:CD(SD)IGS PND 42 6 rats per group	100,300,1000 PND 49 for 10 days 0.2-0.4 TP sc	Gavage corn oil	-	-	-	-	-	-
						-	↓ 100,1000 +TP	↓ 1000 +TP	↓ 1000 +TP	-	-
AZOLE											
Prochloraz 	67747-09-5	Vinggaard *et al*., 2002	Wistar PND 28 6 rats per group	250 PND 42 for 7 days 0.5 TP	Gavage	-	↓	↓	↓	NA	NA
						-	-	-	-	NA	NA

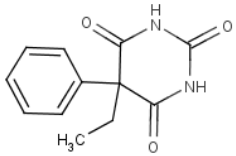
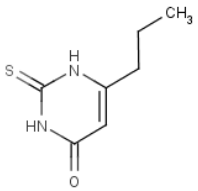
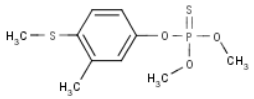
(continued)

Table 9 (continued)

prochloraz	67747-09-5	Vinggaard *et al*., 2002	Wista PND 28 6 rats per group	50,100,200 PND 42 for 7 days 0.5 TP	Gavage Peanut oil	- -	- ↑ All doses +TP	- ↑ All doses +TP	- ↑ All doses +TP	NA NA	NA NA
UREA											
Linuron 	330-55-2	Kang, *et al*., 2004	Sprague-Dawley PND 42 6 rats/group	25,50,100 PND 50 for 10 days 0.4 TP	Gavage corn oil	- -	- ↓ 50,100 +TP	- ↓ 50,100 +TP	- ↓ 100 +TP	- ↓ 100 +TP	- ↓ 50,100 +TP
linuron	330-55-2	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	PND 50 for 10 days 0.4 TP	Gavage stripped corn oil						
TRIAZOLE											
3-amino-1,2,4-triazole (AT) 	61-82-5	Noda *et al*., 2005	BrlHan WIST@Jcl (GALAS) PND 42 6 rats per group	0,40,200,1000 PND 63 for 10 days 0.2 TP	Gavage olive oil	- 	- 	 ↑ 1000; 200, 1000 +TP	 ↑ 40, 200 +TP	- 	-

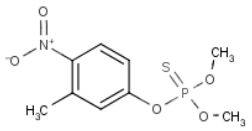
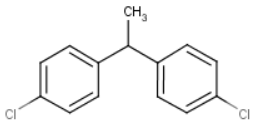
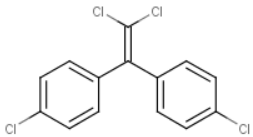
(continued)

Table 9 (continued)

PYRIMIDINE											
Phenobarbital 	50-06-6	Yamada *et al.*, 2004	Crj:CD(SD)IGS PND 42 6 rats per group	125 PND 49 for 10 days 0.2 TP	Gavage corn oil	-	-	-	↓ 125 +TP	-	-
THIONAMIDE											
Propylthiouracil (PTU) 	51-52-5	Yamada *et al.*, 2004	Crj:CD(SD)IGS PND 42 6 rats per group	2.5 PND 49 for 10 days 0.2 TP	Gavage corn oil	-	-	-	-	-	-
ORGANOPHOSPHATE											
Fenthion 	55-38-9	Kitamura *et al.*, 2003	F344 PND35 7 rats per group	25,50 PND 42 for 7 days 0.5 TP sc	Sc panacete	↓ 50	-	-	NA	NA	NA
						-	↑ 25,50 +TP	↑ 25,50 +TP	NA	NA	NA

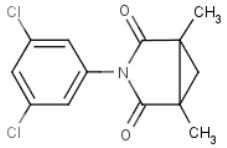
(continued)

Table 9 (continued)

ORGANOTHIOPHOSPHATE											
Fenitrothion 	122-14-5	Sohoni *et al*., 2001	Alp-k:ApfSD PND 42 5 rats per group	15 PND 50 for 10 days 0.2 or 0.4 TP sc with 10/15 fenitrothion	1 gavage and 1 sc dose/day HPMC	- -	- -	- -	- -	- -	- ↑ 10 +TP
ORGANOCHLORINE											
DDE 	3547-04-4	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	5,16,50,160 PND 50 for 10 days 0.4 TP	Gavage stripped corn oil	-	↓ 50,160 +TP	↓ 50,160 +TP	↓ 50,160 +TP	↓ 160 +TP	↓ 50,160 +TP
p,p'-DDE 	72-55-9	Yamada *et al*., 2003	Crj:CD(SD)IGS PND 42 6 rats/group	100 PND 56 for 10 days	Gavage corn oil	-	↓	↓	↓	↓	↓

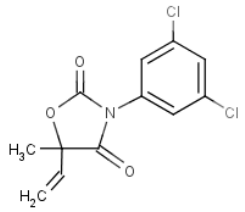
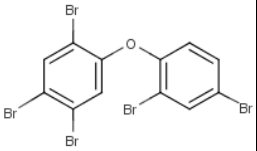
(continued)

Table 9 (continued)

				25,50,100		-	-	-	-	-	-
p,p'-DDE	72-55-9	Kang, *et al*., 2004	Sprague-Dawley PND 42 6 rats/group	PND 50 for 10 days 0.4TP	Gavage corn oil	-	↓ 100 +TP	↓ 50,100 +TP	↓ 100 +TP	-	↓ 100 +TP
DICARBOXIMIDE											
Procymidone 	32809-16-8	Kang, *et al*., 2004	Sprague-Dawley PND 42 6 rats/group	25,50,100 PND 50 for 10 days 0.4 TP	Gavage corn oil	-	↓ All doses +TP	↓ All doses +TP	↓ All doses +TP	↓ 100 +TP	↓ All doses +TP
procymidone	32809-16-8	Kennel *et al*., 2004	Sprague-Dawley PND 46 6 rats per group	3,10,30,100 PND 53-57 for 10 days 0.4 TP	Gavage MC 500	-	↓ 30,100 +TP	↓ 30,100 +TP	↓ 100 +TP	-	↓ 100 +TP
Procymidon	32809-16-8	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	3,10,30,100 PND 50 for 10 days 0.4 TP	Gavage stripped corn oil	-	↓ 10,30,100 +TP	↓ All doses +TP	↓ All doses +TP	↓ 10,30,100 +TP	↓ All doses +TP

(continued)

Table 9 (continued)

Vinclozolin 	50471-44-8	Kang, *et al*., 2004	Sprague-Dawley PND 42 6 rats/group	25,50,100 PND 50 for 10 days 0.4 TP	Gavage corn oil	-	-	-	-	-	-
						-	↓ 50,100 +TP	↓ 50,100 +TP	↓ 50,100 +TP	↓ 100 +TP	↓ 50,100 +TP
vinclozolin	50471-44-88	Ashby *et al*., 2004a	Alpk:APfSD PND 42 6 rats per group	PND 50 for 10 days 0.4 TP	Gavage						
					stripped corn oil						
ORGANOBROMINE											
DE-71 	32534-81-9	Stoker *et al*., 2005	Wistar PND 42 6 rats per group	30,60,120, 240 PND 53-61 0.4 TP sc	Gavage corn oil	-	↓ All doses	↓ 60,120, 240	↓ 240	↓ 240	↓ 120,240
						-	-	-	-	-	-

(continued)

Table 10. Mode of Action (MOA) of Some Chemicals Tested in the Hershberger Assay

Androgens	**Progestins**	**Antiandrogens**	**Estrogens**	**Wt. Change in Only 1 Organ**	**Thyroid Modulators**	**Negative in Assay**
T	Cyrotenone acetate*	Flutamide	17 β-Estradiol	↑ Seminal Vesicle Wt. When used with TP [p-)tert-pentyl)phenol]	PTU	Bisphenol A
TP	Megesterol acetate	DE-71	Tamoxifen	↓ LABC Wt. 4-n-amylphenol p-dodecyl-phenol	PB	Tamoxifen
Methyl T	Nomegestrol acetate (progesterone derivative)	**Fungicides-** Fenarimol Vinclozolin Prochloraz	Diethylstibestrol (DES)		*P,p'*-DDE	Atrazine
T17-dichloroacetate	6α-methyl-17α-hydroxy-progesterone	**Pesticides-** Methoxychlor Procymidone Linuron Iprodione Chlorolinate *p,p'*-DDE (AR antagonist)			3-amino-1,2,4-triazole (AT)	4-Cyclohexylphenol
T benzoate	Spirolactone	**Insecticides-** Fenitrothion				
Trans-androstanediol						
Cis-androstanediol						

Table 10 (continued)

Androgens	**Progestins**	**Antiandrogens**	**Estrogens**	**Wt. Change in Only 1 Organ**	**Thyroid Modulators**	**Negative in Assay**
17-β-hydroxy-5α-androstan-3-one						
5α-androst-2-en-17β-ol						
DihydroT						
Trenbolone						

* partial androgenic activity

Table 11. Examples of Chemicals That Appear to Bind to the AR but Have Not Been Tested in the Hershberger Assay

AA560-antiandrogen

Allylestrenol-antiandrogen

Androstanolone

Androstenedione

Casodex-antiandrogen

Cimetidine-antiandrogen

dehydroepiandrosterone

Dexamethasone

Drospirenone-antiandrogen (progestogen)

ICI 176,334-antiandrogen

Medroxyprogsterone acetate (MAP)

megestrol acetate

Methylandrostenediol

Nomegestrol acetate

19-nortestosterone cyclopentylpropionate

Permethrin-pyrethroid (insecticide)

RU 58642-antiandrogen

T undeconoate-androgen

TZP-4238-antiandrogen

WIN 49596-antiandrogen (AR antagonist)

8.0 DISCUSSION AND CONCLUSIONS

8.1 Discussion

There has been an increasing concern regarding the potential adverse effects of various environmental contaminants. Colburn *et al.* (1993) hypothesized that prenatal and postnatal exposure to EDCs could result in damage to wildlife and humans. Since then, many incidences of decreased reproduction in wildlife have been found. These include morphologic abnormalities, egg shell thinning, population declines, sex reversal, impaired offspring viability, altered hormone levels, and changes in socio-sexual behavior (Fox, 2001).

Assays that are capable of identifying the ability of chemicals to act as EDCs have been suggested by OECD and EDSTAC and are now being standardized and validated by OECD and EPA EDSP. Validation can be defined as the determination of the reliability and relevance of a test for a specific purpose (ECVAM, 1995; ICCVAM, 1997; OECD Guidance Docment No. 34, 2005; Clode, 2006). Although *in vitro* assays can identify substances with endocrine altering modes of action, the pharmacodynamics and pharmacokinetics of endocrine metabolism indicate the need for *in vivo* assays in the overall assessment of possible EDC. Hershberger *et al.* (1953) analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones. In the 1970s and 1980s, the discovery of the AR and the first compounds that acted as antagonists of the receptor, such as cyproterone acetate, was followed by modification of the assay to address antagonistic activity. A fixed dose of a reference agonist (*e.g.,* TP) is administered to several groups of animals that also receive a set of doses of the purported antagonist. The reliability of studies can be confirmed by showing that the weights of the ASO of rats given TP (or a test material acting as an AR agonist) are increased over those of rats given the vehicle alone, and the organ weights of rats receiving FLU (a known potent AR antagonist) or a test material acting as an AR antagonist plus TP are lower than those given TP alone.

The Hershberger and uterotrophic assays are part of the Tier 1 screening battery recommended by the EDSTAC. These assays are the most widely used short-term, *in vivo* assays designed to screen for chemicals that may be endocrine disruptors, and have been (uterotrophic) or are being (Hershberger) validated under the auspices of the OECD. Appendix B gives a comparison of the two assays.

There are some chemicals, for instance technical-grade and formulated deltamethrin, which are not able to elicit anti-estrogenic or anti-androgenic responses in either of these assays (uterotrophic or Hershberger) but do test positive in *in vitro* transcriptional assays (Andrade *et al.,* 2002).

The Hershberger assay has been found to be informative with chemicals that have an effect on the AR. However, there have been studies which indicate that the (anti)androgen potency, according to receptor binding assays, does not completely correspond to potency according to the Hershberger assay (Yamasaki, 2004). Charles *et*

al. (2005) tested seven chemicals in the *in vitro* assays of AR transactivation, AR binding, and the *in vivo* Hershberger assay. Five of the seven anti-androgenic test materials produced positive results in all three assays. Two of the chemicals (MXC and BAP) differed in their responses, but it has not been established firmly whether anti-androgenicity is the primary mechanism of action for these chemicals. For instance, in the case of MXC, its profile of hormonal effects differs from both estrogenic and anti-androgenic materials (Gray *et al*., 1999). It is presumed that other compounds will have complex interactions with the endocrine system that cannot be categorized into a single mode of action. Thus, negative Hershberger assay results may suggest that interaction with the AR may be too simplistic and/or limited to explain the more complex interactions seen *in vivo*. Metabolism and other factors, such as pharmaco/toxicokinetics present in *in vivo* tests, are thought to account for some of these discrepancies.

Atrazine inhibits T production in male rats following peripubertal exposure (Friedmann, 2002). Atrazine affects the pituitary/hypothalamic axis, and no endocrine disruptor properties are found using the Hershberger assay, since the AR ligand and receptor are not involved.

The results of *in vitro* and *in vivo* screens, such as the Hershberger assay, do not always agree. For instance, progesterone is negative *in vitro* but active in the Hershberger assay. This may be due to the *in vivo* conversion to active androstenedione/T. Estrogens may also be active in the Hershberger assay, and ERβ is highly expressed in the prostate. Since hormones other than androgens have effects in the Hershberger assay, it is not entirely specific for androgens (Zacharewski, 1998).

Sonneveld *et al*. (2005) have shown that the Allen-Doisy (uterotrophic) assay requires a much lower amount of estrogenic compound administered to the animal to activate the estrogen receptor than for androgens activating the AR in the Hershberger assay. In fact, for the weaker androgens, the activating dose might not be reached. This may mean that for assessing chemical risk analysis of chemicals, using relatively weaker compounds, the Hershberger assay might not be the best test system (Charles *et al*., 2005).

8.2 Conclusions

8.2.1 Advantages

The Hershberger assay has been undergoing validation and has been shown to be robust, reliable and reproducible as a screening assay for the detection of androgen and anti-androgen effects. It appears to be sensitive and specific to androgen-mediated alterations and capable of detecting weak androgen antagonists such as *p,p'*-DDE and linuron (Lambright *et al*., 2000; O'Connor *et al*., 1999). The assay appears to function as an i*n vivo* androgenic screen (i.e., AR agonists, AR antagonists, and preventing the T conversion to the more potent DHT).

The tissues evaluated in the Hershberger assay are the natural targets for androgens, the tissue response in the assay is relatively rapid, the tissue weights provide quantitative endpoints, a limited number of animals is necessary, and no special facilities or equipment are required. These advantages, combined with the fact that the assay has been shown to measure relevant biological responses and be sufficiently sensitive, robust, and reproducible to detect androgenic and anti-androgenic activity, provide support for the use of this assay as an EDC screen.

The Hershberger assay is also being examined for its ability to detect thyroid function modulators (Noda *et al*., 2005; Yamada et. al., 2004). Because of the known presence of ARs in the thyroid gland of mammals (Pellietier, 2000; Banu *et al*., 2002), the thyroid is speculated to be a target of androgenic compounds. T also has a stimulatory effect on the expression of the TSH mRNA (Ross, 1990), and T administration results in a significant decrease in serum T3 and T4 levels in the 15-day intact male assay (O'Connor *et al*., 2000). By enhancing the Hershberger assay with the addition of thyroid hormone determinations and thyroid histopathology, the assay appeared to be reliable for screening not only (anti)androgenic chemicals but also thyroid modulators. It has also been shown that the reliability of the Hershberger assay for assessing (anti)androgenicity is not confounded by alteration of thyroid homeostasis (Yamada *et al*., 2004).

8.2.2 Problems

One of the main problems with *in vivo* mammalian test methods has been the lack of relevant, reliable, and reproducible data due to different testing strategies, reference chemicals, and data interpretation.

There is an intrinsic variability for the developmental parameters used in the Hershberger assay and an absence of a universal rodent control database for these parameters. This, coupled with the problems of variability of protocols and extrapolation of rodent effects to humans, creates the obvious pitfalls to overcome as much as possible before acceptance of the assay for screening possible endocrine-active chemicals. This means that standardization and validation are vital to the future use of the Hershberger assay being used as a screening assay. These actions are currently underway.

Ashby (2002) also suggested establishing a range of sensitivity hierarchies for each of the EDC screening assays. He felt this will help to decrease the apparent discrepancies seen in the literature for some chemicals that have been tested.

Since the male endocrine system is so complex (including components such as the hypothalamus, pituitary, testis, thyroid, adrenal and pancreas), the Hershberger assay may not be sufficient to completely characterize the absence of endocrine activity (agonist or antagonist) of some chemicals on the male reproductive system structures and/or functions. There are numerous ways in which chemicals can produce interference in addition to effects on the AR. Short-term assays may not be able to detect interference mechanisms such as those affecting the hypothalamic-pituitary-gonadal axis. Also, the

available established assays use a limited number of end points, and significant information gaps exist for other potential targets in the endocrine system.

A complementary battery of *in vitro* and *in vivo* assays would appear to be valuable to fully characterize the absence of endocrine action of some chemicals. The data seem to indicate the need for utilizing a weight of evidence approach when assessing anti-androgenicity (Charles *et al*., 2005).

There is also a need to detect additive and/or synergic effects of these chemicals since most are not found singly in the environment but in mixtures. The Hershberger assay needs to be further tested for screening of mixtures.

Furthermore, the strongest response seen in the EDC screening assay may not turn out to be the most relevant response for human or wildlife assessment purposes (Ashby, 2002).

Although it is truly difficult to represent the complexity of the endocrine system in either cell-based assays or animal models to detect possible endocrine disruptors in humans (Baker, 2001), the use of validated *in vitro* and *in vivo* screens, including the *in vivo* Hershberger (and uterotrophic) assay, goes a long way in screening the thousands of chemicals (as well as mixtures) in development, in commerce, and in the environment to prevent or remove risk of exposure to endocrine-active chemicals from present and future generations of humans and the other organisms which share the planet Earth.

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APPENDIX A

SAMPLE PROTOCOL

TITLE: **Hershberger Assay: Testosterone Proprionate Dose Response Curve**

SPONSOR:

TESTING FACILITY:

PROPOSED STUDY DATES (in-life):

APPROVED BY:

Name Study Monitor Company	Date
Name Study Director Company	Date
Name Sponsor's Representative Company	Date
Name Quality Assurance Manager Company	Date

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I. RATIONALE, PURPOSE, AND BACKGROUND

Myotropic activity of anabolic steroids and androgenic activity of anabolic steroids can be differentiated with the Hershberger bioassay (Hershberger et al., 1953).

Testosterone supplementation in castrated male rats has been shown to increase bulbocavernous/levator ani weight ratio (BL/LA) and seminal vesicle, prostate, and Cowper's gland absolute organ weight (Ashley et al., 2000). Antiandrogens (including flutamide, dibutyl phthalate, finasteride, and vinclozolin) have been shown to reduce the absolute weight of androgen-sensitive organs such as the Cowper's gland, seminal vesicles, and prostate. Taken together, these data suggest that the Hershberger bioassay can be used to detect inhibitors of male sex hormone anabolism and antiandrogens, as well as androgens.

The rodent Hershberger assay was first described in 1953 (Hershberger et. al., 1953). Since that time, it has been used primarily in the pharmaceutical industry. The Hershberger assay is an *in vivo* short-term assay for chemicals that have the potential to act like endogenous sex hormones. It measures, as endpoints, changes in specific tissues that normally respond to endogenous hormones. The information generated by use of the assay can be used to build on that already available (e.g., from relevant *in vitro* screens that are used to narrow the field of chemicals that may need longer-term animal testing).

The rodent Hershberger assay evaluates the ability of a chemical to show biological activity consistent with the agonism or antagonism of natural hormones that have masculinizing effects. These hormones are known as androgens (e.g., testosterone). Accessory sex glands and accessory sex tissues are dependent upon androgen stimulation to gain and maintain weight during and after puberty. If endogenous sources of androgen are removed, exogenous sources of androgen are necessary to increase or maintain the weights of these sex accessory tissues. The sex accessory glands and tissues for this protocol are the: ventral prostrate (VP), seminal vesicles (SV) plus coagulating glands (CG), levator ani plus bulbocavernosus muscles (LABC), glans penis (GP), and Cowper's (or bulbourethral) glands (CP).

This protocol uses sexually immature male rats, castrated at peripuberty by removal of testes and epididymides (orchidoepididyectomized). In most laboratory strains, such as the Sprague-Dawley, Long Evans, or Wistar rats, peripuberty is expected to take place at approximately 6 weeks of age, within an expected age range of 5-7 weeks. Peripuberty is marked by prepuce separation. Testosterone proprionate (TP) will initiate prepuce separation so that the GP can be weighed. At the peripubertal stage of sexual development, the GP and other androgen-dependent sex accessory tissues are sensitive to androgens, having both androgen receptors and appropriate steroidogenic enzymes. The advantage of using this age of rodent is that the sex accessory tissues have a high sensitivity and small relative weight which both help to minimize variation in responses between individual animals.

Little is known about the response of individual sex accessory tissues to exogenous chemicals that may cause androgenic effects, although it has been shown that the male sex accessory tissues have

different sensitivities to androgens and other steroid hormones (Ashby and Lefevre, 2000). This differential sensitivity has been used historically and continues to be used to this day in the pharmaceutical industry by companies searching for chemicals that are anabolic but not androgenic or estrogenic. One example of differential sensitivity is the LABC muscles that lack the 5-alpha reductase enzyme. These muscles lack the ability to convert testosterone to its active form, dihydrotestosterone. Weight increases of the LABC, without concomitant weight increases in the VP, CG, and SV glands (which contain 5-alpha reductase), may reflect a myogenic rather than an androgenic response.

A. Principle of Test

The rodent Hershberger assay is based on changes in weight of male sex accessory tissues in sexually immature castrated male rats. Test substances may stimulate or, in the presence of a reference androgen, inhibit the stimulated development of sex accessory tissues.

The test substance is administered in graduated doses to several groups of male rodents for a number of consecutive days. Measurement of the weight of sex accessory tissues provides information on the androgenic nature of a chemical, however it can also provide additional information on whether effects are due to the effects on the androgen hormone receptor *in vivo* or on other relevant biochemical mechanisms (e.g., effect on other enzymes involved in the production of sex hormones such as 5-alpha reductase).

In addition to the sex accessory tissues, body weight gain is a mandatory measurement to provide information on the general health and wellbeing of the animals. In the initial validation work, liver weight at necropsy is also a mandatory endpoint, as some test substances may appear to be anti-androgenic by inducing an increased metabolism of TP by the liver. This may be indicated by an increase in liver size.

B. Androgen Agonists

To test for androgen agonists, a test substance is administered to immature castrated rats for ten consecutive days. TP is administered by daily subcutaneous injection. TP provides the positive control in studies with substances of unknown androgenic activity. The vehicle provides the negative control.

C. Androgen Antagonists

To test for androgen antagonists, the test substance is administered to immature castrated rats for ten consecutive days together with TP. Administration of TP alone is used as the negative control where treatments are compared to for antiandrogenic activity. The weights of the sex accessory tissues after co-administration of the test chemical and reference androgen are compared with the weights of tissues from this control group.

II. GENERAL

A. Sponsor and Personnel

Sponsor: Name and address

Study Monitor: Name and address

Sponsor's Representative: Name and address

Testing Facility: Name and address

Personnel:

Study Director:

Animal Research Facility Veterinarian:

Study Coordinator:

Laboratory Animal Sciences:

Materials Handling Facility:

Quality Assurance Unit:

Additional personnel may be added and will be included in the final report.

B. Proposed Calendar

Dates are approximate; actual calendar will be included in the project records.

Starting Date of Acclimation (animals arrive at RTI):

Starting Date of Test Chemical Administration:

Starting Date of Mating Period:

Proposed Date for Completion of In-Life Phase (last pnd 21):

Proposed Date for Submission of the Draft Report (data audited):

Proposed Date for Return of Sponsor's Comments to RTI:

C. Alteration of Design

Alterations of this protocol by the Study Director may be made with agreement of the Sponsor as the study progresses and documented by protocol amendment. Examples of such alterations are dates of necropsy, length or timing of exposure to the drug, drug dose level, additional organs for weighing, collection of blood for hormone analyses, and additional laboratory studies.

D. Institutional Animal Care and Use Committee (IACUC) Approval

Approval from the IACUC will be obtained for this study prior to any animal testing (see Section III.C below).

E. Quality Assurance

This study will be conducted under OECD GLP Standards.

III. MATERIALS AND METHODS

A. Test Animals

Species/Strain: Rat; CD (Crl:CD®[SD]BR)

Supplier: Charles River Laboratories, Raleigh, NC. All animals will be castrated at approximately 42 days of age. All animals will be delivered by truck at least seven days before the study. Transport containers will be ventilated, escape-proof rodent shipping crates.

Rationale: The Sponsor has requested the use of live animals. Alternative test systems are not available for assessing the effects of a test substance in the Hershberger assay. The CD® rat is the species of choice by the Sponsor.

Number and Sex: Fifty (50) immature male rats will be ordered for the study. Thirty-six males will be used for the dose response curve. This number includes 14 extra males to provide sufficient animals to obtain animals within the mean $\pm$ 20% body weight requirement of the Sponsor. Arrival at the Animal Research Facility (ARF) will occur in one shipment.

Age and Weight: The males will be approximately six to seven weeks of age on receipt date. Actual weights will be recorded in the study records within two days of arrival. Animals with body weights outside the $\pm$ 20% range may be removed from the study at the discretion of the Study Director.

Quarantine: Animals will be quarantined for approximately 7 days prior to the start of treatment. They will be observed daily for general health status, ability to adapt to the husbandry conditions, and will be released from quarantine by the ARF Veterinarian or designate prior to placement on study.

B. Husbandry Conditions

Housing: During the study, test animals (immature or castrated) will be co-housed, 2 -3 per cage, in solid-bottom, polycarbonate cages with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ) and Sani-Chips® cage litter (P.J. Murphy Associates). All animals will be housed in the ARF for the duration of the study. The animal rooms are air-conditioned, and temperature and relative humidity are continuously monitored by the Barber-Colman Network 8000 System VER 4.4.1 (Barber-Colman Company, Loves Park, IL). The target environmental ranges will be 22ºC (+ 3º C) for temperature and 30-70% (ideally 50-60%) relative humidity, with 12 hours of light per day and 12 hours of dark.

Diet: Pelleted Purina Certified Rodent Chow® #5002 (Purina International [PMI], Richmond, IN) will be available *ad libitum*. Feed will be stored at approximately 60-70°F prior to use and will be used for no longer than 120 days after the milling date. Samples of the diet will be retained for possible analysis by the Sponsor.

Water: Water (tap water; source: City of _____ Department of Water Resources, City, State) will be available *ad libitum* by plastic water bottles with butyl rubber stoppers and stainless steel sipper tubes. Contaminant levels of the water are measured at regular intervals by the supplier per EPA specifications. Documentation of these analyses will be inspected by the Study Director and maintained in the study records. It is anticipated that contaminant levels will be below the maximal levels established for potable water and will not affect the design, conduct, or conclusions of this study.

C. Identification, Quarantine, and Animal Care Approval

Identification: Animals will be uniquely identified, prior to initiation of dosing, by individual ear tags. Animals will be given random numbers and assigned to treatment based upon body weight and random numbers in a randomized complete block approach (SOP _____).

Limitation of Discomfort: Surgery will be done under isoflurane gas anesthesia. Discomfort or injury to animals will be limited in that if any animal becomes severely debilitated or moribund, it will be terminated by CO_2 asphyxiation. All necropsies will be

performed after terminal anesthesia with CO_2. Animals will not be subjected to undue pain or distress.

Culled Animals: Extra animals that arrive with the shipment that will not be used in the study will be euthanized or reassigned. Records will be kept documenting the fate(s) of all animals received for the study.

D. Test Substance and Vehicle

1. Testosterone Proprionate

Identity, purity, stability, and composition of the test substance have been determined by the manufacturer (see Certificate of Analysis for data regarding identity, purity, and composition, which will be provided with the chemical by the Supplier/Sponsor).

Test Substance Trade Names:	Testosterone proprionate
Vehicle:	Stripped corn oil
Supplier:	_____
CAS No.:	57-85-2
Molecular Weight:	420.6
Lot No.:	To be provided by Supplier or Sponsor
Description:	To be provided by Supplier or Sponsor
Formula:	$C_{28}H_{36}O_3$
Solubility:	Insoluble in water; soluble in alcohol ether, pyridine, other organic solvents, and in vegetable oils
Bulk Storage Conditions:	Desiccated at room temperature
Estimated Quantity:	10 grams
Dosing Solution Stability:	To be provided by Supplier or Sponsor
Dosing Solution Storage:	Room temperature

Safety Precautions: Human exposure to the dosing solution will be restricted by the use of gloves, disposable laboratory clothing (coats or jumpsuits, booties), and safety glasses or goggles. When handling the neat chemical, protective clothing, gloves to prevent skin contact, and safety glasses or goggles are minimum required equipment.

2. Vehicle: Stripped Corn Oil

CAS No.: 8001-30-7

Supplier: Sigma Chemical Company

Lot Number: To be determined

E. Dose Formulation and Analysis

1. Preparation of Formulations and Storage

Dosage formulations for injection will be prepared by _____. The test compound to be used in this study will be TP (0.1, 0.2, 0.4, 0.8, and 1.6 mg/kg/day). The vehicle control will be stripped corn oil and will be prepared by _____.

2. Analysis of Formulations

Dosing formulations will be saved for possible analyses for concentration from aliquots of doses used in the study. Samples will be stored at room temperature until analysis.

IV. EXPERIMENTAL DESIGN

A. Study Design

The goal is to characterize the dose response of the sex accessory tissues in castrated male rats to the reference androgen, TP (Groups A-E, n=6 per group, Table 1). Animals will be dosed with TP by subcutaneous injection on the dorsal surface of the animal. The maximum limit on the volume administered per animal is approximately 0.5 ml/kg body weight per day. The animals will be dosed in the same manner and time sequence for ten consecutive days at approximately 24-hour intervals. The dosage level will be adjusted for changes in body weight.

Table 1. Experimental Design: Agonist Response

Group	Treatment
Group 1 - Vehicle Control	Vehicle
Group 2	TP: 0.1 mg/kg/day
Group 3	TP: 0.2mg/kg/day
Group 4	TP: 0.4 mg/kg/day
Group 5	TP: 0.8 mg/kg/day
Group 6	TP: 1.6 mg/kg/day

The volume of dose and time that it is administered will be recorded on each day of exposure. Animals will be assigned to treatment groups by means of randomized complete block approach and stratified such that treatment groups have equivalent mean body weights.

B. Experimental Procedures: Treatments

1. General Condition and Symptoms

Clinical examinations will be conducted and recorded at least once daily throughout the course of the study. Mortality checks will be done once daily. These cage-side observations will include, but not be limited to, changes in skin and fur, eyes, mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior. Any animal found dead will be removed and disposed of without further data analysis. Any mortality of animals prior to necropsy will be included in the study record together with the reasons.

2. Body Weight

Body weights will be recorded once during quarantine (for randomization), at time of surgery, at daily treatments (for calculation of dosing volume), and at necropsy to monitor body health and verify lack of overt general toxicity. A body weight loss of greater than 10% or reduced body weight gains will be considered as an indicator that general toxicity has resulted. Group means and standard deviations will be calculated.

3. Feed and Water Consumption

Feed consumption will be generally observed and any significant events recorded (e.g., animals not eating or drinking). Water and food consumption will not be measured. A sample of the diet will be collected for possible future analysis at the request of the Sponsor.

4. Administration of Compound

The males will be injected subcutaneously with TP or vehicle once daily for ten consecutive days.

C. Experimental Procedures

1. Necropsy

Animals will be used to evaluate the effect(s) of TP on sex accessory tissues in castrated male rats. Approximately 24 hours after the last administration of the test substance, blood will be collected by cardiac puncture (optional) and the rats will be euthanized by CO_2 inhalation and then exsanquinated.

The order in which the animals are necropsied will be a block design, such that one animal from each of the groups is necropsied (in a random, within-block fashion) before necropsy of the second animal from each group. In this way, all the animals in the same treatment group are not necropsied at once.

If the necropsy of each animal requires more total time than is reasonable for a single day, necropsy will be conducted on consecutive days. In this case, the work would be divided so that necropsy of two to three animals per treatment per day takes place on the first day, with dosing and necropsy being delayed by one day in the other animals.

The sex accessory tissues will be excised and their weights determined (see #3 below) for comparison with the weights of sex accessory tissues from the vehicle control group.

Carcasses will be disposed of in an appropriate manner following necropsy.

2. Histology

The sex accessory tissues will be preserved in 10% neutral buffered formalin and stored. Histology may be done at a later date at the request of the Sponsor for additional compensation.

3. Sex Accessory Tissue Weights

The sex accessory tissues will be excised and their weights determined, for comparison with the weights of sex accessory tissues from the vehicle control group or reference TP group (in the case of antagonist response). The sex accessory glands and tissues to be weighed are the: VP, SV plus CG, LABC, GP, and CP. The liver will also be excised and weighed. After excision and weighing of the ventral prostate, it will be fixed for 24 hours in 10% neutral buffered formalin (4% formaldehyde) and weighed again. The LABC will be weighed separately and then added. This allows the calculation of ratios, as these tissues are purportedly affected differently by different androgens (Hershberger et al., 1953).

D. Statistical Analyses

The sex accessory tissues will be excised and their weights determined (see above) for comparison with the weights of sex accessory tissues from the vehicle control group or reference TP group (in the case of antagonist response). Weights and other information will be placed in proper blanks on electronic spreadsheets furnished to the Sponsor.

V. RETENTION OF SPECIMENS AND RECORDS

_____will be responsible for data management until the end of the study. Study record data and reports will be sent to the Sponsor at the completion of the study, where they will be retained according to OECD retention requirements. Specimens will be maintained in formalin for one year from acceptance of the final report. If histology is not requested by the Sponsor within that year, the tissues will be discarded with the permission of the Sponsor's Representative.

VI. RECORDS TO BE MAINTAINED

Receipt of Chemical, Certificate of Analysis and other Chemistry Records

Dosing Formulation and Dose Administration Records

Animal Receipt Records, Animal Room Log Sheets

Temperature and Humidity Records for Animal Rooms

Quarantine Animal Health Surveillance Records

Randomization and Assignment to Study

Male Body Weights

Clinical Signs

Necropsy-Male Sex Accessory Tissue Weights and Gross Pathology Findings

Tissue Samples

VII. REPORTING

A. Status and Interim Reports

Informal status reports will be given via e-mail or telephone during the course of the study and documented in the study record. Time points of interest include initiation of dosing and completion of necropsy.

B. Draft and Final Reports

The Study Director, prior to the issuance of the final report, will issue a draft final report to the Study Monitor. The final report format will correspond to that specified for data reporting of drugs to the extent possible for this study. The final report, including individual data tables and complete statistical analyses of data, will be submitted to the Sponsor's Representative following acceptance of the draft report. The final report will include:

Abstract

Introduction

Experimental Design

Materials and Methods

Narrative Discussion of Parameters Evaluated

Discussion

Conclusions

References

Summary Data (means $\pm$ standard errors) by Dose Group and Time Points

Individual Data Tables

Statistical Analysis

Appendices: Protocol and Amendments, if any

Feed and Water Analysis

VIII. REFERENCES

Ashby, J., and P.A. Lefevre (2000). Preliminary evaluation of the major protocol variables for the Hershberger castrated male rat assay and for the detection of androgens, antiandrogens and metabolic modulators. *Reg Tox Pharm.* 31, 92-105.

Hershberger, L., E. Shipley, and R. Meyer (1953). Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. *Proc. Soc. Exp. Biol. Med.* 83, 175-180.

OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1, Organisation of Economic Co-operation and Development, Paris 1998. OECD Principles of Good Laboratory Practice (as revised in 1997).

APPENDIX B

COMPARISON OF UTEROTROPHIC VERSUS HERSHBERGER ASSAY

Comparison of Uterotrophic Versus Hershberger Assay (page 1 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
Current versions	Intact, sexually immature female Ovariectomized, sexually mature female	Intact, sexually immature male Castrated, sexually immature male Castrated, sexually mature male
Evaluation species	Rat, mouse	Rat, mouse
Initial developer(s)	Allen and Doisy (1923, 1924); Bülbring and Bern (1935)	Korenchevsky (1932); Eisenberg and Gordan (1950); Hershberger *et al*. (1953)
Endogenous sex steroid	17β-estradiol (E2)	Testosterone (T) and dihydrotestosterone (DHT) from T by 5α-reductase
Site of synthesis	Thecal and granulosa cells of the follicle in the ovaries	Interstitial cells of Leydig in the testes
Receptor	Estrogen receptor α and β (ER)	Androgen receptor α, β, γ (AR)
Response organs	Weight of uterus usually without luminal fluid (weighed wet and/or dry) Absolute weight (mg or g) or relative weight (mg or g per 100 g or kg terminal body weight)	Five accessory sex organs: Weight of intact prostate (now usually ventral and dorsolateral prostate lobe weights separately); paired seminal vesicles and coagulating glands; paired Cowper's (bulbourethral) glands; levator ani/bulbocavernosus (LABC) muscle; glans penis, and, in the intact animals only, epididymides. Absolute weight or relative to terminal body weight
Procedure	A. Intact immature female Prior to puberty (for mouse, begin on PND 21/22; for rat, begin on PND 25), daily administrations of test chemical sc, po, iv, ip, diet, etc.) for 3-5 consecutive days. Necropsy 24 hours after last dose and weigh the uterus. Group sizes vary by author.	A. Intact immature male Prior to puberty (for mouse, begin on PND 24-25; for rat, begin on PND 32-38), daily administration of test chemical (sc, po, iv, ip, diet, etc.) for 5-7 days. Necropsy 24 hours after last dose and weigh the five specified accessory sex organs. Group sizes vary by author.

Comparison of Uterotrophic Versus Hershberger Assay (page 2 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
Procedure	B. Ovariectomized immature female (OECD method of choice; OECD series on Testing Assessment No. 38, 2003) Prior to puberty, ovariectomize females (no need to wait before administration of test material). Administer test material for 3-5 consecutive days, necropsy at last dose, and weigh the uterus.	B. Castrated immature male (OECD method of choice; Owens *et al*., 2006) Prior to puberty, castrate males (for OECD validation [Owens *et al*. 2006], castrate the males on PND 42 since castration prior to PND 40 prevents complete PPS). Wait 12 days for T/DHT-dependent tissues to regress. Then, administer test material for 10 consecutive days. Necropsy 24 hours after last dose and weigh the five specified accessory sex organs.
	C. Ovariectomized, mature female Postpubertal adult female is ovariectomized at >60 days old. Wait 0-14 days post-ovariectomy for E2-dependent tissues to regress. Then, administer test material for 3-5 consecutive days. Necropsy 24 hours after last dose and weigh the uterus.	C. Castrated, mature male Postpubertal adult male (>70 days old) is castrated. Wait 14-28 days post-castration for T/DHT-dependent tissues to regress. Then, administer test material for 10 consecutive days. Necropsy 24 hours after last dose and weigh the five specified accessory sex organs.

Comparison of Uterotrophic Versus Hershberger Assay (page 3 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
To detect	Estrogenic activity: Compare the weight of the uterus after ovariectomy or prepuberty (with no treatment) to the weight of the uterus after repeated administration of test material. If the uteri from treated females are significantly heavier than the uteri from untreated ovariectomized or prepubertal females, then the test material is estrogenic.	Androgenic activity: Compare the weights of the sex accessory organs after castration or prepuberty (with no treatment) to the weights of the five specified organs after repeated administration of test material. If the weights of the five specified organs from treated males are significantly higher than the five specified organ weights from the untreated castrated or prepubertal males, then the test material is androgenic.
To detect	Anti-estrogenic activity: Same as above except dose the prepubertal or ovariectomized female with a potent estrogen (E2 or ethinyl estradiol [EE]) and the test material concurrently. Necropsy 24 hours after last dose. Weigh the uteri from vehicle control (no treatment), positive control (E2), test material only, and test material plus E2 groups. If the test material is an anti-estrogen, then the increase in uterine weight with E2 plus test material is significantly less than that with E2 alone; uteri from the group treated with test material only are compared with uteri from the vehicle control group and should be equivalent (i.e., no growth), and the uterine weight from the E2 only exposed group should exhibit the maximal response.	Anti-androgenic activity: Same as above except dose the prepubertal or castrated males with a potent androgen (e.g., testosterone propionate [TP], methyl-testosterone [mT], etc.) and the test material concurrently. Necropsy 24 hours after last dose. Weigh the accessory sex organs 24 hours after last dose, from the vehicle control (no treatment), positive control (TP, mT, etc.), test material only, or test material plus TP (or mT) groups. If the test material is an anti-androgen, then the specified organ weights with T and test material will be significantly lower than the organ weights with T alone; the organ weights from the group treated with test material are compared with the weights for the vehicle control group and should be equivalent (i.e., no growth); the five specified organ weights from the TP (or mT) only exposed group should exhibit the maximal response.

Comparison of Uterotrophic Versus Hershberger Assay (page 4 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
Background review document (BRD)	OECD Series on Testing and Assessment No. 38, detailed background review of the uterotrophic bioassay, March 2003	BRD in Progress
Phase 1:	Kanno *et al*., 2001	Owens *et al*., 2006
Phase 2:	Kanno *et al*., 2003	In preparation (draft report)
Phase 3:	Publication in preparation	In preparation (draft report)
Potent agonists	Ethinyl estradiol, DES, estriol, estrone, clomifene (selective estrogen receptor modulator; SERM)	Trenbolone acetate, methyl T, TP, androstanolone
Weak agonists	Methoxychlor (one metabolite has anti-androgenic activity), genistein, diadzein, octylphenol, nonylphenol, bisphenol A, o,p'-DDT, zearalanol	Mesterolone, flyoxymesterone
Potent antagonists		Flutamide, bicalutamide, cyproterone acetate
Weak antagonists	Tamoxifen, raloxifene (SERMs)	Linuron, vinclozolin, procymidone, p,p'-DDE, cyproterone
Agonist mechanism	Pre- and postpubertal uterus has ER (estrogen receptors). When an estrogenic compound (i.e., an estrogen agonist) is administered, it is recognized by and binds to the ER. The presence of the agonist ligand on the ER initiates a cascade of gene transcriptional activation, resulting in inhibition and growth of the organ.	The pre- and postpubertal accessory sex organs have ARs. When an androgenic compound (i.e., an androgen agonist) is administered, it is recognized by and binds to the AR. The presence of the agonist ligand on the AR initiates a cascade of gene transcriptional activation resulting in the growth of the accessory sex organs.

Comparison of Uterotrophic Versus Hershberger Assay (page 5 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
Antagonist mechanism	When an anti-estrogenic compound (i.e., an estrogen antagonist) is administered, it is also recognized by and binds to the ER (*i.e.,* it acts as a ligand). The presence of the antagonist ligand on the ER blocks the cascade of gene transcriptional activations and also blocks any available endogenous estrogen (depending on the relative concentrations) from binding to the ER and triggering the cascade.	When an anti-androgenic compound (i.e., an androgen antagonist) is administered, it is also recognized by and binds to the AR (*i.e.,* it acts as a ligand). The presence of the antagonist ligand on the AR blocks the cascade of gene transcriptional activations and also blocks any available endogenous androgen (depending on the relative concentrations) for binding to the AR and triggering the cascade.
Strengths of the ovariectomized/castrated animal model	The ovariectomized prepubertal or adult female is a robust specific assay with only one mechanism: through binding to the ER. It is standardized and validated by OECD (Kanno *et al*., 2001, 2003). It detects both strong and weak estrogen agonists and antagonists. It works in various strains of mice and rats, under differing environmental conditions (feed, bedding, caging, temperature, relative humidity, housing, etc.), as long as all groups are exposed to the same conditions.	The castrated prepubertal or adult male is a robust, specific assay with only one mechanism: through binding to the AR. It is standardized and validated by OECD (Owens *et al*., 2006; Gray *et al*., 2005). It detects both strong and weak androgen agonists and antagonists. It works in various strains of mice and rats, under differing environmental conditions (feed, bedding, caging, temperature, relative humidity, housing, etc.), as long as all groups are exposed to the same conditions.

Comparison of Uterotrophic Versus Hershberger Assay (page 6 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
Weaknesses of the ovariectomized/castrated animal model	It does not detect estrogenic or anti-estrogenic compounds which do not act through the ER (e.g., those that affect steroidogenesis), but it does detect aromatase inhibitors since the ovary is the site where aromatizable androgens are converted to estrogens by aromatase. It cannot act as a "decision node" since it evaluates only one mechanism.	It does not detect androgenic or anti-androgenic compounds which do not act through the AR (e.g., those that affect steroidogenesis), tbut it does detect 5α-reductase inhibitors and metabolic modulators. It cannot act as a "decision node" since it evaluates only one mechanism.
Strengths of the intact animal model	This assay is a robust test that evaluates many mechanisms. Use of the intact pubertal animal allows detection of agents that act "anterior" to the ovary (e.g., hypothalamus, pituitary), act on the ovary, or act "posterior" to the ovary (e.g., end-organ responses, distribution, metabolism, elimination, etc.). It does detect aromatase inhibitors (see above). It can be used as a "decision node" since it evaluates a number of mechanisms. The use of the intact animal also has animal welfare advantages.	This assay is a robust test that evaluates many mechanisms. Use of the intact pubertal animal allows detection of agents that act "anterior" to the testis (e.g., hypothalamus, pituitary), act on the testis, or act "posterior" to the testis (e.g., end-organ responses, distribution, metabolism, elimination, etc.). It does detect 5α-reductase inhibitors (see above), It can be used as a "decision node" since it evaluates a number of possible mechanisms. The use of the intact animal also has animal welfare advantages.
Weaknesses of the intact animal model	Many mechanisms are evaluated but are not identified; additional assays/tests must be run to identify mechanism, if necessary.	Many mechanisms are evaluated but are not identified; additional assays/tests must be run to identify mechanism, if necessary.

Comparison of Uterotrophic Versus Hershberger Assay (page 7 of 7)

Parameter	Uterotrophic Assay	Hershberger Assay
Window of Responsiveness	In the immature, intact female rat, there is a window of maximum sensitivity and responsiveness to estrogen between PND 18 and 26. Prior to PND 18, the uterus is insensitive to maternal estrogens, and there is a high circulating level of α-fetoprotein (AFP). AFP declines rapidly after birth with the nadir reached on PND 16-17. The window of sensitivity closes at puberty when there is a burst of E2 from the ovaries and a consequent increase in baseline uterine weight (and loss of sensitivity to exogenous estrogens). Approaching puberty, variability in mean group uterine weight is increased. Loss of optimal conditions without surgical (or chemical) intervention begins at ~ PND 26 in the female rat (OECD, 2003). In the ovariectomized prepubertal or adult female, the tissues are responsive and sensitive to estrogens but (except for minor steroid synthesis [~8%] in the adrenal glands) there are no endogenous estrogens. Therefore, the uterus is sensitive and responsive to exogenous estrogens prior to puberty and after surgical or chemical ovariectomy.	The same situation exists in the intact, immature male, with sensitivity and responsivity optimum (maximum) prior to puberty (which begins in the rat after PND 35 and in the mouse after PND 25). In the intact male, puberty is the time of rapidly increased T from the testes and consequent increase in baseline weights of the accessory sex glands (and loss of sensitivity to exogenous androgens). Please note that the weanling assay appears to be approximately equivalent to the castrate assay with DDE and linuron (with four of six validation laboratories reporting to date; W. Owens, personal communication). Approaching puberty, variability in the mean weights of these organs is increased. Loss of optimal conditions occurs at puberty without surgical (or chemical) intervention (at ~ PND 38 in the male rat). In the castrated prepubertal or adult male, the tissues are responsive and sensitive, but (except for minor steroid biosynthesis [~8%] in the adrenal glands) there are no endogenous androgens. Therefore, the accessory sex organs are sensitive and responsive to exogenous androgens prior to puberty and after surgical or chemical castration.