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Detailed Background Review of the Uterotrophic Bioassay

Summary of the Available Literature in Support of the Project of the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) to Standardise and Validate the Uterotrophic Bioassay

Environment Directorate
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The work of the OECD related to chemical safety is carried out in the **Environment, Health and Safety Programme**. As part of its work on chemical testing, the OECD has issued several Council Decisions and Recommendations (the former legally binding on Member countries), as well as numerous Guidance Documents and technical reports. The best known of these publications, the **OECD Test Guidelines**, is a collection of methods used to assess the hazards of chemicals and of chemical preparations. These methods cover tests for physical and chemical properties, effects on human health and wildlife, and accumulation and degradation in the environment. The OECD Test Guidelines are recognised world-wide as the standard reference tool for chemical testing.

More information about the Environment, Health and Safety Programme and its publications (including the Test Guidelines) is available on the OECD's World Wide Web site (see page 7).

The Environment, Health and Safety Programme co-operates closely with other international organisations. This document was produced within the framework of the Inter-Organisation Programme for the Sound Management of Chemicals (IOMC).

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, FAO, WHO, UNIDO and the OECD (the Participating Organisations), 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. UNITAR joined the IOMC in 1997 to become the seventh Participating Organisation. 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.

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PREAMBLE

This detailed background review paper provides a summary of the relevant literature (up to September 2001) relevant to the standardisation and validation of the rodent uterotrophic bioassay. The rodent uterotrophic bioassay is being validated as part of the OECD Test Guidelines Programme.

The rodent uterotrophic bioassay is based on the principle that the uterus is under the control of oestrogens 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 uterine growth.

The objective of the OECD work on the uterotrophic 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 female sex hormones. More specifically the rodent uterotrophic bioassay is intended to identify chemicals that act like oestrogen agonists or antagonists. The assay, once validated, is intended to be used as a short-term assay within an overall testing strategy for the detection and assessment of potential endocrine disrupters.

Dr William Owens, a member of the OECD's Validation Management Group for the Screening and Testing of Endocrine Disrupters for Mammalian Effects (VMG-mam), prepared this detailed background document on behalf of the Group.

The document describes the scientific basis for the uterotrophic bioassay, including:

- Biological basis and mode of action;
- History of the development and use of the uterotrophic bioassay;
- The two primary versions for the uterotrophic bioassay (sexually immature female and ovariectomised sexually mature female);
- Procedural variables of the uterotrophic bioassay that apply to both versions;
- Performance characteristics of the uterotrophic bioassay;
- Experience of using the uterotrophic bioassay with different test substances; and
- Background pharmacodynamic and toxicity information for selected weak oestrogen agonists.

A draft of this document was circulated for review to the VMG-mam on 9 July 2001 with a deadline for comment of 17 August 2001. All comments received by that date have been addressed in this version. In addition the original author has added information that has become available since circulation to the VMG-mam.

The detailed background document will provide one of the essential references for independent peer review of the validation of the uterotrophic bioassay.

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EXECUTIVE SUMMARY

i) The purpose of this detailed background review paper is to provide a summary of the literature available as of September 2001 in support of the standardisation and validation of the rodent uterotrophic bioassay. Preparation for the validation of a test method includes a number of tasks, e.g., standardisation of a protocol and standard operating procedures (SOPs), testing the transfer of the protocol to get reproducible results among qualified laboratories, testing of intra- and inter-laboratory variation over time, etc. (Balls *et al.*, 1990, 1995; Bruner *et al.*, 1996; Curren *et al.*, 1995; ICCVAM, 1997; OECD, 1998b). This document examines the scientific literature relevant to the protocols for the uterotrophic bioassay based on a procedure using three consecutive days of test substance administration to either intact sexually immature, or ovariectomized (OVX) young adult laboratory rodents. The intended purpose of the uterotrophic bioassay is to identify chemicals that act like oestrogen agonists and/or antagonists *in vivo*. The assay, once validated, is intended to be used as a short-term assay in an overall testing strategy for the detection and assessment of potential endocrine disrupters. Such substances may then require additional tests for adverse reproductive and developmental effects.

ii) The rodent uterus and its rapid and dramatic growth in response to oestrogen during the natural oestrous cycle are the basis for the uterotrophic bioassay. The mode of action for oestrogen, and similar substances, begins by its acting as a ligand and binding to the oestrogen receptor (ER). The binding event initiates (agonist) or inhibits (antagonist) a cascade of molecular, biochemical, and physiological events in the uterus. This cascade, beginning with oestrogen-mediated gene transcription, culminates in the growth of the uterus, which is measured by increased uterine weight.[1] A uterine weight increase, compared to an untreated control, can be used to indicate whether a chemical exhibits the characteristics of an oestrogen agonist or antagonist *in vivo*. The natural response of the uterus to oestrogen during the oestrous cycle, the molecular action of oestrogen and related substances as ligands for the ER, and the cascade of events leading to uterine growth are discussed in **Chapter 1. Biological basis of the uterotrophic bioassay and mode of action**.

iii) The uterotrophic bioassay has been used historically for the pharmaceutical development of oestrogen agonists and antagonists. Pharmaceutical development originally used the assay to detect substances with a relative binding affinity (RBA) to the ER that was similar to the natural ligand, 17β-oestradiol. Another intended application of the uterotrophic bioassay is, however, to detect weak oestrogen agonists and antagonists. Weak agonists and antagonists are defined here as having RBA's less than 1% of 17β-oestradiol (log RBA <0). The history of the development and use of the assay, including a number of procedural variations, is discussed in **Chapter 2. History of the development and use of the uterotrophic bioassay.**

iv) Two versions of the uterotrophic bioassay, the sexually immature female rat and the OVX adult female rat, appear qualitatively equivalent based upon the available literature. The literature supports administration of the test substance between postnatal day 18 (pnd 18) and 26 (pnd 26) in the immature rat version. This provides a window of sensitive and maximum response to oestrogens. Thus, for the immature version, the age of animals should be carefully specified in order to conduct the assay during this temporal window. The ovariectomized (OVX) version should allow sufficient time for regression of the uterus, approximately 14 days after ovariectomy, for a sensitive and maximum response. The OVX animals should be monitored for possible incomplete ovariectomy, which could change control values and

[1] As noted below, the uterotrophic assay is conducted using either sexually immature or ovariectomized females, so that the uterine weight is low and stable. This precludes the use of intact, adult female animals due to the significant and rapid changes in uterine weight that occur during the rodent oestrous cycle.

reduce sensitivity in test animals. In both versions, a procedure of administering three daily consecutive doses, followed 24-hours later by necropsy for measuring uterine weights, appears sufficient to detect possible oestrogen agonists and antagonists. The basic details of both versions and the basis for concluding that the versions appear equivalent are discussed in **Chapter 3. The primary versions for the uterotrophic bioassay**.

v) The procedural variables for each version for the uterotrophic bioassay have been identified from the literature. The literature provides background information on the influence of the strain of animal used. The influence of strain on the assay appears to be small in mice and rats. Other variables include routes of administration, choice of vehicle, and tissue dissection and preparation procedures, *e.g.*, precautions to prevent the desiccation of the uterus, equipment, and technical skills need to perform the assay. There are two primary endpoints, the wet and blotted uterine weights that, respectively, include and exclude the uterine luminal fluid. The blotted uterine weight has been more widely used and is less variable than the wet weight. Historically, only the body of the uterus has been weighed. However, inclusion of the cervix in the weight of the uterus allows the retention of the luminal fluid. As the cervix is nearly as responsive to oestrogen as the uterus itself, this will not impair the overall performance of the assay. Procedural variations such as fixation of the uterus before weighing, or using an additional endpoint such as the oven-dried uterus weight, are reported in the literature. Any advantages that these variations might confer have not been fully documented. Statistical procedures used to analyse the assay data have also varied. Finally, there is evidence that phytoestrogens in some laboratory diets can influence the responsiveness of the uterotrophic bioassay. Incidents of sporadic increases in uterine weight due to phytoestrogens have been recorded, but the problem does not appear widespread. Most current diets appear to be adequate for use as long as phytoestrogen levels are below 200 μg, as genistein equivalents, per gram of diet. Therefore, phytoestrogen analyses of the diet, or testing the uterine baselines and responses of animals to dietary lots, may be useful as a precaution. The details of the various procedures involved in the uterotrophic bioassay, including the diet, are discussed in **Chapter 4. Procedural variables of the uterotrophic bioassay**.

vi) It is important that a widely used assay is repeatable, specific, uses the minimum number of animals, and has its limitations identified. There are conflicting reports in the literature concerning the reproducibility of the uterotrophic bioassay. The reported cases of non-reproducibility commonly occur where a minimal 20-40% increase in uterine weight was found to be statistically significant. Group-to-group variation in control (untreated) uterine weights have been documented that would be sufficient to occasionally generate false positive results.[2] Similar low percentage uterine weight increases could also be observed for a positive substance at the low, uncertain end of the dose-response curve. One suggested remedy to distinguish a positive from a negative substance is that a positive should display a dose-response curve for at least two doses, while achieving uterine weight increases >40% over the controls at the high dose. Positive results with the uterotrophic bioassay alone, however, may not be definitive that a substance is an oestrogen agonist or antagonist. In order to improve the assay's overall specificity and to confirm the positive nature of an uterine weight increase, three strategies have been suggested: 1) to screen candidate substances by performing precursor assays, such as ER binding; 2) to provide for complementary and confirmatory endpoints that can be used concurrently, such as histological changes in the vaginal or uterine epithelium; or 3) to use both strategies. The uterotrophic bioassay appears to be efficient in the use of animals and can be successfully conducted with as few as six animals per dose group. The overall use of animals in any overall testing strategy could be further reduced by assessing the structure of unknown substances as possible ligands for the ER, or conducting *in vitro* screens, *e.g.*, receptor-binding affinity or other *in vitro* assays responsive to oestrogen ligands before conducting the

[2] A false positive is defined as a substance that is incorrectly identified as an oestrogen agonist or antagonist based upon an increase in uterine weight (this would include acting directly as the parent substance or indirectly through metabolic activation).

uterotrophic bioassay. The details of repeatability, specificity, animal use, and performance limitations for the assay, as well as possible future improvements, are discussed in **Chapter 5. Performance characteristics of the uterotrophic bioassay**.

vii) The pharmacodynamics and the pharmacokinetics of oestrogen metabolism clearly indicate a need to use an *in vivo* assay with animals in the overall assessment of possible oestrogen agonists and antagonists. The essential assumption is that the active factor is the concentration in the serum of a free, bioavailable ligand in equilibrium with the intracellular ligand concentration. This portion of an administered substance is then the fraction available to bind the ER. A multiplicity of factors and events affect the serum concentration of the ligand and, thus, its *in vivo* activity in the uterotrophic bioassay. These factors include intestinal and liver metabolism leading to either active or inactive metabolites, intestinal and liver conjugation reactions, biliary and other routes of excretion, specific and non-specific binding to serum proteins, sequestration in other body compartments, *e.g.,* adipose tissue for hydrophobic compounds like most oestrogen agonists, and the receptor concentrations in target tissues. Collectively, these factors support the necessity to assess a test substance in the intact animal. The pharmacodynamics and pharmacokinetics of oestrogen metabolism are discussed in **Chapter 6. Performance of the assay**.

viii) An important question is whether the uterotrophic bioassay results are predictive of adverse effects. Several weak agonists positive in the uterotrophic bioassay have elicited some, but not all, of the effects of 17β-oestradiol in reproductive and developmental bioassays. Other weak agonists in the uterotrophic bioassay have not displayed oestrogenic activity in reproductive or developmental bioassays. In general, the doses at which these adverse effects are observed have been similar to doses giving positive responses in the uterotrophic bioassay when using oral gavage as the route of administration. Therefore, a positive response in the uterotrophic bioassay suggests 1) the need for additional testing for adverse reproductive and developmental effects, and 2) when administered by oral gavage, a possible dietary dose range in chronic, definitive tests that may lead to oestrogen-mediated effects. The available test data for a number of oestrogenic substances, including those used in recent validation work, is compiled and discussed in **Chapter 7. Background pharmacodynamic and toxicity information for selected weak oestrogen agonists**.

ix) The possible use if the uterotrophic bioassay in the testing and assessment of chemicals is discussed in **Chapter 7**

x) The data summarised in this paper provide broad support for the validation and regulatory use of the uterotrophic bioassay as an *in vivo* screen for possible oestrogen agonists and antagonists. In addition:

- Clear evidence supports the binding of a ligand to the ER as an initial step in a cascade of molecular, biochemical, and physiological events that culminate in uterine growth. Increased uterine growth is measured gravimetrically in the uterotrophic bioassay.

- The extensive history of the uterotrophic bioassay supports the ability of the assay to evaluate the oestrogenic potential of substances, even weak oestrogen agonists with log RBAs less than 0 and greater than 3.

- The two major versions for the uterotrophic bioassay, the intact sexually immature rat and the ovariectomized sexually mature rat, appear to be equivalent.

- The major procedural variables for the uterotrophic protocol are known.

- Many laboratories have the technical skill, equipment, and facilities to conduct the uterotrophic bioassay.

- The overall reproducibility and specificity of the uterotrophic bioassay appears to be adequate, and the limits of its application to different classes of test substances have been demonstrated. With regards to specificity, modest increases in uterine weight (20-40%) at high doses present the possibility that a false positive result may occur. Criteria for accepting data, *e.g.*, maxima for acceptable vehicle control uterine weights, together with clear guidance for interpretation, may be useful.

- Pharmacodynamics and pharmacokinetic factors in the intact animal can modify the activity of a test substance. This supports 1) the need to use animals in a tiered, hierarchical framework, and 2) the need to use a relevant route of administration for each test substance.

- There is a general correspondence between the uterotrophic bioassay and subsequent testing outcomes for adverse effects.

- Several substances positive in the uterotrophic bioassay have produced oestrogen-mediated effects in reproductive and developmental assays.

- Other substances positive in the uterotrophic bioassay have not caused oestrogen-mediated effects in reproductive and developmental assays, *i.e.*, some false positives occur.

- At doses where no evidence for adverse effects has been found in robust reproductive and developmental assays, the uterotrophic bioassay has been negative by a similar route of test substance administration.

- Structure-activity relationships and *in vitro* assays appear able to identify substances with an oestrogenic mode of action as candidates for the uterotrophic bioassay, thereby minimising the use of resources and animals.

xi) In summary, the available data support the fitness of the uterotrophic bioassay to identify those substances which may act though an oestrogen mode of action and warrant consideration of further testing for adverse effects.

CHAPTER 1: BIOLOGICAL BASIS OF THE UTEROTROPHIC BIOASSAY, AND MODE OF ACTION

1. This Chapter summarises the biological bases of the uterotrophic bioassay and its mode of action. In particular the following points are addressed:

- Uterine structure and the oestrous cycle in laboratory rats and mice.
- The basis for oestrogen's biological effects in the uterotrophic bioassay.
- The structure of an ER ligand as it relates to its receptor-binding affinity.
- The binding affinity of an ER ligand and subsequent uterotrophic activity.
- The molecular and cellular events that occur between ligand binding to the ER and the appearance of a uterine weight increase.

2. The biological and scientific basis for the uterotrophic bioassay is the central role of oestrogen in the natural oestrous cycle. The tissues of the female reproductive tract - the uterus, cervix, and vagina - respond rapidly to oestrogen with cell division and tissue growth, leading to weight increases. The weight increases of these tissues provides an inherent biological measurement for the possible oestrogenic activity of administered test substances. In rats and mice, endogenous 17β-oestradiol stimulates this tissue growth response within a period of approximately two days. The entire cycle of growth and regression is complete in 4-5 days. The time-frame for conducting the uterotrophic bioassay roughly corresponds to this time, and measures increases in uterine tissue weight after 3 consecutive days of test substance administration.

3. The uterotrophic bioassay also presents the opportunity to assay antagonist, or anti-oestrogenic, activity. The test substance and a potent reference oestrogen are administered together. The inhibition of the uterine weight increase in the test animals is measured relative to the control animals which only receive the reference oestrogen. Any inhibition of the oestrogen-mediated weight gain is presumed to be mediated by the anti-oestrogen.

Uterine Structure And The Oestrous Cycle In Laboratory Rats And Mice

4. The uterus in rats and mice is composed of three basic tissue layers: the outer myometrium, the middle stroma, and the inner epithelium plus epithelial glands that penetrate the stroma. These tissues each respond differentially to oestrogen.

5. When viewed in transverse section, the two horns of the uterus of rats and mice are basically cylindrical bodies composed of three, concentric anatomical layers. The outer layer is the myometrium. This layer is composed of an external sheet of longitudinal muscle, a central sheet of connective tissue, and an internal sheet of circular muscle, and is responsible for uterine contractions. The vascular stroma comprises the middle layer of the uterus, and is primarily a connective tissue. The innermost layer is the luminal epithelium, which includes a number of glandular structures. The epithelium supports the implantation of the fertilised ovum and is essential for contact with the placenta to support the developing foetus. The possible relationships of these different tissues in the oestrogen-mediated response of the uterus are discussed in the following section.

6. The initial growth and differentiation of the uterine tissues appears to be oestrogen-independent. These tissues are immature at birth, and they differentiate and develop post-natally. There is a period of rapid growth and cellular differentiation, including the postnatal development of uterine epithelial glands, which penetrate the stroma. The differentiated uterus is achieved by postnatal day (pnd) 15.[3]

Oestrogen and the oestrous cycle in the laboratory rodent

7. The endogenous steroidal oestrogen, 17β-oestradiol, is the essential endocrine signal that controls the cell division and growth of several tissues in the mammalian female, e.g., uterus, cervix, vagina, and mammary glands. Of these, the reproductive tract tissues; uterus, cervix, and vagina, undergo rapid cyclic growth and regression during the oestrous cycle. In laboratory rats and mice, the entire cycle of growth and regression is 4-5 days in length. In perspective for the uterotrophic bioassay, the uterus does not reach either 1) a minimum weight, because circulating endogenous oestrogen levels are always present, or 2) a maximum potential weight, because administration of additional oestrogen increases the uterine weight still further.

8. The uterus, then, presents an opportunity for a short-term assay to identify the oestrogenic activity of exogenously administered test substances. The necessary condition is a non-functional hypothalamic-pituitary-gonadal axis to ensure a sensitive and consistent uterine response to both exogenous oestrogens and to anti-oestrogens. The increase in uterine weight is measured after 3 consecutive days of test substance administration. For the measurement of anti-oestrogens, a reference dose of a potent oestrogenic compound is administered to both test and control groups of animals. In the test animals, the test substance is simultaneously co-administered. The rationale is that an anti-oestrogen will inhibit the action of the potent oestrogen, thereby reducing the growth of the uterus relative to the control animals.

Brief description of the hormonal control of the oestrous cycle

9. The oestrous cycle is controlled by the hypothalamic-pituitary-gonadal endocrine axis. The endocrine hypothalamus[4] secretes gonadotropin-releasing hormone (GnRH) into the specialised hypophyseal portal circulation. This capillary system carries the GnRH directly to the target anterior pituitary gland without dilution in the general circulation. There basophilic cells with GnRH receptors respond and secrete the two primary hormones regulating gonadal function into the general circulation, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). After distribution, both FSH and LH act via cell surface receptors to activate second messenger cascades to up-regulate or down-regulate target genes and processes in the target cells.

10. The primary site of 17β-oestradiol synthesis is the ovary, although several tissues appear to produce low levels of 17β-oestradiol that may act locally, *e.g.*, bone and the male reproductive tract (Hess *et al.*, 2001; Labrie *et al.*, 1997; Sasano *et al.*, 1997; Simpson *et al.*, 2000). During dioestrus, FSH regulates ovarian follicle maturation and stimulates follicular thecal and interstitial cells to produce and secrete androgens, which are precursors to 17β-oestradiol. Adjacent follicular granulosa cells take up these androgens. FSH simultaneously stimulates the follicular granulosa cells to produce the aromatase enzyme, which converts available androgens into 17β-oestradiol. The 17β-oestradiol is released both into the follicle and into the general circulation. From the general circulation, 17β-oestradiol affects the primary target organs, the uterus, cervix, and vagina. The circulating 17β-oestradiol also closes the feedback loop with the hypothalamic-pituitary-gonadal endocrine axis, ensuring that homeostasis is

[3] Additional details describing early uterine development and its estrogen independence can be found in Chapter 3.

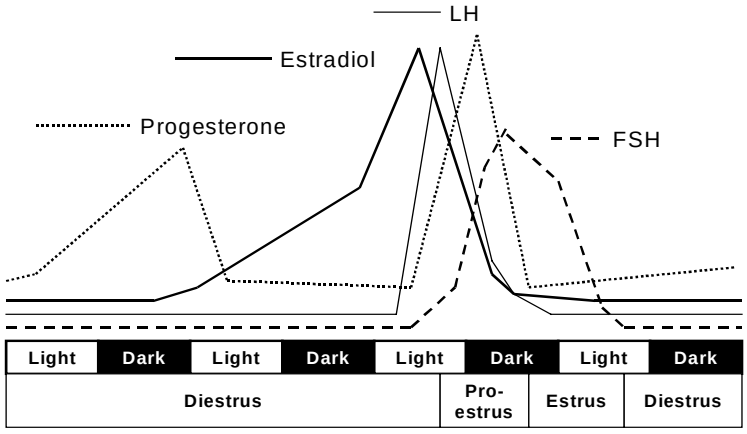
[4] The GnRH secretion is also under modifying control from higher brain centers, as the secreting cells are the targets of neurons releasing dopamine, norepinephrine, and γ-aminobutyric acid.

maintained. However, GnRH secretion is also under modifying control from higher brain centers as the hypothalamic secreting cells are the targets of neurons releasing dopamine, norepinephrine, and γ-aminobutyric acid.

11. The hormonal patterns in the oestrous cycle are depicted in **Figure 1.** The circulating quantities in serum are small. FSH, LH, and progesterone can be analytically detected in nanograms per ml, while circulating oestrogen levels can be detected at picograms per ml. The period of increasing 17β-oestradiol levels, and the resulting cell division and tissue growth responses of the uterus and vagina, occur within 2 days.[5] Administration of chemicals for 3 days would then exceed the natural response time for the growth phase of the uterus to endogenous oestrogen. For the interested reader, Smith *et al.* (1975) recorded levels of these circulating hormones during the oestrous cycle of rats at approximately two-hour intervals.

12. Thus, the uterus appears to be an ideal tissue for detecting oestrogenic effects: its natural response time is relatively short (~2 days), its gravimetric weight increase is easily measured, its sensitivity can be increased in the absence of endogenous oestrogens (low baseline weight), and the magnitude of its potential response is at least several-fold. Because the uterine weight increase is one that occurs naturally, the uterotrophic bioassay measures a natural biological response.

Figure 1. Representation of the hormonal changes during the 4-day rat uterus cycle in rats with a time progression of approximately 12-hour light and dark periods.



The Basis for Oestrogen's Biological Mode of Action in the Uterotrophic bioassay

13. The mode of action of 17β-oestradiol is to bind the ER as an agonist ligand. This ligand-receptor interaction is the essential initiating event for biological activity leading to uterine cell division and growth.

14. Several lines of evidence support that ligand binding to the ER initiates tissue responses: 1) the presence of ERs in the responding uterine tissues; 2) the absence of a uterotrophic response to oestrogens

[5] See Davis *et al.* (2001) for data on the cycle length and endocrinology in rat (Sprague-Dawley and F344) and mouse (CD-1 and B6C3F1) strains.

in mice when the ERα gene is functionally 'knocked out;' and 3) the inhibition of the action of oestrogens by specific oestrogen antagonists.

15. Oestrogen antagonists compete with oestrogen agonists for receptor binding sites. The anti-ER complex is inactive, fails to initiate further biological action, and can reduce or abolish biological activities such as uterine growth.

16. There is emerging evidence that, although the primary signal for uterine growth occurs through the ER, secondary apocrine and paracrine signals, based on epidermal growth factor (EGF) and other growth factors, may be relevant. For example, one suggested signaling pathway is that the uterine stroma, which is under oestrogen control, stimulates epithelial growth via paracrine production and secretion of EGF.

Nuclear receptor superfamily of genes

17. The ER is one member of a family of evolutionarily related proteins referred to as the nuclear receptor superfamily.[6] Nuclear receptors play a fundamental role in the modulation of metazoan gene transcription and mediate their activity through specific signal ligands. The nuclear receptors display a common, conserved functional structure (Mangelsdorf *et al.*, 1995). An *N*-terminal domain is involved in modulating non-ligand dependent transcriptional activation (activation factor-1 or AF-1) and may contain several phosphorylation sites. A central domain is the binding site for the DNA response elements through two structural zinc fingers. A *C*-terminal region is involved in both ligand binding and interactions with essential ER-coactivator proteins (*cf.* Gronemeyer and Laudet, 1996). This ligand-binding region structurally forms a cavity, so that the protein receptor completely envelops its cognate ligand in the binding process.

18. The basic outline of how nuclear receptors control gene transcription has been illuminated in the past decade or so. To briefly describe the multiple steps in these control events for the subset of nuclear receptors for steroid ligands, the steroid receptor is sequestered in a receptive state to receive the ligand by binding to a 'chaperone' complex composed of heat shock protein 90 and other proteins. This forms an apo-receptor (Buchner, 1999; Pratt and Toft, 1997). When the apo-receptor binds with a ligand, it is released from this chaperone complex. The activated steroid receptors appear to then form homodimers.[7] The activated ligand-receptor homodimer then binds to specific DNA activation elements (DNA base sequences) upstream of the target genes. Evidence has recently emerged that if the activated homodimer fails to bind to DNA, it is vulnerable to ubiquitin-based proteolysis via the 26S proteosome (Kopf *et al.* 2000; Laney and Hochstrasser, 1999; Lange *et al.* 2000). This is consistent with a rapid fall in the number of receptors in a cell when exposed to a receptor ligand, and would plausibly provide temporal control of the signal.

19. The DNA bound receptor complex then exerts transcriptional modulation of the target genes by performing several functions. The first function is to recruit essential transcriptional cofactors (see McKenna *et al.* 1999; Moras and Gronemeyer, 1998; Xu *et al.* 1999, for reviews). These specifically recruited cofactors perform several roles. One role is chromatin remodelling to make the DNA accessible to the basal cell transcription machinery, *e.g.*, acetylation of histones to open the DNA structure. This permits the recruitment of the general cell RNA transcriptional machinery to the TATA box promoter for

[6] These are distinct from cell surface transmembrane receptors that relay external signals to the cytoplasmic side of the membrane using second messenger mechanisms.

[7] Other nuclear receptors combine with other members of the receptor superfamily to form heterodimers, which apparently allows further refined control of transcriptional signals in either a positive or negative fashion.

the target gene. Another role is the phosphorylation of certain members of the general transcriptional complex to further activate and modulate the rate of target gene transcription.

20. The nuclear receptors display a common, conserved functional structure (Mangelsdorf *et al.* 1995). An *N*-terminal domain is involved in transcriptional activation, a centrally located domain is involved in binding the essential DNA control elements, and a *C*-terminal region is involved in ligand binding and other interactions essential to gene transcriptional activation (*cf* Gronemeyer and Laudet, 1996). The *C*-terminal binding region forms a cavity, which appears to literally and completely envelope the ligand in the binding conformation. In addition to the ER, considerable work has shown a strong relationship between the overall tertiary structure of the ligand binding region, the ligand binding role of specific amino acids in the sequence, and the ligand specificity of the receptor (see, *e.g.*, Egea *et al.*, 2000; Géhin *et al.*, 1999).

21. The steroid receptor family is a discrete subgroup of the nuclear receptor family and is expressed in vertebrates (Baker, 1997; Escriva *et al.*, 1997; Laudet, 1997; Mangelsdorf *et al.*, 1995; Thornton, 2001). The steroid receptor family includes oestrogen, androgen, progesterone, glucocortisone, and other receptors. The structure and mode of action of the nuclear receptors and steroid receptor subgroup have been reviewed by Beato *et al.* (1996), Tsai and O'Malley (1994), and recently by Weatherman *et al.* (1999).

Oestrogen receptor (ER)

22. The oestrogen first binds to the ER. Briefly, the active ligand-receptor complex binds to DNA sequences or response elements specific for the ER, and up-regulates or down-regulates the transcription of specific genes. The end result of gene transcription modulation is a biological response of the target tissue, such as uterine cell division and growth, in response to oestrogen.

23. The experiments of Jensen and Jacobsen (1962) first suggested that the specific binding of oestrogen occurred in oestrogen target tissues. The rat uterus and vagina were able to take up and retain either i.v. or s.c. injected [^{3}H]-17β-oestradiol in a tissue-selective fashion (see **Figure 2**). The data suggested that the observed compartmentalisation in target tissues was based on a specific, non-covalent binding of the oestrogen. The history of further early research confirming a specific receptor protein, and the interaction of the ligand-receptor complex with DNA, has been reviewed (Gorski *et al.*, 1968; Jensen and DeSombre, 1973).

The presence of ERs in target tissues

24. 17β-Oestradiol has multiple actions beyond its effect on the growth and differentiation of the uterus and the vagina, including sexual behaviour, maintenance of bone density, effects on circulating cholesterol and lipid levels, and development of the male testis and accessory sex tissues. The complete, individual molecular mechanisms for these multiple actions of 17β-oestradiol are incompletely known. The essential point is that ERs have been found in all associated target tissues, and are necessary for these tissues to respond to endogenous or administered oestrogens.

25. In addition to the original ER identified in the uterus, a second, β, form has recently been discovered in the rat (Kuiper *et al.*, 1996), mouse (Tremblay *et al.*, 1997), and human (Mosselman *et al.*, 1996). Both receptors are closely related in amino acid sequence and follow the basic structural features of steroid receptors, such as the DNA binding domain and the ligand binding *C*-terminal domain. As summarised in **Table 1,** the tissue distributions of the two ERs differ (Kuiper *et al.*, 1996, 1997) with the original (α-) receptor being significant in the ovary, testes, epididymis, and pituitary, and the β-receptor

significant in the ovary and prostate. The same workers have also explored the binding affinities of the different rat ERs for a spectrum of chemicals. While the binding affinities differ amongst the chemicals, the differences were rarely greater than an order of magnitude (Kuiper *et al.*, 1997).

Figure 2. Concentration of radioactivity in rat tissues after single subcutaneous injection of 0.098 μg of 17β-oestradiol-6,7-^{3}H in 0.5 ml saline (from Jensen and Jacobsen, 1962)

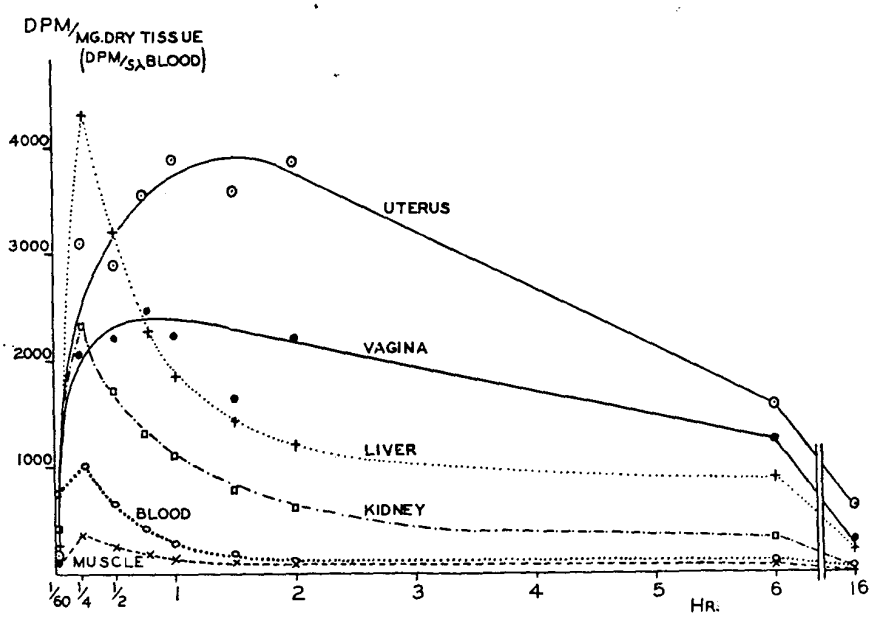


Table 1. Tissue distribution and cellular levels of ER forms in the rat.[a]

Tissue	ER-α	ER-β
Ovary	Significant	Significant
Uterus	Significant	Moderate
Testes	Significant	Moderate
Prostate	Limited	Significant
Epididymis	Significant	Limited
Pituitary	Significant	Limited
Hypothalamus	Limited	Limited
Liver	Limited	- -
Kidney	Moderate	- -
Bladder	Limited	Moderate
Adrenal	Moderate	- -
Lung	- -	Moderate

[a] See Kuiper *et al.* (1996, 1997) for details

ER knock-out mice

26. The gene for the α-ER has been disrupted and rendered non-functional in a strain of ER-α knock-out (α-ERKO) mice (Korach *et al.*, 1996; Lubhan *et al.*, 1993).[8] From the initial experiments, a pattern of pleiotrophic effects apparently related to ERα in each sex were apparent. Foremost, both male and female homozygous α-ERKO animals survive to adulthood and undergo an apparently normal development of the reproductive tract *in utero*. However, male homozygous α-ERKO mice were subsequently infertile. More detailed reproductive functional tests, sperm function tests, tissue weights, and histology in males were reported by Eddy *et al.* (1996). Testes size in young adults was reduced and histological examination shows that the seminiferous tubules were dysmorphogenic, with few germ cells. The severity of these conditions appeared to be progressive over time, possibly due to reduced fluid resorption in the efferent ductiles. Sperm counts in homozygous α-ERKO males were ≤10% of controls. Weights of accessory tissues such as the epididymis, seminal vesicles, coagulating glands,, and prostate were normal. Histologically, these tissues appeared also to be normal (Eddy *et al.*, 1996), as were testosterone and serum levels of LH and FSH. A decrease in bone density, which is maintained by 17β-oestradiol was, however, observed. These findings are consistent with a reported human case with a functional mutation in the ER gene in a male subject (Korach *et al.*, 1996; Smith *et al.*, 1994). Recent detailed investigations with α-ERKO mice have revealed a series of subtle-to-severe developmental effects in male reproductive tract tissues normally expressing the ER-α (Hess *et al.*, 2000).

27. Female homozygous α-ERKO mice are infertile and do not undergo pubertal development. The α-ERKO appears to abolish molecular and phenotypic expressions of all uterine cell division and growth responses to 17β-oestradiol. Molecular and cellular responses, such as oestrogen up-regulation of uterine oestrogen responsive genes such as lactoferrin, the progesterone receptor, glucose-6-phosphate dehydrogenase, and morphological changes in the uterine epithelium, were all abolished (see subchapters below). Mitotic uptake of [^{3}H]-thymidine into DNA as a signal of cell replication in the uterus and vagina was also abolished (Couse *et al.*, 1995; Korach *et al.*, 1996). Finally, the uterine weight increase in the uterotrophic test using three repeated high doses of three agonists (40 μg/kg 17β-oestradiol, 1 mg/kg hydroxytamoxifen (OH-tamoxifen),[9] and 40 μg/kg diethylstilbestrol) was convincingly abolished (Korach *et al.*, 1996). Hormonal analyses indicated an inability of the hypothalamic/pituitary/gonadal axis to achieve homeostasis without the α-ER (Couse *et al.*, 1995; Scully *et al.*, 1997). Bone density was also decreased in females.

28. The β-ER knock-out mouse, lacking a functional ER-β, has also been generated and studied (Crege *et al.*, 1998). Females are phenotypically normal, with pubertal maturation of sexual organs including the uterus, vagina, and mammary glands, as well as normal oestrus cyclicity. The animals were fertile and bore litters. However, the litter size was reduced. Additional experiments indicate that follicle maturation and ovulation are impaired. This is consistent with the localisation of the ER-β in the granulosa cells of the ovarian follicles (Kuipfer *et al.*, 1997). No impairment of male fertility, reproductive function, or reproductive development, was observed in these experiments.

29. Information gained from the ER knock-out mice have been supplemented by 1) case studies of human mutations in the aromatase gene. which necessary as the final step in 17β-oestradiol synthesis to

[8] The precise construction of the knockout gene should be consulted as some have asserted that a potentially active protein product may still be produced, although the weight of the evidence does not support this assertion (see Couse and Korach, 1999).

[9] Hydroxytamoxifen is an oestrogen agonist, not antagonist, in the mouse which is one of the key apparent differences between the rat and the mouse.

convert testosterone to 17β-oestradiol (*cf* Carani *et al.*, 1997), and 2) murine knock-outs of the aromatase gene (Fisher *et al.* 1998). These case observations and aromatase knock-outs consistently support the phenotypic outcomes of the two ERKO mouse strains summarised above.

30. Work with both ERKO strains continues, as well as experiments to assess double knock-outs of both receptors (mice null for both the α and β ERs). These additional experiments have been reviewed by Couse and Korach (1999).[10] In addition, both the ER-α and ER-β findings are consistent with recent investigations of the role of 17β-oestradiol in the male reproductive tract, as summarised by Hess *et al.* (2000) and Williams *et al.* (2001).

Anti-oestrogens as ER antagonists

31. Receptor-mediated processes are susceptible to inhibition by receptor antagonists. Given the hormonal importance of 17β-oestradiol, substances with antagonist or anti-oestrogen activity have been long sought by the pharmaceutical industry. Such interest has yielded several substances that are potent receptor antagonists of oestrogen.

32. These antagonists exhibit high affinity for the ER and competitively inhibit the binding of the native 17β-oestradiol ligand and its subsequent biological activity. These substances severely reduce or abolish the uterine weight increase response when co-administered with sufficient 17β-oestradiol, diethylstilboestrol (DES), or other oestrogenic substances, to induce a robust uterotrophic response (*cf.* Duncan *et al.*, 1963; Holtkamp *et al.*, 1960; Lerner *et al.*, 1958, for early anti-oestrogen reports). Some chemicals, such as tamoxifen, also exhibit a low level of oestrogenic activity when administered alone to the rat, leading to the designation of these substances as a partial agonists/antagonists (*cf.* Jordan *et al.*, 1978; Wakeling and Slater, 1980; Wakeling and Valcaccia, 1983). Other substances, however, show no apparent agonist activity and are designated pure antagonists (*cf.* Jordan and Gosden, 1983; Wakeling *et al.*, 1983, 1991; Wakeling and Bowler, 1988).

Evidence for a subsequent paracrine transmission of the oestrogen signal within the uterus

33. There is evidence to suggest that 17β-oestradiol, via the ER, can possibly mediate the secretion of paracrine signals from original target cells that stimulate mitosis in adjoining tissues. Experiments in the 1980s suggested a role for the epidermal growth factor (EGF) in the oestrogen signalling pathway leading to uterine growth (Gardner *et al.*, 1989; Mukku and Stancel, 1985). Work with ER-α knock out mice, however, showed that even in tissues lacking the receptor, some growth could still be induced by EGF (Curtis *et al.*, 1996). Recent experiments have dissected reproductive tract tissues into stromal and epithelial components, followed by transplantation of tissue elements into the kidney capsule, in order to study the basics of signalling between the tissues. This technique allows the testing of specific wild type cells from one component of the uterus or vagina against knock-out cells with other components (Buchanan *et al.*, 1999; Cooke *et al.*, 1997; Hom *et al.*, 1998; Kurita *et al.*, 1998). In the case of EGF, the stromal cells must contain an active ER-α while the epidermal cells must contain an EGF receptor; the ER-α is unnecessary to stimulate epithelial mitosis. In EGF receptor knock-out mice, the mitotic stimulus of oestrogen is reduced in the stroma. These data then suggest both a direct and an apocrine action of oestrogen on the uterine stroma, and a paracrine action of stromal EGF on the epithelium.

[10] More recent work has appeared after this review, for example, see Dupont *et al.* (2000) with expanded studies on single knockout α-receptor and β-receptor mice as well as a double knockout of both α- and β-receptors that allows the study of compensatory roles between the two receptor isotypes.

34. In addition to EGF, several experiments also suggest a role for insulin-like growth factor I as a secondary signal for oestrogen-mediated uterine growth (Huynh and Pollak, 1993; Klotz *et al.,* 2000; Murphy *et al.,* 1987; Richards *et al.,* 1998). Tissue dissection experiments similar to those with EGF have not yet been conducted to elucidate this possibility.

35. Finally, other experiments suggest modulation and, possibly, even activation of the ER-α by non-ligand or ligand independent pathways, *e.g.,* direct kinase phosphorylation in the AF-1 region. The AF-1 region is rich in serine residues, and evidence suggests that the ER is activated by phosphorylation in this region by the mitogen activated protein kinase (MAPK) pathway, leading to the recruitment of transcriptional co-activators (see Freedman, 1999; Kato *et al.,* 1995; Webb *et al.,* 1998, and references therein). Phosphorylation in the AF-1 region appears to further positively modulate ligand binding-induced transcriptional activation in the AF-2 region. Given the need for rapid and effective modulation as well as compensation, multiple signalling pathways directed at the ER's control of mitosis may not be surprising. However, if ligand-independent pathways mediated through the ER exist, then the specificity of the uterotrophic bioassay for 17β-oestradiol-mediated modes of action will be reduced.

The structure of an ER ligand and its relationship to receptor-binding affinity

36. An increasing body of data relates the structure of an oestrogen ligand to its ER-binding affinity. These data provide the basis for understanding the role of key structural features of the ligand, and to make predictions of binding affinity based on ligand structure. An example is the near universal structural feature of an unhindered hydroxyl on a phenyl ring as a prerequisite for receptor-binding affinity. This structural feature is clearly analogous to the 3-hydroxy on the phenyl-A ring of 17β-oestradiol. Structural models provide a basis to identify possible receptor binding candidates, estimate their binding affinity, and their role as agonists or antagonists, without the use of animals. Together with other *in vitro* methods, the versions can enable a reduction in animal use in any overall testing strategy.

Receptor binding data

37. A body of literature exists on the affinity of ER-α for numerous structural ligands. Binding data have often been gathered in conjunction with uterotrophic data since the 1970s (see **Table 2,** below). A single dataset for ER affinity, comprising over 230 structurally diverse positive and negative compounds, has been recently published (Blair *et al.,* 2000; Branham *et al.,* 2000). These data have been compared with several other ER affinity data sets (e.g., Kuiper *et al.,* 1997; Waller *et al.,* 1996). Collectively, these data strongly suggest that structural features of a ligand can be used to qualitatively predict binding affinity to the ER.

QSAR concepts

38. There has been a historical objective to qualitatively explain and predict ligand binding affinities on the basis of structure-activity relationships. Several structural aspects of ER ligands are apparent (see reviews by Anstead *et al.,* 1997; Duax *et al.,* 1985; Jordan *et al.,* 1985):

- Analogous structures to the 3-hydroxyl and the phenyl-A ring of the 17β-oestradiol are key elements in the binding affinity of most ligands.

- This hydroxyl should be unhindered at the *ortho* position, and hydrophobic groups at the *meta* and *para* positions tended to improve binding affinity.

- A second hydroxyl analogous to the 17β-hydroxyl, and an optimum inter-oxygen distance analogous to the 3-hydroxyl and the 17β-hydroxyl spacing, contribute to high affinity binding and are often absent in lower affinity ligands.

- The intermediate region separating the oxygens is largely hydrophobic in nature. The presence of polar and ionisable groups in this region generally reduce the binding affinity, while substitution of hydrophilic amino acids in the ligand binding region of the receptor protein reduces affinity of a number of substances.

- A preferred molecular volume optimum and shape are also apparent.

Table 2. Studies employing ER affinity binding assays in conjunction with uterotrophic bioassays.[a]

Immature, intact rat	Acton *et al.* (1983; Allen *et al.* (1980); Arcaro *et al.* (1999); Ashby *et al.* (1999a); Bhavnani & Woolever (1991); Bhavnani *et al.* (1998); Cano *et al.* (1986); Chander *et al.* (1991); Connor *et al.* (1996); DeSombre *et al.* (1988); di Salle *et al.* (1990); Ferguson & Katzenellenbogen (1975); Gabbard & Segaloff (1983a,b); Gazit *et al.* (1983); Gould *et al.* (1998); Hammond *et al.* (1979); Hayes *et al.* (1981); Hostetler *et al.* (1996); Jones *et al.* (1984); Jordan (1976); Jordan & Gosden (1983); Jordan *et al.* (1977,1978); Kallio *et al.* (1986); Katzenellenbogen & Ferguson (1980); Katzenellenbogen *et al.* (1979); Lan & Katzenellenbogen (1976); Markaverich *et al.* (1988); Pento *et al.* (1988); Robertson *et al.* (1982); Routledge *et al.* (1998); Ruenitz *et al.* (1983a,b); Ruh *et al.* (1995); Saeed *et al.* (1990); Schmidt & Katzenellenbogen (1979), Segaloff & Gabbard (1984), Sharma *et al.* (1990a,b); Van de Velde *et al.* (1994); Wade *et al.* (1997); Wakeling & Bowler (1988); Whitten *et al.* (1992)
Immature, OVX rat	Black *et al.* (1983); Markaverich *et al.* (1995); Ng *et al.* (1994); Zacharewski *et al.* (1998)
Adult, OVX rat	Jones *et al.* (1979); Katsuki *et al.* (1997); Olson & Sheehan (1979); Omar *et al.* (1994, 1996); Rosenberg *et al.* (1993); Routledge *et al.* (1998); Santell *et al.* (1996)

[a] In most studies, the RBA data were the basis to select promising compounds for placement in the uterotrophic bioassay. For references, see Annex.

The three dimensional structure of the ligand-receptor complex

39. The x-ray crystallography of the α-ER with the bound ligands 17β-oestradiol, raloxifene (RF), DES, and hydroxytamoxifen (HO-TAM) has provided detailed insight into the ligand-receptor interaction and conformation (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Tanenbaum *et al.*, 1998). First, the data show that the oestradiol 3-OH interacts via two stereospecifically oriented accepting and donating hydrogen bonds, with both an essential water molecule and the amino acids Glu353 and Arg394 of the receptor. The 17β-OH, however, forms a single donor hydrogen bond with amino acid His524 of the receptor. The primary hydroxyl of other chemicals (RF, DES, HO-TAM) were bound in the conserved Glu353 / Arg394 /water molecule complex. The DES also bound to the His 524, but the HO-TAM did not. Second, the striking conformational plasticity of the receptor becomes apparent because the

interaction of numerous hydrophobic amino acid side chains and the resulting van der Waals interactions are not identical between the native 17β-oestradiol and other ligands. Third, differences in the ability to interact with the essential co-activation factor, AF-2 protein, become apparent (see Barry *et al.,* 1990; Kumar *et al.*, 1987). RF and HO-TAM appear to disrupt the AF-2 binding site by moving the placement of helix 12 of the receptor[11], which suggests that the action of anti-oestrogens is to displace helix 12 from an interaction critical to transcription of the target gene. A similar ligand binding structure and effect on the co-activation site through helix 12 has been observed with the recent x-ray crystallography analysis of the β-ER with genistein and raloxifene (Pike *et al.*, 1999).

40. The structural work has been extended to, 1) an analysis of peptides mimicking the nuclear receptor binding motif on the steroid receptor coactivator protein families, using both the α- and β-ERs and a variety of ligands including agonists, partial agonists, and antagonists (Paige *et al.,* 1999), and 2) an analysis of ligands specific in their activity with the α- and β-ERs that effect interaction with steroid receptor co-activators (Kraichely *et al.* 2000). These experiments strongly support that ligand binding induces a change in position of the ER's helix 12 that influences the external surface topology of the ER, and, thereby, controls the interaction with the essential steroid receptor co-activators. The ligand-induced variation in the ability to recruit essential transcriptional cofactors would plausibly explain the phenomena of partial agonists, while the inability of a ligand to properly position helix 12, thereby interrupting ER-cofactor interactions, would explain antagonism.

Application of QSAR models

41. Knowledge of structure-activity relationships provides the basis to support quantitative estimates for ligand binding without the use of animals. Several investigators have developed models to first identify candidates based on structural alerts and, second, to estimate the ligand affinity using various QSAR paradigms (Bradbury *et al.,* 2000; Mekenyan *et al.,* 1999; 2000; Shi *et al.,* 2001a,b). In one model, the investigators use x-ray crystallography of the receptor with several ligands to refine the affinity estimates and to remove errors. This model has been applied to 58,000 commercially-produced chemicals, indicating that approximately 6,000 are candidates for further investigation (Hong *et al.,* 2001). Thus, this QSAR approach of identifying and prioritising candidates and eliminating large numbers of chemicals as possible receptor ligands, leads to a more efficient and targeted use of the uterotrophic bioassay. Such an approach would eliminate the use of significant numbers of animals that would be otherwise consumed by assaying chemicals with no receptor-binding activity.

The binding affinity for the ER, and uterotrophic activity

42. The evidence suggests that the binding affinity of an oestrogen ligand to its receptor may be qualitatively associated with its uterotrophic activity. Binding affinity, however, does not consider molecular interactions of the ligand-receptor with other transcription factors, DNA response elements, etc., and is unrelated to adsorption, distribution, metabolism, or excretion in the intact animal. Despite these limitations, the available data support a general association between binding affinity and 1) other *in vitro* activities, and 2) the *in vivo* activity expressed in the uterotrophic bioassay. This suggests that a combination of receptor-binding affinity, other *in vitro* data, information on pharmacodynamics and pharmacokinetics, and information on related substances, could assist in substance selection and prioritisation for the uterotrophic bioassay.

43. The relationship between the relative binding affinity (RBA) to the ER and the uterotrophic activity of a substance has only recently been explored for weak oestrogen agonists. Weak oestrogen

[11] Raloxifene also interrupts a stabilizing interaction between Asp 351 of helix 3 and an amide in the peptide backbone of helix 12, in addition to the steric displacement.

agonists are defined as having RBA's of two or more orders of magnitude less than 17β-oestradiol. Such order of magnitude differences often leads to the use of a logarithmic scale for the relative binding affinity. That is, if 17β-oestradiol's RBA=100 (Log RBA = 2), then the RBA for a weak oestrogen agonist would be ≤ 0 (Log RBA ≤ 0).

44. For substances with high RBAs, there is a long history of presuming an association between the receptor-binding affinity and uterotrophic activity (see **Table 2**). Substances were selected in pharmaceutical development on the basis of high RBA values, and those promising substances were then assayed for uterotrophic activity. Generally, uterotrophic activity was found to be in rough proportion to the RBA. The use of *in vitro* assays before the *in vivo* uterotrophic bioassay conserved animals and provided rapid results. Investigators explicitly recognised that the use of binding affinity alone failed to consider the process of adsorption, distribution, metabolic activation, or excretion (ADME) in the intact animal. Simultaneously, examples of substances that needed metabolism, such as methoxychlor and tamoxifen, were emerging to introduce some further caution for extrapolating using *in vitro* results as the sole predictor of uterotrophic activity (Bulger *et al.*, 1978; Fromson *et al.*, 1973; Jordan *et al.*, 1977).

45. Coldham *et al.* (1997), and others have recently investigated the association between RBA, the results of other *in vitro* screening assays, and the uterotrophic bioassay. The data suggest that a general, but imperfect, association exists despite the lack of standardization among any of the assays. Fang *et al.* (2000) have reviewed and assembled the binding affinity data for comparison to other *in vitro* data. They used the RBA data set from their own laboratory and combined it with data sets from other laboratories (see Kuiper *et al.*, 1997; Waller *et al.*, 1996). Their review shows relatively strong associations between receptor-binding affinity and the results of other *in vitro* assays. These investigators have extended their work to the association of structural alerts and *in vitro* biological activity with *in vivo* uterotrophic results from the literature. Again, a strong pattern of general association is found (H. Fang, personal communication and manuscript in preparation). However, Colham *et al.* (1997) and Kuiper *et al.* (1998) have shown that the association is imperfect, and may vary between the α and β forms of the ER.

46. This suggests that the combination of RBA, other *in vitro* data, information on pharmacodynamics and pharmacokinetics, and information on related chemicals would be expected to assist in the selection and prioritisation of substances for the uterotrophic bioassay. These assays should not be expected to be consistent in their absolute ranking of individual chemicals or consistently accurate in their predictions of *in vivo* activity.

Molecular and Cellular Events Leading to Uterine Weight Increases

47. After the initial interaction of the ligand with the receptor, a temporal sequence of events begins with DNA transcription. This sequence includes the synthesis of specific messenger RNAs, the increased synthesis of a number of proteins, changes in cellular morphology, and DNA synthesis and replication in certain tissues. All of these events are abolished by antagonists and in α-ERKO mice, making them consistent with an ER-mediated mode of action.

ER Distribution

48. Several investigators have studied the distribution of the ER in the uterine tissues. The original data of McCormack and Glasser (1980) indicated that ER concentrations are higher in the epithelial cells of the uterus compared to the stroma, and lower in the myometrial cells. Subsequent experiments have generally supported these findings (*cf.* Korach *et al.*, 1988), suggesting that the epithelium, followed by the stroma, were the primary oestrogen target tissues in the uterus. Detailed tissue dissection and

transplantation experiments suggest a secondary, paracine, signal between the stromal and epithelial responses.

Oestrogen Action on the Uterus: Molecular Events

49. The use of the increase in uterine weight as an endpoint is an integration and culmination of a sequence of complex molecular, cellular, and tissue events in the uterus. The sequence is initiated by the transcription of genes into messenger RNA. This process has been conceptually divided into an early phase (0-6 hours after oestrogen administration) and a late phase (12-24 hours after the initial oestrogen dose). The hallmarks of the later phase are the mitotic events necessary for uterine growth. After 24 hours, the sequence then culminates as measurable uterine tissue growth. (Clark and Peck, 1979; Clark and Markaverich, 1983). The sequence of early and late events in response to oestrogen treatment is given in **Table 3**.

50. The molecular events leading to RNA transcription have been described above (the initial interaction of the receptor-ligand, the dissociation of the ligand-receptor from chaperone proteins, dimerization of the receptor-ligand, the association of the dimer complexes with to specific DNA response element sites, the recruitment of steroid receptor co-activators, and the modulation of DNA transcription at the target genes). These complex events lead to the increased rate of synthesis of mRNAs from oestrogen target genes (this is synonymous with early phase events). The synthesis of several marker proteins follow; these include lactoferrin, creatinine kinase, uterine peroxidase, the progesterone receptor, and several proteins involved in regulating the cell cycle, *e.g., c-fos*. Simultaneously, cellular concentrations of other proteins, such as the ER, decline. Examples of these different markers are given in **Table 4**.

51. The evidence supports that the increased synthesis of these markers is consistent with an ER-mediated mode of action. Several of the references cited in **Table 4** demonstrate that the increases in synthesis are inhibited by the administration of anti-oestrogens. Couse *et al.* (1995) have also shown that several molecular marker mRNAs and proteins, e.g., progesterone receptor and lactoferrin, are not induced in α-ERKO mice. The evidence is further supported by the identification of oestrogen response elements (receptor binding sites on the DNA) upstream of specific target genes (*cf.* lactoferrin, Liu and Teng, 1992; Shi and Teng, 1994).

Table 3. Early and late uterotrophic events in response to oestrogen treatment (from Clark and Markaverich, 1983)

Supportive or metabolic events	Biosynthetic events
A. *Early Uterotrophic Responses* (first occurring 0 - 6 hr after oestrogen injection)	
1. Hyperemia	1. Increased lipid synthesis
2. Histamine metabolism	2. Increased activity of RNA polymerase I and 11
3. Eosinophil infiltration	3. Synthesis of the induced protein (IP) or ornithine decarboxylase and its mRNA
4. Water imbibition	4. Increased synthesis of glucose-6-phosphate dehydrogenase
5. Albumin accumulation	
6. Increased electrolytes	

Table 3 (continued). Early and late uterotrophic events in response to oestrogen treatment (from Clark and Markaverich, 1983)

7. Lysosome labilization 8. Increased cyclic nucleotides, prostaglandins, and associated enzyme activation 9. Increased glucose metabolism and associated enzyme activity 10. Increased uptake of RNA and protein precursors 11. Increased calcium influx	5. Increased chromatin template activity and RNA polymerase initiation sites 6. Increased synthesis of histone and non-histone proteins 7. Synthesis of proteins signaling cell replication, *e.g., c-fos* 8. Increased synthesis of lactoferrin and uterine peroxidase
B. *Late Uterotrophic Responses* (beginning 12 - 24 hr after oestrogen injection)	
1. Many of the functions listed above continue for many hours after oestrogen treatment	1. Increased general and specific protein and RNA synthesis 2. Continued stimulation of RNA polymerase activity 3. Increased synthesis or changes in histone and non-histone proteins 4. Cellular hypertrophy and cell division (hyperplasia) *5.* DNA synthesis and mitosis

Table 4. Biomarkers of early phase events in response to oestrogen administration in the uterus of rats and mice *in vivo* or uterine cells *in vitro*.

Marker	**Reference**
IP or creatinine kinase	Cummings & Metcalf (1995); Katzenellenbogen & Gorski (1972)
Uterine peroxidase	Anderson *et al.* (1975); Lyttle and DeSombre (1977); Newbold *et al.* (1992)
Progesterone receptor	Aronica & Katzenellenbogen (1991); Connor *et al.* (1996); Cotroneo & Lamartiniere (2001); Gould *et al.* (1998)
Lactoferrin	Jefferson *et al.* (2000); Markey *et al.* (2001); Newbold *et al.* (1997), Teng (1995)
c-fos and other cell cycle regulatory factors	Ghahary & Murphy (1989); Nelson *et al.* (1992); Nephew *et al.* (1993,1995); Papa *et al.* (1995); Takahasi *et al.* (1994); Weize & Bresciani (1988).

Oestrogen Action on the Uterus: Cellular Events

52. In addition to molecular and biochemical responses to oestrogen, the morphology of uterine target cells is transformed in response to oestrogen. These morphological changes involve endothelial and stromal tissues, and can be assessed histologically. The most common morphological change measured is epithelial cell height, which is often supplemented by other measures such as epithelial gland cell height, stromal thickness, etc. The luminal epithelium responds by differentiating and extending into a columnar shape. The longitudinal height of the epithelial cells increase as much as five-fold (measured in micrometers).

Oestrogen Action on the Uterus: Mitotic Events

53. Cell division is necessary for uterine growth in response to oestrogens. Based on the observations of earlier workers, Allen *et al.* (1937) used colchicine to definitively demonstrate that a single dose of 17β-oestradiol resulted in a burst of cell division, as measured by mitotic figures in the epithelium of the rat uterus. This led to a number of studies on oestrogen-induced cell division and replication. For example, Leroy *et al.* (1969) used [^{3}H]-thymidine to demonstrate the differential responses of uterine and vaginal tissues to oestrogen. Kaye *et al.* (1972) also used [^{3}H]-thymidine to define both the time course and the dose response of uterine mitotic events to a single dose of oestrogen in immature, 20-day old, rats. The peak of mitotic division occurred approximately 24 hours after the 17β-oestradiol dose. The epithelial mitotic response was greatest, followed by the stroma and the myometrium (**Figure 3).** The dose response for the 24-hour time point in each tissue is shown in **Table 5**. The relative increases in uterine weights were recorded at 24 hours at the same doses used in **Table 5**. Significant increases in uterine weights were first seen at the 5 ng dose (~0.15 μg 17β-oestradiol per kg). This suggests that the histological observation of an increased number of mitotic figures, particularly in the uterine epithelium, is concordant with the increase in uterine weights.

Figure 3. Mitotic index increase after injection of 5 μg of 17β-oestradiol into 20-day old rats. Epithelium (solid circles, solid line), stroma (solid triangles, solid line) and myometrium (open circles, dotted line). [From Kaye et al., 1972.]

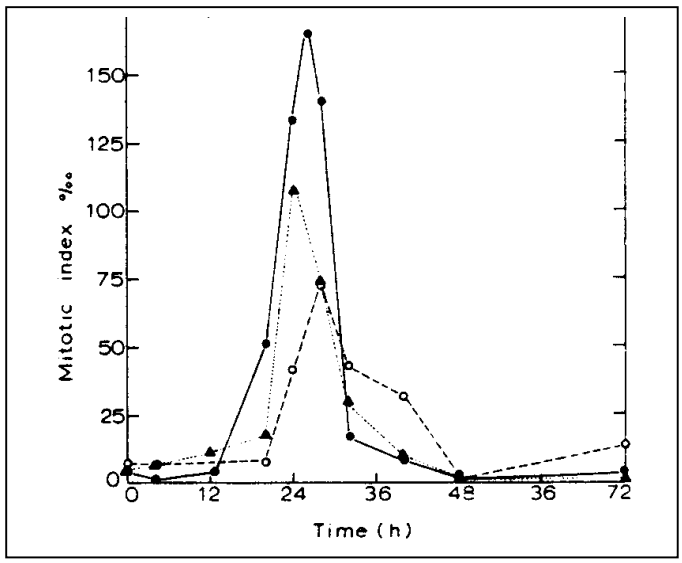


Table 5. Stimulation of cell division by 17β-oestradiol.

Dose		Mitotic index			
(ng)	(μg/kg)[a]	Epithelium	Gland	Stroma	Myometrium
0	0	3	1	10	8
0.05	0.0015	4	1	13	10
0.5	0.015	2	0	6	8
5	0.15	66	3	69	60
50	1.5	65	1	67	33
500	15	104	13	58	52
5000	150	164	8	60	58

[a] Estimate based on authors' statement that the average body weight of the rats was 33 g.

54. Experiments showing similar results have been conducted by a number of laboratories, including Clark (1971), Galand *et al.* (1971), Gorski *et al.* (1977), Ham *et al.* (1970), Kirkland *et al.* (1979), Martin and Finn (1968), and Martin *et al.* (1973). Several of these experiments include the mouse and ovariectomized rat versions, showing a consistency across uterotrophic versions. These mitotic indicators are inhibited by oestrogen agonists and are absent in α-ERKO mice, supporting an ER-mediated mode of action. Bromodeoxyuridine (BrdU) has, since the late 1980s, largely replaced tritiated thymidine for measuring cell mitosis. Experiments assessing uterine weight increases with BrdU labelling and other markers of cell proliferation, along with other uterine markers, include those of Ashby *et al.* (1999), Carthew *et al*. (1999a,b), Markey *et al.* (2001), Newbold *et al.* (2001a). and Tinwell *et al*. (2000).

55. A review of the literature shows that with potent agonists such as 17β-oestradiol and DES, the minimal effective doses for the molecular, , and mitotic markers and the traditional uterine weight increase, are similar. The strength of the responses (*i.e.*, a high maximum value for various measures), a high response slope, and the wide dose ranges used, confound the interpretation about whether the molecular and cellular markers are indeed more sensitive than the standard uterine weight increase. With weak oestrogen agonists, the evidence is equivocal. Weak oestrogen agonists will sometimes, but not always, elicit responses of other potential oestrogen markers, in parallel with the traditional uterine weight increase. The interpretation of these data are hindered by the weaker response (maximum value) relative to 17β-oestradiol, moderate to shallow slope of the responses, the wide variety of different markers employed in the experiments, the lack of procedural standardization for the markers, and the relatively wide ranges of doses employed. Therefore, there are some inconsistencies in the results, where the same marker may precede the uterine weight increase at a lower dose in one case, appear at the same dose in another case, and fail to respond in still another case.

56. Several recent experiments with weak oestrogen agonists, particularly bisphenol A, can be used to evaluate the possible relationships between the various markers and the increase in uterine weight.

Tinwell *et al.* (2000b) used a potent agonist, DES, and a weak agonist, bisphenol A (BPA). Administration of 10 μg/kg/day DES by either subcutaneous injection or oral gavage resulted in parallel increases in uterine weight and both epithelial and endometrial cell heights (**Table 6**). However, the results with BPA were less consistent. Statistically significant increases in uterine weight occurred without a parallel increase in either epithelial and endometrial cell height (**Table 6**). Using BrdU labelling, DES administration resulted in a significant increase in uterine weight. The labelling index increased in epithelium and stroma in all three experiments, and in epithelial glands in two of three experiments. With BPA, epithelial cell labelling was consistent with a uterine weight increase following s.c. administration (5 experiments). BrdU labelling was significantly higher with two oral gavage doses, although a significant uterine weight increase was absent. The labelling of glands and stroma were even less consistent. Bisphenol A produced a uterine weight increase by the s.c. route were in the second phase of the OECD validation program. However, the oral gavage doses in these experiments were in the range of the minimal effective dose in the OECD program. This would suggest that these experiements were conducted in the most uncertain region of the dose responsive curve and that a greater variability in achieving statistical significance would indeed have been expected.

57. In experiments with single doses of oestradiol benzoate and coumestrol, Tinwell *et al.* (2000b) used three rat uterotrophic versions (intact immature, OVX immature, and OVX young adult). They observed statistically significant increases in epithelial and endometrial cell heights at 60 mg/kg/d coumestrol via s.c. administration, but only for epithelial cell heights at 80 μg/kg/d oestradiol benzoate. They also observed statistically significant increases in BrdU labelling for uterine epithelium, endometrial glands, and stroma in all versions with the coumestrol dose. Oestradiol benzoate gave a statistically significant labelling responses in all tissues with the immature OVX, only in glandular epithelium with the intact immature, and in none of the tissues with the young adult OVX. Uterine, vaginal, and cervical gravimetric weight increase patterns were similar; strong in the both immature versions (3-4 fold) and weaker in the young adult OVX, particularly with oestradiol benzoate. Therefore, while the tissue, histopathological, and mitotic responses gave similar patterns in the three versions, there was no ideal correlation.

58. Similar experiments in mice and rats with bisphenol A have recently been published by Markey *et al.* (2001) and Matthews *et al.* (2001), respectively. In the experiments of Markey *et al.* (2001), the 100 mg/kg/d maximum dose of BPA was at the lower part of the dose-response curve. Administration to the immature mice began on pnd 23, so that some increased variability from early puberty would be expected in some animals (see **Figure 7B**, and note the higher than average uterine weights in **Table 7**), possibly reducing the uterine responsiveness. In addition to the absolute uterine weight increases, additional measurements included the uterine weight relative to body weight, vaginal opening, the relative area of several tissues in the uterus, epithelial cell height, lactoferrin content, and, as a marker of mitosis, proliferating cell nuclear antigen. The experiments included control animals and animals implanted with osmotic pumps and the delivery vehicle (DMSO). Regrettably, the pump/DMSO controls were not included in the statistical analyses in their Tables 1, 2, and 3, and the unoperated controls and the pump/DMSO controls were pooled in Figure 1 of their paper. Markey *et al.* (2000) concluded that their work argued against the use of the mouse uterotrophic bioassay and, instead, for more sensitive endpoints. However, looking more closely at results in **Table 7**, the 'more sensitive' endpoints are no more than two-fold, based on 100 mg/kg/d for the absolute uterine weight increase; 75 mg/kg/d for the epithelial cell height, lactoferrin, and PCNA marker in the glandular epithelia. Matthews *et al.* (2001) observed similar dose-related responses for the uterine weight increases and the morphological markers (BrdU labelling was not performed in these experiments). While there was no evidence for increased sensitivity in experiments with oral administration, the morphological markers did respond at 100 mg/kg/d in the s.c. experiments, where the uterine weight increase was 20-25%, but not statistically significant. A more detailed review of these data has been conducted by Ashby (2001b). Importantly, for the purposes of

comparison, the actual dose responses for the uterine weight increases between the mouse and the rat were so similar that there appears to be no evident species difference in sensitivity.

59. Cotroneo and Lamartiniere (2001) have recently conducted experiments using Sprague-Dawley rats with a set of oestrogenic responses, including two tissue level responses, *i.e.*, maintenance of endometriosis implants and increases in uterine weights; and three molecular markers; ER-α levels, and levels of both progesterone receptor isoforms. The oestrogen agonist, genistein, was administered both subcutaneously and in a semi-purified diet that did not contain phytoestrogen. The serum of levels of both total genistein and free (unconjugated) genistein were measured using LC-MS. The results are shown in **Table 8**. Similar responses occur when the serum levels of free, unconjugated genistein approach >500 nM levels. In the subcutaneous injection experiments, where higher levels of free genistein were achieved, the tissues responses correspond with results for decreases in the ERα and increases in progesterone receptor B. However, increases in progesterone receptor A did not occur with the reference oestrogen or show a response linked to the increasing genistein dose. In the dietary experiments, progesterone receptor A and B levels increased, but the ERα did not respond. These observations using dietary 1000 mg/kg genistein levels would appear to be in the lower portion of the dose-response curve, *i.e.*, in the NOEL/LOEL transition region, where increasing variability in the ability of any endpoint to provide a statistically significant response would be expected to occur.

60. In conclusion, a number of molecular and biochemical markers, histological changes, and mitotic events appear to be consistent with increased uterine weights - an ER-mediated mode of action. These molecular, histopathological, and mitotic responses have been in qualitative concordance with the uterotrophic responses observed. In some cases, the data suggest that these other responses may be slightly more sensitive. However, the protocols for the molecular, morphological, and mitotic responses have not been standardised, their reliability and reproducibility have not been systematically studied with a number of diverse weak oestrogen agonists, nor have they not been tested with careful attention to their responsiveness at close dose intervals, and so the limited experimental consistency is reasonable. The available data further suggest that any concordance or increased responsiveness with the additional endpoints would be just as variable in the lower part of the dose-response range with weak oestrogen agonists, and would imply that a full dose-response curve should be evaluated to be more confident about any increased sensitivity.

Table 6. Comparison of blotted uterine weight responses to cellular morphological changes and bromodeoxyuridine (BrdU) labeling in mice treated with bisphenol A (BPA) and diethylstilbestrol (DES) (from Tinwell *et al.*, 2000b).

Expt. Route	Compound	Dose (/kg/day)	No. of Animals	Uterus Blotted Weight (mg ± SD)	Cell Hypertrophy (μm± SD)		% Cells BrdU Labelled		
					Epithelium	Endometrium	Epithelium	Glands	Stroma
A	Arachis oil	5ml	25	7.7 ± 2.1	12.0±2.1	110.1 ± 30.9	4.7 ± 4.9	23.4 ± 11.1	5.6 ± 3.8
s.c. Injection	BPA	20μg	25	7.7 ± 1.9	11.5 ± 1.5	114.0 ± 25.3	6.7 ± 4.9	21.1 ± 9.5	5.5 ± 3.9
		200μg	25	7.8 ± 2.0	11.0 ± 1.4	135.4 ± 85.2	5.9 ± 5.4	21.9 ± 8.5	5.1 ± 4.4
		200mg	25	9.6 ± 1.2**	12.0 ± 1.2	120.0 ± 19.8	44.4 ± 18.3**	50.2 ± 11.2**	17.4 ± 13.3**
	DES	10μg	10	49.9 ± 5.5**	31.7 ± 1.9**	155.2 ± 40.5*	66.7 ± 12.4**	52.6 ± 6.8**	37.9 ± 17.9**
B	Arachis oil	5ml	12	8.7 ± 2.5	12.1 ± 2.8	112.2 ± 37.6	13.3 ± 16.2	31.4 ± 13.1	11.4 ± 13.3
s.c. Injection	BPA	200μg	12	10.3 ± 2.0	11.6 ± 1.5	110.6 ± 21.4	16.6 ± 10.2	26.4 ± 8.8	13.5 ± 8.2
		500μg	12	9.1 ± 2.4	11.3 ± 1.8	101.9 ± 16.6	13.4 ± 12.2	34.3 ± 16.3	12.0 ± 5.1
		1mg	12	9.6 ± 2.6	11.1 ± 1.1	118.6 ± 26.8	15.8 ± 8.8	34.2 ± 10.4	11.8 ± 3.8
		5mg	12	11.0 ± 2.0**	11.6 ± 1.3	122.1 ± 14.2	21.5 ± 11.8*	35.3 ± 9.3	14.5 ± 5.5
		10mg	12	9.6 ± 1.4	11.2 ± 1.3	111.2 ± 27.4	20.2 ± 11.2	40.4 ± 12.6	15.2 ± 8.7
		50mg	12	10.9 ± 1.4**	12.4 ± 1.1	114.8 ± 13.7	32.7 ± 15.2**	50.1 ± 11.1**	17.6 ± 9.4*
		100mg	12	11.3 ± 1.8**	12.9 ± 1.8	111.3 ± 19.5	29.3 ± 14.7**	43.8 ± 10.6*	12.0 ± 7.1
		200mg	12	11.9 ± 1.4**	13.8 ± 1.8*	129.1 ± 25.4	66.2 ± 12.5**	54.9 ± 14.7**	15.1 ± 14.9
	DES	10μg	12	64.0 ± 14.0**	32.7 ± 4.0**	289.8 ± 57.2**	74.2 ± 14.5**	42.4 ± 13.6	34.8 ± 11.8**
C	Arachis Oil	5ml	12	9.3 ± 3.0	10.4 ± 1.4	126.1 ± 42.4	11.7 ± 8.6	41.7 ± 18.9	10.3± 6.8
Oral Gavage	BPA	500μg	12	9.7 ± 1.9	10.2 ± 1.2	135.8 ± 26.3	16.0 ± 11.0	36.5 ± 13.8	17.8 ± 17.2
		1mg	12	10.1 ± 2.8	10.7 ± 0.8	138.0 ± 24.9	22.6 ± 21.4	49.1 ± 18.1	17.3 ± 12.3
		5mg	12	10.0 ± 1.8	10.0 ± 0.8	120.9 ± 20.4	19.4 ± 15.2	46.3 ± 10.4	22.3 ± 12.5*
		10mg	12	10.3 ± 1.8	10.6 ± 0.9	128.2 ± 21.5	18.2 ± 9.5	37.7 ± 16.3	26.1 ± 17.8
		50mg	12	9.5 ± 1.2	10.4 ± 1.5	130.0 ± 23.6	17.5 ± 12.1	46.4 ± 11.3	18.5 ± 9.4
		100mg	12	9.5 ± 2.0	9.9 ± 1.3	125.2 ± 23.6	18.8 ± 12.0	35.8 ± 12.3	16.9 ± 10.2
		200mg	12	9.9 ± 1.4	10.2 ± 1.3	136.2 ± 18.1	32.9 ± 11.5**	53.3 ± 10.3	27.4 ± 12.8**
		300mg	12	10.6 ± 2.6	11.1 ± 2.8	130.5 ± 17.0	56.6 ± 26.2**	64.3 ± 20.4**	28.1 ± 11.4**
	DES	10μg	10	38.8 ± 5.1**	27.1 ± 2.9**	225.4 ± 43.2**	84.0 ± 6.8**	62.2 ± 14.4**	43.8 ± 14.5**

Data were assessed for statistical significance using ANOVA; * : $p<0.05$; ** : $p<0.01$

Table 7. Comparison of the different endpoint measurements after administration of bisphenol A (Markey *et al.*, 2001)

Dose of Bisphenol A [a] Endpoint	1 mg/kg/d	5 mg/kg/d	50 mg/kg/d	75 mg/kg/d	100 mg/kg/d
Absolute uterine weight increase[b]	16.08 ± 1.84	15.51 ± 1.89	19.13 ± 1.40	23.74 ± 1.94[b]	29.08 ± 2.87[c]
Epithelial cell height	15.83 ± 0.76	16.46 ± 0.60[c]	16.13 ± 0.47	20.43 ± 0.97[c,d]	23.46 ± 0.74[c,d]
Lactoferrin expression	17	0	33	100	100
PCNA glandular epithelia	1.79 ± 1.15	1.18 ± 0.47	2.36 ± 0.71	7.67 ± 1.26[c]	8.71 ± 0.60[c]

[a] All markers were significantly different with 5 μg/kg/d 17β-oestradiol.
[b] Per the comment in Markey *et al.* (2001) on the pnd 23 age, note that the uterine weights are higher than expected for immature animals in Attachment 1 (of Markey *et al.*, 2001) and questions if a statistical difference may have been observed at 75 mg/kg/d in younger animals.
[c] Statistically different from either unoperated control (see Tables 1, 2, and 3 of Markey *et al.*, 2001)
[d] Statistically different from pooled unoperated control and operated with DMSO vehicle control (see Fig 1 of Markey *et al.*, 2001).

Table 8. Endometriosis, uterine weight, and other biomarker measures, with concurrent analysis of serum, total, and free genistein levels (Cotroneo and Lamartiniere, 2001)

Subcutaneous Route of Administration					
	Vehicle	**Estrone 1 mg/kg/d**	**Genistein 5 mg/kg/d**	**Genistein 16.6 mg/kg/d**	**Genistein 50 mg/kg/d**
Uterine to body weight ratio	100[a]	265**	160	185**	340**
Endometriosis implant survival	0	100	0	100	100
α-ER level	100	79*	102	14**	38**
Progesterone receptor A	100	180	320*	330*	135
Progesterone receptor B	100	275**	250**	210**	320**
Serum total genistein (nM)	4 ± 2	Not done	450 ± 180	1380 ± 250	5090 ± 700
Serum free genistein (nM)	Not done	Not done	Not done	662 ± 94	2243 ± 477
Dietary Route of Administration					
	AIN-76A control	**AIN-76A 250 mg/kg Genistein ~ 25 mg/kg/d**		**AIN-76A 1000 mg/kg Genistein ~ 100 mg/kg/d**	
Uterine-to-body weight ratio	100	20		190**	
Endometriosis implant survival	0	0		0	
ERα level	100	No significant effect		No significant effect	
Progesterone receptor A	100	110		190**	
Progesterone receptor B	100	125		290**	
Serum total genistein (nM)	49 ± 23	1115 ± 552		2031 ± 271	
Serum free genistein (nM)	Not done	138 ± 9		466 ± 35	

[a] Tissue and molecular responses are normalised against the vehicle controls as 100%, in contrast to the genistein levels.

* significantly decreased; ** $p < 0.05$; *** $p < 0.01$

CHAPTER 2: HISTORY OF THE DEVELOPMENT AND USE OF THE UTEROTROPHIC BIOASSAY

61. This chapter summarises the history of the development of the uterotrophic bioassay and its use. The chapter is organised around the following points:

- The history of the development and use of the oestrogen bioassays using the uterus and vagina

- A comparison of the historical use of the assay and its proposed use for identifying possible weak oestrogen agonists and antagonists

62. Several investigators developed the principles of the uterotrophic bioassay in the 1930s as a rapid bioassay for oestrogenic activity. This was a period of intense investigation, attempting to isolate native oestrogen, and to identify its structure and source within the body. This effort required a biological assay to trace activity during purification procedures and to analyse biological fluids and samples. The basic papers in the early development of uterine weight-based assays are summarised. Two basic versions emerged, the sexually immature female and the ovariectomized, sexually mature female. Both versions use multiple, consecutive dose administrations. The methods used in these early reports varied from laboratory to laboratory, but the different methods did not apparently affect the ability to detect oestrogenic activity.

63. From the 1930s through the 1960s, the uterotrophic bioassay was the primary means to screen the pharmacological activity of possible oestrogen therapies. The search for clinical anti-oestrogens led to a modification of the uterotrophic bioassay for that purpose in the 1960s and 1970s. In this modification, a potent oestrogen was co-administered with the test anti-oestrogen to assess a possible *reduction* in the expected uterine weight increase.

64. In the 1970s, the testing paradigm shifted with the development of a receptor-binding assay. Candidate substances were first identified by *in vitro* tests such as the ER-binding assay. High receptor-binding affinity was presumed to be associated with high oestrogenic or anti-oestrogenic potency. After testing with the receptor-binding assay, the *in vivo* response was examined in the uterotrophic bioassay, thereby conserving resources and time. On the basis of the uterotrophic results, additional small-scale animal tests could then be initiated, where warranted, to investigate the utility of the substance(s).

65. Recently, the paradigm has shifted once more. The objective of the assay became the identification of weak agonists and new, rapid *in vitro* assays (e.g., oestrogen-sensitive reporter genes in yeast and cultured cell lines) have emerged as screens for the uterotrophic bioassay.

Historical background

66. Fellner (1913) demonstrated that alcohol-ether extracts, particularly extracts of the ovaries, could induce oestrogenic changes in ovariectomized rabbits, including uterine growth and vaginal responses. Herrman (1915) then isolated active fractions from corpea lutae and placentas. Both investigations demonstrated the need to trace the active substance during fractionations and, thus, required a rapid biological assay.

67. Allen and Doisy (1923, 1924) developed an assay to measure the vaginal cornification and keratinization response in the rat. This biological assay allowed the identification of the Graafian follicle as the ovarian source of the female hormone, identification of estrone and 17β-oestradiol as the major hormones in urine, and allowed MacCorquodale *et al.* (1936) to confirm that 17β-oestradiol was the

native oestrogen, by extracting the follicular liquor from nearly four tons of porcine ovaries. Another aspect was the structure-activity relationship of oestrogens. Using the biological assays, Sir Charles Dodds with co-workers published a series of papers that demonstrated that a wide structural spectrum of substances, including those with non-steroidal structures, could impart oestrogen activity. These substances were often synthesised and tested as homologous structural series (see Campbell, 1940; Cook *et al.,* 1934; Dodds and Lawson, 1936, 1937; Dodds *et al.,* 1938).

Short-term uterine responses using a single dose: the 6-hour Astwood Assay

68. While investigating the time sequence of changes in the uterus to oestrogen, Astwood (1938) developed a single-dose test based on the rapid imbibition of water by the uterus. The Astwood assay used immature rats (21-23 days of age) and sacrificed the animals 6 hours after substance injection. The full time course of the experiments and the corresponding responses were published. The Astwood assay has fallen into disfavour because the imbibed water response is primarily an effect of relatively potent oestrogens, or of the application of weak oestrogen agonists at high doses.

Uterine weight increase assays using multiple doses: Development of the uterotrophic bioassay

69. Several laboratories in the 1930s identified the increase in a uterine weight as an endpoint for oestrogenicity. To achieve maximum uterine growth, multiple, consecutive injections of a substance over several days were used to develop the response. All of these experiments may be considered the predecessors of the current uterotrophic bioassay. These experiments established the quantitative endpoint of increased uterine weight as the standard for measuring oestrogens, in comparison to the more subjective examination and scoring of the vaginal lining.

70. Bülbring and Burn (1935) related how they had become dissatisfied with the Allen and Doisy vaginal assay and its qualitative scoring, so they sought a robust quantitative response. They undertook to develop an uterine assay analogous to one for the male sex accessory organs (a predecessor of the Hershberger assay) . This latter method, directed at androgens, used the responses of the prostate and the seminal vesicle in immature, castrated male rats, and a consecutive series of daily, test substance administrations (Korenchevsky *et al.*, 1932). In Bülbring and Burn's uterotrophic procedure, immature rats were ovariectomized, allowed to rest for 2 days, and then injected s.c. with estradop or other test substances for 4 consecutive days. The uteri were dissected 48 hours after the last injection and fixed in Bouin's solution for 24 hours. After the fixed uterus was blotted between dry filter papers, it was weighed. The subsequent data were reported on a relative basis as mg uterine weight per 100 grams body weight. For the assay of unknown substances, groups of six rats were used.

71. Dorfman *et al.* (1935) used the intact, immature rat and began daily s.c. injections on pnd 25; continued the injections for 5 days; and followed with necropsy 24 hours after the last injection. Although not specified, the uterine weights suggest that the uteri were intact and still contained the interluminal fluid. A direct comparison was made of the responsiveness of the uterine weight increase versus the keratinization of the vaginal lining. The uterine weight increase was found to be more responsive at low doses for some of the substances tested. Groups of five animals were routinely used.

72. Levin and Tyndale (1937) used the intact, immature mouse. Mice were treated by s.c. injection for 3 consecutive days starting on pnd 21, and sacrificed 20-24 hours after the last injection. Extending the time of necropsy to ~48 hours after the last injection often resulted in a marked 60-70% reduction in the uterine weight increase that had been observed at 20-24 hours. The published methods describe that organs were removed, dissected, stripped of mesentery and fat, the uterus was freed of interluminal fluid by pressure against a dry filter paper, and then weighed. Animal group sizes varied from experiment to experiment.

73. Lauson *et al.* (1939) used intact, immature rats. As with Bülbring and Burn (1935), they were dissatisfied with the qualitative aspects of the vaginal assay and suggested that larger numbers of animals were necessary to obtain a degree of accuracy equivalent to the uterine weight assay. Animals were treated by s.c. injection for 3 consecutive days starting on pnd 22 or 23 and sacrificed ~24 hours after the last injection. If the substances were injected as aqueous suspensions, then twice daily injections were used. If the substances were injected in oil vehicle, then only one injection per day was used. The results suggest that the different administration procedures were equivalent. The uteri were weighed both before and after the interluminal fluid was expressed using moistened filter paper. Lauson *et al.* (1939) sometimes used groups of over 10 rats. They also made several related observations: 1) when the uterine weight response for a substance was very robust and increased approximately three-fold over controls, premature vaginal opening appeared in some individuals; 2) the maximum blotted response of estriol was only about half that of oestradiol and estrone; and 3) estriol resulted in little accumulation of interluminal fluid compared to oestradiol and estrone (with the latter compounds the wet uterine weights exceeded 300 mg and blotted weights exceeded 90-100 mg). These were the first recorded observations of significant differences in the patterns of the uterine responses among individual chemicals, other than differences in the minimal effective dose.

74. Evans *et al.* (1941) used intact, immature mice. The animals were treated for three consecutive days and necropsied on the 4th day, 18 hours after the last injection. The starting age of the animals was not given, but the body weights were between 6 and 8 grams. Subcutaneous injections were given twice per day, and the group size was five animals. Vehicle controls and a dose-response for a reference oestrogen, estrone, were run in parallel with test substances. The reference dose-response was made necessary by the observation of some variation of the reference responses over time among experimental results.

Development and application of the uterotrophic bioassay after the 1930s

75. The basic uterotrophic bioassay has changed little since the 1930s. The majority of laboratories have used the intact, immature version and favored the rat as the test species. Procedural variations are numerous, including route of administration (i.e., p.o., s.c., i.v., i.p., and dietary administration), differences in the number of consecutive days, the weighing of the uterus with imbibed fluid (wet) in some publications and without (blotted) in others, and the use of absolute weights in most cases and relative to body weight in others. The reference oestrogen also varied from researcher to researcher. The primary use of the assay from 1940 to about 1990 has been the pursuit of oestrogens as possible pharmaceuticals, studies of structure-activity relationships, and the elucidation of the oestrogen mode of action. In the 1970s, however, with the discovery of the ER, the receptor-binding assay was routinely used to screen test substances prior to using animals in the uterotrophic bioassay. Typically, only substances demonstrating a significant affinity for the receptor, e.g. 1% of the native ooestradiol affinity (RBA $\geq$ 0.01 or Log RBA $\geq$ 0), were taken forward to the uterotrophic test. A number of publications using the receptor-binding affinity test in conjunction with the uterotrophic bioassay are noted in **Table 2**, along with the uterotrophic model used.

76. During the same time period, significant research efforts were underway to develop pharmaceutical antioestrogens, and the uterotrophic bioassay was modified for this purpose. The underlying principle was to inject sufficient reference oestrogen to achieve a near maximum response in the uterine weight. Doses of the putative anti-oestrogen were then co-administered with the selected reference oestrogen dose, and the reduction in uterine weight relative to the oestrogen reference controls was measured. The results of one set of experiments are shown in **Figure 4** (Wakeling and Bowler, 1988). Tamoxifen, when administered alone, will increase the uterine weight similar to an agonist. However when it is co-administered with the reference oestrogen, it does not completely abolish the

uterine weight increase, but only reduces the response to the level consistent with its own agonist activity. In contrast, ICI 160,325 (**Figure 4**, left, lower B panel) and ICI 163,964 and 164,275 (**Figure 4**, right) show no agonist activity when administered alone, and completely abolish the action of the reference oestrogen, oestradiol benzoate. These results illustrate that tamoxifen is a partial agonist/antagonist while the three ICI chemicals are full antagonists.

Recent Developments since 1990

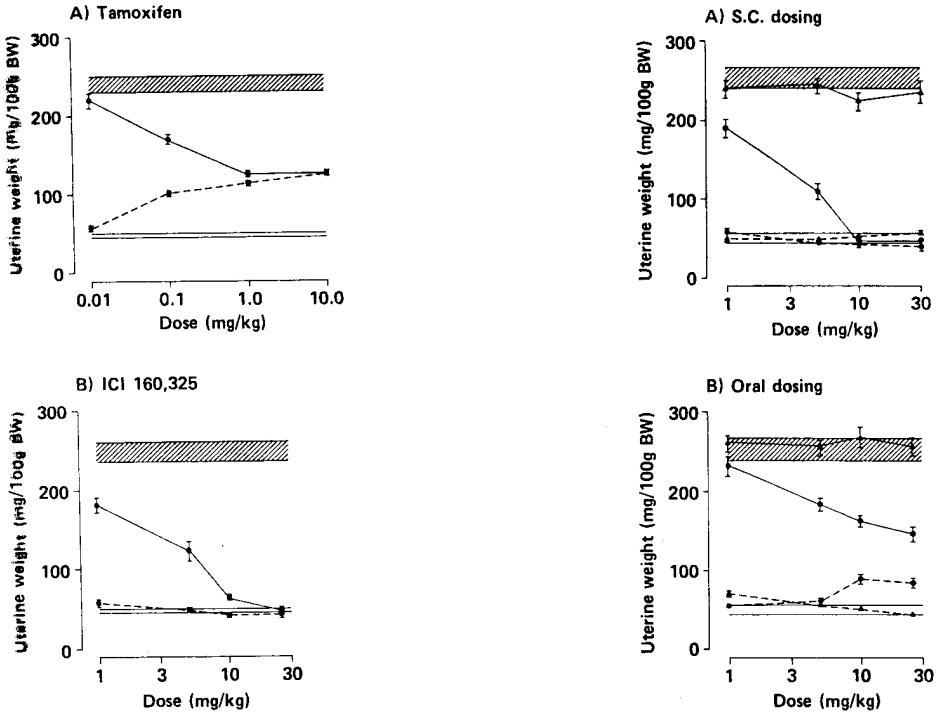
77. Recently, additional changes have occurred in how the uterotrophic bioassay is used. First, with the rising interest in weak and very weak agonists (RBA 0.01-0.00001, or Log RBA 0 to -3), the uterotrophic bioassay has been increasingly used to assess the biological response of low affinity chemicals. This has required the use of doses far in excess of those used for classical oestrogens, i.e., in the 10-1000 milligram per kg per day range. At the same time, new *in vitro* assays have been developed that can screen substances prior to using the uterotrophic bioassay, and which may complement the standard receptor-binding assay. These new *in vitro* assays include yeast strains and cultured cell lines transfected with plasmids carrying the ER and a reporter gene incorporating an oestrogen DNA response element (see review by Zacharewski, 1997). These techniques, particularly with yeast, have recently been used to screen up to hundreds of chemicals at a time (Miller *et al.,* 2001; Nishihara *et al.,* 2000).

Figure 4, left. Uterotrophic and anti-uterotrophic effects of tamoxifen (A, top graph) or ICI 160,325 (B, bottom graph) in immature rats.

Animals received 3 daily doses of vehicle alone (open horizontal bar), 0.5 µg ooestradiol benzoate s.c. alone (hatched horizontal bar), or increasing doses of tamoxifen or ICI 160,325, s.c., alone (dashed line) or together with ooestradiol benzoate (solid lines). Bars on each point represent the standard error of the mean. Where no bar is present, the errors were smaller than the symbols (from Wakeling and Bowler, 1988).

Figure 4, right. Comparative uterotrophic and anti-uterotrophic activity of ICI 163,964 and ICI 164,275 in immature rats. (A) Subcutaneous and (B) oral administration

ICI 163,964 alone (circles with dashed line) or with oestradiol benzoate (circles with solid line); ICI 164,275 alone (triangles with dashed line) or with oestradiol benzoate (triangles with solid line). The same protocol was used as in the left-hand figure.



CHAPTER 3: THE PRIMARY VERSIONS FOR THE UTEROTROPHIC BIOASSAY

78. This chapter summarises the two primary versions for the uterotrophic bioassay: the intact, sexually immature female and the ovariectomized, sexually mature female, and covers the following points:

- The major versions for the uterotrophic bioassay
- The intact, immature rat version and its important variables
- The ovariectomized (OVX) rat version and its important variables
- A comparison of the intact, immature rat version and the young adult OVX rat versions

79. The responsiveness of the uterus to administered oestrogens depends upon the elicitation of a maximum difference in uterine weight between control and test animals. This requires a minimum uterine weight in control animal(s), which hinges upon the need for the animal to be lacking a functional hypothalamic-pituitary-gonadal axis, to ensure a sensitive and consistent uterine response.

80. Two versions provide these conditions. The first is the intact, sexually immature rat prior to puberty and the accompanying surge in ovarian oestrogen production. The second is the sexually mature female after removal of the ovaries (ovariectomy), with adequate time provided for the uterus to regress to a lower weight. The rat has been the most studied species, and is the major species used for regulatory toxicological studies of reproduction and development.

The Intact, Immature Rat Version And Its Important Variables

81. The intact, immature rat has been the primary version for the uterotrophic bioassay. This version is constrained by a window of maximum sensitivity and responsiveness to oestrogens between pnd 18 and pnd 26 (where pnd 0 is the day of birth). There are two early postnatal factors that control when the window of oestrogen responsiveness opens and the version can be used. The first is the initial insensitivity of the uterus to the natural oestrogens and high circulating levels of α-foetoprotein (AFP). However, AFP rapidly declines after birth, and the immature animal becomes highly responsive to natural oestrogens after pnd 16-17. The second factor is animal husbandry. The immature animals are small, only just weaned, and are subject to stress when being handled and, because of their size, there are possible difficulties in dosage administration. When these factors are combined, the conditions argue that administration should occur after pnd 18 in the rat or pnd 16 in the mouse.

82. The window of sensitivity is closed by entry into puberty when the surge in endogenous oestrogen level production occurs. The action is twofold. First, there an increase in baseline uterine weights, thereby reducing assay responsiveness. Second, the variability in the mean group uterine weight increases. The loss of optimal conditions without surgical intervention (OVX) begins about pnd 26 in the rat.

83. Thus, in the immature rat, it appears that substance administration should not commence before pnd 18 and, with three consecutive days of administration, should end with necropsy no later than pnd 25. Further, potent oestrogens such as ethinyl oestradiol (EE) and DES, which bind AFP with less affinity than 17β-oestradiol, are the favoured candidates for reference oestrogens.

Timing of Uterine Oestradiol Sensitivity

84. A body of literature indicates that the rodent uterus goes through three periods of postnatal development: 1) a period of oestrogen-independent development (pnd 0-16 in the rat); 2) a quiescent period (pnd 17-26 in the rat); and, 3) after pnd 26, increasing development as levels of endogenous 17β-oestradiol stimulate growth that signals the beginnings of puberty. The quiescent period corresponds to the window of sensitivity to conduct the uterotrophic bioassay in the immature animal. The following studies support this conclusion.

85. Price and Ortiz (1944) assessed the changes in organ and tissues weights in female and male rats from birth to puberty in response to six consecutive daily injections of three substances: equine gonadotropin, testosterone propionate, and oestradiol benzoate. **Table 9** shows the percentage change in ovarian and uterine weights using these substances. Without ovariectomy, the peak period of responsiveness, based on a percentage increase in uterine weight of > 400%, is pnd 20 for both direct oestradiol and indirect gonadotropin stimulation. Histology of the ovaries for the gonadotropin groups indicated that they were producing endogenous oestrogens. The uterus also responded to testosterone propionate, although histological examination indicated that the developmental pattern of the endometrium (stroma and epithelium) was not consistent with a classical oestrogenic response.

86. Katzenellenbogen and Greger (1974) assessed uterine responsiveness from pnd 5 through 23 using two early uterine biomarkers for oestrogen: oestrogen-induced protein and increased glucose metabolism, and the classical increase in (blotted) uterine weight. They observed that some oestrogen responses could be induced at pnd 5, if the endpoint was the quantity of protein produced per uterine cell. However, for 3 consecutive doses, the maximum percentage increase in uterine weight was not reached until pnd 20-22 (**Figure 5**).

87. Branham *et al.* (1985) measured the development of the immature rat uterus from pnd 1 to 32 by assessing epithelial gland development and the relative uterus-to-body weight. Their data show that by pnd 16 uterine development is complete. The uterus then becomes quiescent for approximately ten days. The relative uterine weight declines in this period as the animals gain body weight. At pnd 28, uterine weight relative to body weight begins to accelerate as the animals approach puberty (**Figure 6**).

88. Ogasawara *et al.* (1983) measured uterine weight and the baseline level of DNA synthesis in both intact and OVX mice from pnd 1 through pnd 50. Their data show three distinct periods in the development and maturation of the immature uterus (**Figure 7**). The initial rising slope of uterine weight increase takes place from pnd 1-15 in parallel with a high level of DNA labelling with [^{125}I]-iododeoxyuridine incorporation. This phase occurs in both OVX and intact animals, further supporting the oestrogen independence of this developmental phase, as observed by Branham *et al*. (1985). There is a similar period of quiescence during pnd's 18-25.

89. Recently, Schlumpf *et al.* (2001) published detailed daily measurements of body weight and uterine weight from pnd 20 to pnd 32. These data further reinforce the observation of a quiescent uterus until pnd 25 and, afterwards, the gradual increase in the mean uterine weight and increased group uterine weight variability as the animals enter puberty. Thus, between pnd 25 and 30, a new phase begins in intact animals as the uterine-to-body weight ratio and DNA labelling rise again in parallel. In contrast, the OVX animals do not respond to oestrogen at pnd 25, supporting the oestrogen dependence as puberty begins and endogenous oestrogen levels rise.

90. Thigpen *et al.* (1987a) measured the time course of uterine weight growth in mice. In addition, they plotted the individual data points to provide insight on variability within each group (**Figure 7B**).

The variability increases sharply from pnd 22 through 28 as the animals begin to enter puberty. Recalling that mice mature a few days earlier than rats, these differences suggest variability and greater standard deviations in the uterotrophic test if substance administration were to begin later than pnd 22 or 23. This source of variability in the uterotrophic bioassay has, in fact, been suggested when rats receive their initial administration on pnd 22 and are sacrificed on pnd 26 (Christian *et al.,* 1998). These authors present the data in tables for three rat strains. On histological examination, the high outliers showed patterns consistent with Thigpen *et al.* (1987a), *i.e.,* some variation in the oestrogenic stimulation of the uterus in some individuals entering the early stages of puberty.

91. **Table 10** shows further evidence for the suggested age-related responsiveness of the uterus using a dose range of 17β-oestradiol at pnd 1-5, pnd 10-14, and pnd 20-24 (Branham *et al.,* 1985). At the latter time, animals had become more responsive to 17β-oestradiol than at earlier ages (**Figure 8**). It is important to note that the same dose induces the uterine weight increase regardless of the day the dosing began. The absolute and the relative responses increase dramatically at pnd 20-24.

92. In conclusion, the literature shows that the uterus has completed development by pnd 14-16. A window for a robust response opens about pnd 18 and lasts until pnd 25 or 26 in the rat. The window closes as individuals begin to enter puberty, with a rise in endogenous circulating oestrogens and an increase in uterine weight. This results in a rapid increase in the variability within the group as well as a rise in the mean baseline uterine weight. In the mouse, the literature supports the window for maximum biological response starting around pnd 16 and closing at pnd 22.

The Role of α-Foetoprotein

93. One factor controlling the oestradiol sensitivity of the immature uterus is the circulating levels of α-foetoprotein (AFP). AFP binds 17β-oestradiol specifically, and with high affinity. Circulating levels of free, unbound 17β-oestradiol are then reduced, and consequently the uterine response is reduced. AFP declines sharply after birth. The relevant literature includes:

- Raynaud (1973) who demonstrated a rapid decline in AFP binding capacity in the serum from five days before birth to near disappearance on pnd 25.

- Linkie and LaBarbera (1979) who used radioimmunoassays to measure the quantities of both AFP and albumin in serum of pnd 19-28 rats, showing an exponential decline in AFP levels.

- Germain *et al.* (1978) who measured the serum/tissue ratio of [^{3}H]-17β-oestradiol in pnd 2 and 26 or 28 day rats. On pnd 2, oestradiol was primarily bound in the serum. On pnd 28, binding in the serum was very limited while specific uptake of oestradiol into the uterus was now high. This can be estimated using the area under the curve in the paper of Germain *et al.* (1978).

- The uterotrophic experiments of Mizejewski *et al.* (1983) support the inhibitory role of AFP. These workers injected 17β-oestradiol in saline or in the presence of several proteins, including AFP. Injection of the oestradiol with AFP reduced the uterotrophic response in a manner that was AFP dose-dependent, and inhibited an increase in the mitotic index (Mizejewski *et al.,* 1983).

94. Payne and Katzenellenbogen (1979) measured the binding affinities of several oestrogens to both AFP and the uterine cytosol ER. Their data indicated significant differences in binding profiles.

The uterine cytosol receptor was more promiscuous than AFP, binding a range of substances. AFP was more selective for 17β-oestradiol. However, relative binding affinities lower than 0.01 were not measured, and all tested ligands had steroidal structures. The additional binding affinity work of Garreau *et al.* (1991) and Milligan *et al.* (1998) also show that AFP is more specific for 17β-oestradiol and the steroidal oestrogenic structure, and thus support the conclusion that interference with most weak agonists should be minimal.

95. Sheehan and Branham (1987) measured the relative response of the uterus to three oestrogens in both the immature rat and the OVX young (60 days) adults at three time points (**Table 10**). The response of the infantile animal was markedly less. However, the response of the immature version was less different when compared adult animals treated with EE or DES.

96. Collectively, these data support the hypothesis that AFP may interfere with the analysis of 17β-oestradiol and other oestrogens to which it binds. This interference is proportional to the AFP binding affinity of the reference oestrogen and declines rapidly after birth. In conclusion, the high postnatal levels of AFP must decline sufficiently to obtain a consistent robust response. As AFP binding appears far more structurally specific than the ER, modest AFP levels do not appear to be a concern for weak agonists. Administration can then commence about pnd 18. The high differential affinity for 17β-oestradiol, however, suggests either EE or DES be used as the reference oestrogen in the immature version.

Husbandry

97. With immature animals, animal husbandry issues arise that limit the use of very young animals. Laboratories, if they lack breeding facilities, must order the animals. Typically, the young are received while still nursing with their dam or a foster dam. Prior to pnd 17, the animals are very young and possibly vulnerable, *e.g.*, not obtaining adequate nourishment on their own after weaning. For example, Turnbull *et al.* (1999) obtained animals at pnd 15 and immediately weaned them. Dietary treatment began on pnd 17 and continued for four days, avoiding any additional handling and administration stress due to s.c. injection or oral gavage. The authors attributed several deaths in the DES control groups to the young age and early weaning. In conclusion, animal weaning, handling stress, etc., suggest that pnd 18 is the earliest day to use the immature, intact rat for the uterotrophic bioassay.

Table 9. Percentage changes in ovarian and uterine weights using consecutive daily doses of equine gonadotrophin, testosterone proprionate, and oestradiol benzoate, administered at different ages to the female rat (from Price and Ortiz, 1944)

Treatment	Age in days		Num.	Controls	Treated	Ovary	Uterus
	Start	Autopsy				%	%
Equine gonadotropin	0	6	44	8	15		- .06
10 R.U. daily for 6 days	4	10	38	7	19	60	82
	8	14	40	6	20	173	265
	14	18	46	9	20	254	561
	20	26	26	6	10	2928	675

Table 9 (continued). Percentage changes in ovarian and uterine weights using consecutive daily doses of equine gonadotrophin, testosterone proprionate, and oestradiol benzoate, administered at different ages to the female rat (from Price and Ortiz, 1944)

Treatment	Age in days		Num.	Controls	Treated	Ovary	Uterus
	Start	Autopsy				%	%
	30	36	31	6	12	1540	538
	50	56	53	9	20	543	121
Total			**278**	**51**	**116**		
Testosterone propion-ate 0.1 mg daily for 6 days	0	6	43	5	18		25
	4	10	62	7	15	- 4	48
	8	14	88	18	36	- 6	75
	14	18	74	13	27	-27	114
	20	26	34	9	12	2	375
	30	36	58	12	21	0.5	344
	50	56	56	13	24	-13	41
Total			**415**	**77**	**153**		
Oestradiol benzoate 1 R.U. daily for 6 days	0	6	35	8	11		171
	4	10	35	7	16	-16	235
	8	14	57	15	25	-18	258
	14	18	61	14	27	-17	356
	20	26	44	12	15	18	440
	30	36	77	15	27	40	365
	50	56	75	16	33	-8	31
Total			**384**	**87**	**154**		
Grand Total			**1077**	**215**	**423**		

Figure 5. Response of the rat uterus 24 hours after a single injection of 1 μg/10g body weight 17β-oestradiol. (insert shows relative uterus weight increases) (from Katzenellenbogen and Greger, 1974).

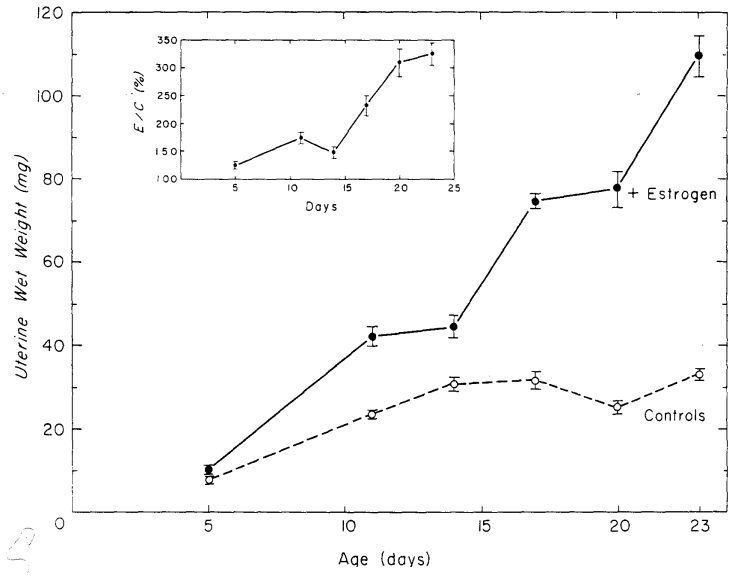


Figure 6. Development of the rat uterus from pnd 1 to 32. Uterine blotted weight (right inner axis, solid circles, solid line), luminal epithelial cell height (right outer axis, open triangles, dotted line), and number of epithelial glands (left axis, open circles, solid lines) (from Branham et al. 1985).

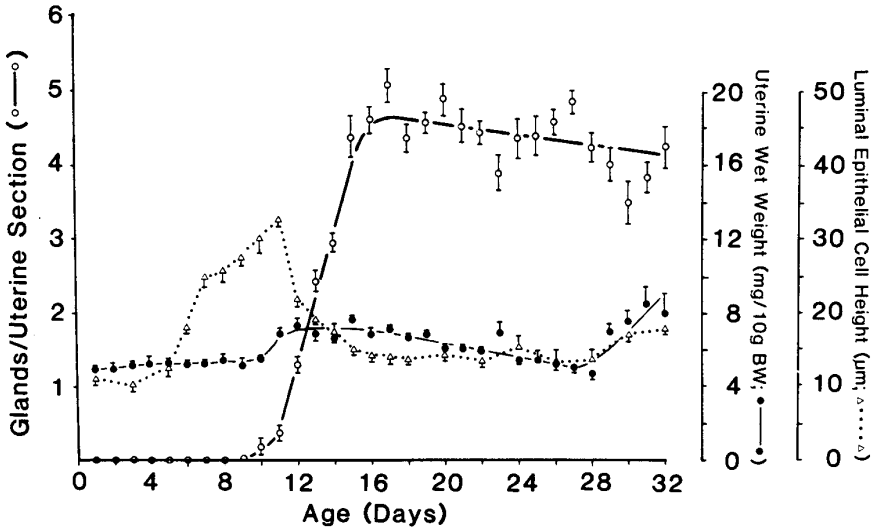
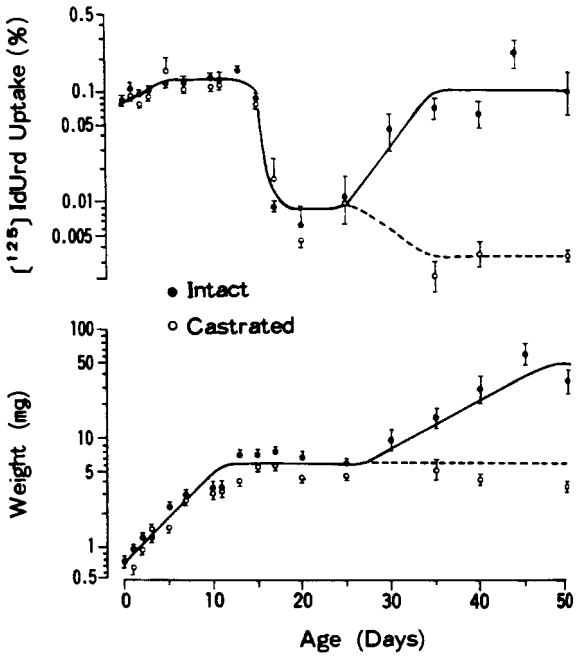


Figure 7. Measurement of uterine weight . Left panel, [^{125}I]Iododeoxyuridine uptake (top) and weight (bottom) of the uterus, in intact and OVX mice. The values are plotted on a log scale. Each point is the mean ± SE of 5-18 mice (from Ogasawara et al., 1983). Right panel, effects of diet on uterine weights of CD-1 mice weaned at 15 days of age. Uterine weights were determined at 15 days and at 2-day intervals from 20 to 28 days. Average of 15 mice/group (from Thigpen et al. 1987a).



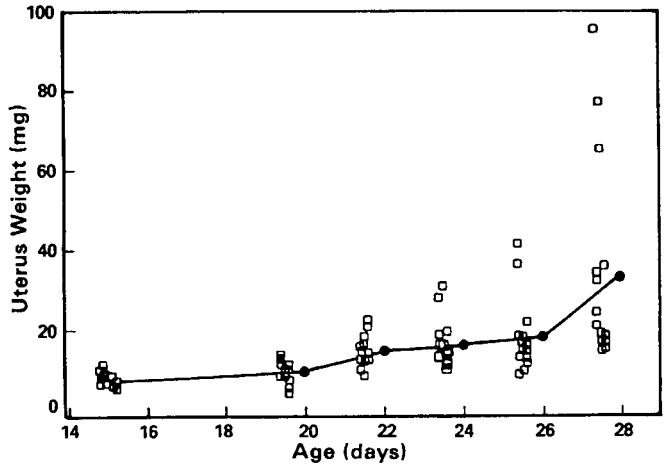


Figure 8. 17β-Oestradiol related responsiveness of the uterus as measures by the number if epithelial glands and by measure meant of uterine net weight in response to 5 consecutive days of increasing doses of oestradiol. Uterine wet weight (A) and gland number (B) in response to five consecutive days of increasing doses of oestradiol. Dosing began on day 1 (open squares), day 10 (open diamonds), or day 20 (open triangles) (from Branham et al., 1985).

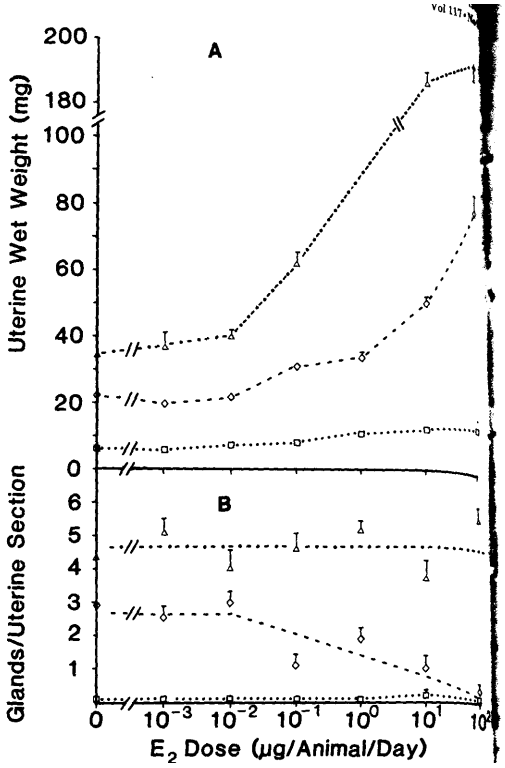


Table 10. Responsiveness of uteri in rats at different ages (from Sheehan and Branham, 1987)

	Oestrogen treatment period[a]		
Age in days	**days 10-14 (infantile)**	**days 20-24 (immature)**	**days 60-64 (adult)**
Oestrogen	ED_{50} (µg/kg/day)		
17β-Oestradiol	900	90	3
EE	50	5	1
DES	70	12	3

[a] Treatments began on the first day for three consecutive days, with necropsy on the fourth day.

The Ovariectomized (OVX) Rat Version and its Important Variables

98. The ovariectomized (OVX), sexually mature rat is an alternative version for the uterotrophic bioassay. To achieve a highly responsive assay, the removal of the ovaries must be complete, and adequate time allowed for uterine tissues to regress. Monitoring, e.g., vaginal smears or observation for ovarian tissue at necropsy, is necessary to ensure that ovariectomy is complete. Allowing 14 days for uterine regression appears to be adequate to produce a responsive version for the uterotrophic bioassay.

99. The uterotrophic bioassay requires a low level of endogenous oestrogens and low group variability to achieve a maximum responsiveness. After puberty, the ovaries, the primary source of endogenous oestrogens, must be surgically removed and sufficient time allowed for the uterine tissues to regress.

100. Ovariectomy must be properly performed and monitored, e.g., vaginal smears or observation for ovarian tissue at necropsy, to ensure that the ovariectomy is complete. Incomplete removal of ovarian tissue will result in marked hypertrophic growth of any remnants of ovary tissue, since FSH and LH stimulation from the pituitary can still occur (noted in the 1930s by Langston and Robinson, 1935). Zacharewski *et al.* (1998) observed in one of two lots of commercially supplied OVX immature rats, that several animals had evident ovarian tissue at necropsy, and uterine weights in other animals were high and suspect. These animals were supposedly ovariectomized at pnd 19 with necropsy on pnd 35. In groups with individuals having suspect, incomplete OVX, vehicle control mean blotted uterine weights were elevated to > 50 mg (Zacharewski *et al.*, 1998). These uterine weights were similar to non-OVX, pubertal animals. Comparisons with data in Table 10 of Zacharewski *et al.*, 1998, indicate that incomplete OVX will lead to anomalous results with high control uterine weights.

101. Limited regression of the uterus would also reduce the overall response of the assay. Langston and Robinson (1935) examined the time course of uterine regression after ovariectomy. However, they used longitudinal, not gravimetric, measurements and a limited number of animals. These researchers noted the need to confirm that no evidence of ovarian activity was present using vaginal smears before using animals, and to conduct a careful examination at necropsy for evidence of any remaining ovarian tissue. At nine and ten weeks after ovariectomy, uterine weight regression was 35 and 36%, respectively, and parallel histological examination showed a flattened epithelium lumen and compact stroma. At two weeks, data are available only from two animals, which showed regressions of 9 and 24% from the control mean.

102. Stob *et al.* (1954) performed a set of experiments in mice measuring the regression of the uterus after ovariectomy. In these experiments, ovaries were removed at 2 - 3 months of age. Mean uterine weight dropped from 74 mg to 8.5 mg over six weeks, with the most rapid drop in the first 2 - 3 weeks (**Table 11**). Santell *et al.* (1997) performed a set of experiments in rats that support the use of a minimum of 14 days between ovariectomy and use in the uterotrophic bioassay (**Table 12**). The data suggest an approximately one third drop in uterine weight 14 days after ovariectomy, in contrast to a continued increase in uterine weights with age.

103. In more detailed experiments, Sheehan *et al.* (1984) OVX young adult female rats (50-80 days) and followed the regression of the uterus and the fall in endogenous oestrogen levels. Over 14 days there was a constant fall in the uterine weight approaching 50%, but this did not reach a stable plateau (consistent with Langston and Robinson, 1935). Within 48 hours of ovariectomy the circulating level of 17β-oestradiol dropped dramatically and remained at a mean of 29 pg/ml throughout the remainder of the experiments. These investigators also followed OVX animals after the removal of oestrogen-containing implants. Uterine weight decreased approximately 20-25% over 5 days after implant removal.

104. **Tables 22** and **23** summarise control uterine weights in rodents following various experimental procedures, and indicates that the most common practice is to allow 10-14 days for regression. The

OECD Phase-1 uterotrophic protocols called for animals to be ovariectomized at about pnd 42, or 6 weeks of age (Kanno *et al.*, 2001). Animals at this age are just starting their estrous cycle (see reviews of Goldman *et al.*, 2000 and Ojeda and Urbanski, 1994, on puberty in female rat). Therefore, the uteri of rats at this age will have been stimulated by the rise in oestrogen and possibly one or more full oestrous cycles. In conclusion, allowing 14 days for uterine regression appears adequate. Monitoring procedures to ensure complete ovariectomy would be a beneficial precautionary measure.

Table 11. Regression in murine uterine weights after OVX (from Stob *et al.*, 1954)

Group #	Time after ovariectomy (wks)	Group size	Mean body weight (range) (gms)	Mean uterine weight (mgs)
1	0	6	23 (21-25)	74.17 ± 11.67
2	1	5	22 (20-27)	22.80 ± 7.89
3	2	5	23 (21-26)	16.56 ± 2.85
4	3	5	23 (22-26)	15.76 ± 0.94
5	4	5	26 (22-28)	16.24 ± 1.66
6	5	5	23 (20-26)	12.28 ± 0.74
7	6	5	24 (17-29)	8.48 ± 0.51

Table 12. Uterine weights in non-OVX and OVX rats at different ages (from Santell *et al.*, 1997)

Procedure for Group	Uterine weight (mg)
Expt. 1	
Not OVX, necropsy day 70	386.6 ± 41.1
OVX day 56, used day 70	76.5 ± 3.2
Expt. 2	
Not OVX, necropsy day 56	136.7 ± 5.5
OVX day 56, necropsy day 70	96.5 ± 3.9

Comparision of the Intact, Sexually Immature and The OVX, Sexually Mature Versions

105. Given the extensive application of both the intact, immature and the adult OVX rat in the uterotrophic bioassay, the question arises: how do these versions compare? Several comparisons are available in the literature. They are not robust comparisons in the sense that they are often not conducted simultaneously, and the number of chemicals and the number of doses were limited. The available literature, while limited, supports the qualitative equivalence of the intact, sexually immature rat and the OVX sexually mature versions in the uterotrophic bioassay.

106. Bickoff *et al.* (1959) compared immature and OVX adult mice using DES in the diet. Their data from a single experiment indicate that the immature mouse may be more responsive than the OVX adult mouse. The uterine weights of immature animals were induced nearly two-fold, while the OVX adult animals showed no increases in uterine weights at the same dose of DES.

107. Dukes *et al.* (1994) used both intact, immature and adult, OVX rats. Intact, immature animals received 3 consecutive days of administration, while dosing of the OVX rats was extended to 7 days in one set of experiments and 14 days in another. Oral gavage and subcutaneous administration were used in both versions. The results for oestrogen antagonism, were qualitatively similar suggesting rough equivalence of the assays, or that the OVX version could achieve equivalence by extending the dosing.

108. The data of Ashby *et al.* (1997a) using raloxifene suggest general agreement and equivalence between the two versions (**Table 13**). However, only one dose of raloxifene was in common between the experiments. Ashby *et al.* (1997b) also conducted experiments with clofibrate. Both the immature and OVX versions were non-responsive to clofibrate at the doses tested.

109. Welch *et al.* (1969) also conducted apparently separate experiments comparing immature and OVX rats, using similar doses of both technical grade DDT and *o,p'*-DDT. The latter is important, as *o,p'*-DDT is considered to be a weak oestrogen agonist. Again, the data (presented in **Table 14**) suggest basic equivalence between the two versions, but only three doses overlap directly.

110. Tinwell *et al.* (2000a) employed three versions to study the reference oestrogen, oestradiol benzoate, and the weak agonist, coumestrol: 1) the intact, immature female rat, 2) the immature, OVX female rat, and 3) the young adult, OVX rat. The uterine responses were similar for both wet and blotted weights in all three versions for both chemicals. The responses of histopathological (epithelium and endometrium cell heights) and mitotic markers (BrdU labelling) were similar, as were vaginal and cervical responses. However, the adult OVX version did not respond with statistical significance at 80 μg/kg/d oestradiol benzoate, and the responses to 60 mg/kg/d coumestrol (3-4 fold increase in the immature versions versus a 0.60-fold increase in the adult OVX version) were lower on a relative basis. Again, the relative response patterns of the vagina and cervix were of the same order (higher in the immature versions).

111. Laws *et al.* (2000) employed both immature and OVX, Long Evans rats, and both s.c. and oral administration, to assess several potent (17β-oestradiol and EE) and weak (methoxychlor, octylphenol, nonylphenol, and BPA) oestrogen agonists. The data indicate overall equivalence between the immature and the OVX versions, although responses in the immature version occurred at slightly lower doses for orally administered EE (0.01 versus 0.1 mg/kg/d) and nonylphenol (50 versus 100 mg/kg/d). Given the wide dose interval for EE, and experimental variability in the case of nonylphenol, significant weight should not be attributed to these findings unless replicated. In conclusion, based on limited data and experimental designs, the intact, immature and adult OVX versions appear qualitatively equivalent.

Table 13. Comparison of immature and OVX rats (from Ashby *et al.*, 1997a)

	Uterus wet weight		
	mg ± SD (n)	P (t-test)	% increase
OVX rats			
Control	81.3 ± 9.2 (7)		
Raloxifene, (0.1 mg/kg, oral)	97.7 ± 12.0 (6)	< 0.01	120.4%
Oestradiol, (0.04 mg/kg, sc)	239.5 ± 21.1 (7)	< 0.01	294.6%
Immature rats			
Control	25.0 ± 4.6 (10)		
Raloxifene, (0.01 mg/kg, oral)	30.9 ± 6.9 (5)	< 0.05	123.6%
Raloxifene, (0.1 mg/kg, oral)	42.1 ± 5.2 (5)	< 0.01	168.4%
Raloxifene, (0.25 mg/kg, oral)	35.4 ± 1.3 (5)	< 0.01	141.6%
Raloxifene, (1.0 mg/kg, oral)	35.4 ± 3.2 (5)	< 0.01	141.6%
Oestradiol, (0.4 mg/kg, oral)	95.9 ± 7.3 (5)	< 0.01	383.6%

Table 14. Comparison of the uterine responses of immature and OVX rats following administration of technical DDT or *o,p'*-DDT (Welch *et al.*, 1969)

	Immature rat		OVX rat	
Dose - i.p. (mg/kg/day)	**Uterine weight**	**% Increase**	**Uterine weight**	**% Increase**
Technical DDT				
Control	20.4 ± 0.4		89.4 ± 4.9	
1	20.6 ± 0.5	1		
5	23.4 ± 1.1	15	119.5 ± 6.8	34
10	28.0 ± 1.1	37		
25	35.0 ± 2.6	72		
50	35.8 ± 1.8	75	146.0 ± 6.4	63
o.p'-DDT				
Control				
0.25	20.1 ± 0.6	Decrease		
1	23.2 ± 0.5	14		
5	34.1 ± 1.1	67	135.4 ± 0.4	51
10	35.5 ± 1.1	74		
50			180.2 ± 10.3	102

CHAPTER 4: PROCEDURAL VARIABLES OF THE UTEROTROPHIC BIOASSAY

112. This chapter summarises the procedural variables of both versions of the uterotrophic bioassay. This is essential to the process to establish the critical features of a protocol, and to standardise it to be well understood and robust, with any limitations clearly identified. This includes a establishing a defined endpoint and a clear understanding of its biological and toxicological meaning. Variables common to both the immature and mature OVX uterotropic assay include the laboratory rodent strain, the route of administration, dissection and tissue preparation for the uterus, measurement of the uterine weight with and without the uterine luminal fluid, the timing of necropsy, and the laboratory diet. The chapter is organised around the following points:

- The laboratory strain's influence on the responsiveness of the assay.
- The different routes of substance administration.
- The choice of vehicle used to administer substances and its possible affects on the assay.
- The materials, equipment, and technical skills needed to perform the assay.
- Dissection and preparation techniques.
- Uterine weight measurement.
- The possible role of desiccation as a source of variability.
- The inclusion of the cervix when dissecting the uterus for weighing.
- Fixation of the uterus before weighing.
- Oven-drying of the uterus before weighing.
- Timing of the necropsy.
- The statistical methods employed for the data analysis.
- Phytoestrogens in the diet and the responsiveness of the assay

Strain of Laboratory Animal

113. The strain of animal used for the uterotropic assay is a potential concern. If different strains have different levels of responsiveness, strain differences may introduce variability among the results from different laboratories. Although small differences in uterine responsiveness have been reported, there is no available evidence that the use of different animal strains would lead to the failure to detect weak oestrogen agonists. The degree of difference observed is small, and responses adequate to detect potent oestrogens were observed in all cases. Most of the comparisons showing differences in responsiveness have used mice. The available experiments with rats do not show any significant strain differences in the uterine response.

The strain of laboratory rodent is known to be a determinant in the toxicological response of rodents to various chemicals (*cf.* Kacew *et al.*, 1995), including reproductive and developmental responses (*cf.* Chapin and Heindel, 1993; Chapin *et al.*, 1996; Goldman *et al.*, 2000). These strain variations are due to numerous genetic, pharmacokinetic, and biochemical factors. There is a concern, therefore, that the strain of laboratory rodent could influence the general responsiveness of the uterotrophic bioassay. Small differences in uterine responsiveness have been sporadically reported in the literature. However, given that existing data involve a limited set of chemicals, available strains, the power of detection of uterine response differs and that the experiments have not been repeated in several laboratories, a general phenomena that certain strains have different sensitivities in the uterotrophic bioassay has not been demonstrated.

Mice

114. There is evidence for limited differences in responsiveness among mouse strains. Both Pedersen-Bjergaard (1939) and Emmens (1939) commented or provided data, respectively, on the variation of the oestrogenic responses in mouse strains. Later, Emmens (1962) suggested that each colony be tested for responsiveness to a known oestrogen reference compound in a dose-response experiment with carefully interspersed doses. Claringbold and Biggers (1955), using vaginal cornification, tested the responsiveness of two inbred mice strains, C_{57} and CBA, and the F_1 hybrids, while investigating previous work indicating differences in strain responsiveness in mice. Using both oestradiol and estrone and both subcutaneous injection and intra-vaginal instillation with each of the four sets of mice, Claringbold and Biggers (1955) showed statistically significant differences by strain as well as by chemical and by route of administration for the dose response slope and median effective dose observed. However, all strains responded in a similar qualitative fashion.

115. Farmakalidis and Murphy (1984a) reported that positive doses of genistein in other laboratories failed to induce an uterine weight increase in their laboratory with the CD-1 mouse. This led them to compare three mouse strains (Farmakalidis and Murphy, 1984b). Data from both papers are extracted and combined in **Table 15**. Genistein was given orally in 4 consecutive daily doses, and uterine weights included imbibed fluid (wet weights, not blotted weights). The CD-1 mice in their earlier experiments received an approximately eight-fold higher dose, but the response was also higher. A more complete dose response for each strain, and the evidence to support strain differences in mice, is limited.

116. Roper *et al.* (1999) used the uterotrophic bioassay on several strains of mice during efforts to identify genetic loci for the uterotrophic response. They observed modest differences in uterine weight increases among three strains using 17β-oestradiol (**Table 16**).

117. A recent comparison of three mouse strains (CD-1, C57, and Alderley Park) showed no strain differences in the uterotrophic responses (Ashby, 2001).

Rats

118. No significant differences in the uterine responses among rat strains has been found in the available literature. However, a number of researchers have been unable to duplicate the findings of colleagues, *e.g.*, Edgren and Calhoun (1961) could not fully reproduce the experiments of Velardo, (1995). Edgren and Calhoun (1961) used both immature and adult OVX animals and various procedures. Satisfactory explanations were not found, so they speculated that one source of potential difference was that their Sprague-Dawley rats were less responsive than the 'Charles River' rat strain used by Velardo.

Christian *et al.* (1998) employed three strains of rats in a detailed study of the uterotrophic bioassay (two Wistar derivatives and a Sprague-Dawley strain). Their data suggest minimal differences in dose response to DES, and other characteristics, amongst the strains used.

119. Odum *et al.* (1999a,b) employed their own Wistar-derived Alderley Park rat strain, a Sprague-Dawley rat strain, and a Noble rat strain, using nonylphenol, a weak oestrogen agonist, as well as oestradiol. The uterotrophic data in the Noble rat is also supported by administration using implanted minipumps (Odum *et al.*, 1991a). The minimal effective dose and the percentage increase in uterine weight at the same dose was approximately the same across strains.

120. On the other hand, Steinmetz *et al.* (1998) investigated possible differential responses in the uterus and the vagina to a weak agonist, BPA, in OVX Fischer 344 rats. When uterine epithelial cell height and wet weight responses were compared between the Fischer 344 and Sprague-Dawley rats (Steinmetz *et al.*, 1998) using silastic implants to administer the test substance, both strains responded to 17β-oestradiol in a similar manner. However, the Sprague-Dawley rats showed no effective cell height response at the estimated dose of 0.3 mg/kg/d. In previous work, the pituitary responses of the F344 strain has been shown to be more responsive to oestrogens, while the uterine responses were similar (Wiklund *et al.*, 1981).

121. Long *et al.* (2000) have also investigated possible differential responses to a weak agonist, BPA, in Harlan Sprague-Dawley and Fischer 344 rats. They found similar rapid clearance in both strains ($t_{½} \cong$ 90 minutes after intraperitoneal injection), similar binding affinities for the ER, and similar stimulation of c-*fos* synthesis, in both strains. The levels of BrdU labeling stimulated by a series of 17β-oestradiol doses in the vaginal epithelium were similar. In contrast to the work of Steinmetz *et al.* (1998), BPA stimulated uterine labeling, which occurred at lower doses in the F344 rat strain, however the different chemicals were not administered in concurrent experiments. Tinwell *et al.* (2000) also encountered difficulties with precise reproduction of experimental data with BPA.

122. McKim *et al.* (2001) compared Sprague-Dawley (SD) and Fischer 344 rats with several oestrogen agonists, both potent and weak, using a the same doses for each substance in the two strains, and including responses to a pure anti-oestrogen, ICI 182,780. The responses of the two strains with EE, DES-dipropionate, and ICI 182,780 were generally similar, with small reductions in the DES-dipropionate response with the Fischer 344 strain, possibly due to variation. With octamethyl cyclotetrasiloxane, however, the SD rat appeared to be slightly more sensitive than the Fischer 344 strain.

123. The central question is whether a general, universal difference in sensitivity exists among rodent strains. Ideally, one would choose to work with a more sensitive strain and to avoid a less sensitive strain. Given the numerous genetic, pharmacokinetic, and biochemical factors, considerable data may be needed to establish a general phenomenon and to distinguish it, for example, from strain differences in the metabolism of a particular chemical. In Phase one of the OECD validation program, a carefully spaced dose response curve for EE was generated for each protocol among 20 laboratories (Kanno *et al.*, 2001) No evidence for strain differences was found. The primary strains used were Sprague-Dawley and Wistar-derived. In conclusion, the existing data are not sufficient to support a generalization for the substantial sensitivity of a particular rodent strain versus others in the uterotrophic bioassay.

Table 15. Responses of different mouse strains to genistein (from Farmakalidis and Murphy, 1984a,b)

Treatment bw grams, uterine wt mg	ICR		B6D2 F_1		B6C3F_1		CD-1	
	Body wt	Uterine wt.	Body wt	Uterine wt.	Body wt	Uterine wt.	Body wt	Uterine wt.
Control	17.58	27.30	10.90	12.66	11.16	13.70	13.1	25.4
7.5 μg/kg/d[a]	16.59	29.90						
12.5 μg/kg/d			11.47	24.34				
11.5 μg/kg/d					11.16	26.00		
80 μg/kg/d							12.1	79.5
Mean Increase		2.60		11.68		12.90		54.1

[a] Doses are calculated from the body weights, as only the dose per mouse was given in the publications.

Table 16. Responses of different mouse strains to 17β-oestradiol (from Roper *et al.*, 1999)

Treatment	C57BL/6J	C3H/HeJ	B6C3 F_1
	mean uterine wt in mg ± s.d. (number of animals)		
Control	17.5 ± 4.7 (13)	19.5 ± 3.4 (11)	17.6 ± 5.5 (9)
17β-Oestradiol (40 μg/kg/d)[a]	60.9 ± 10.9 (33)	42.2 ± 6.9 (27)	57.0 ± 13.6 (26)

[a] Two daily subcutaneous injections followed by necropsy; blotted uterine weights.

Route of Administration

124. The route of administration used in the uterotrophic bioassay affects the dose responses for most substances. In general, the minimal effective dose for most substances is lower by as much as an order of magnitude with subcutaneous injection compared to oral gavage. With the appropriate selection of doses, the response appears to be adequate to detect weak oestrogen agonists by either route of administration. There are exceptions to this generalization, where the minimal effective dose is higher for the oral route of administration, e.g., liver metabolism activates methoxychlor and tamoxifen.

125. A variety of routes of administration have been used with the uterotrophic bioassay: intravenous injection, intraperitoneal injection, subcutaneous injection, intramuscular injection, oral gavage, inclusion in the diet, inclusion in drinking water, and dermal application. These routes are recorded as part of the literature review and are extracted into tables in the Annex. Additional details of metabolism and conjugation, and effects that appear to be route dependent, are provided in Chapters 6 and 7, respectively.

126. Numerous laboratories have observed differences in the uterine response due to the route of administration employed. Odum *et al.* (1997) compared several potent reference oestrogens by both oral gavage and s.c. administration. The s.c. administration led to a lower minimal effective dose. However, the degree of difference between s.c. and oral gavage appears to depend upon the chemical. This and other direct comparisons of the subcutaneous and the oral gavage routes have been extracted into **Table 17**. Cortroneo and Lamartiniere (2001) observed consistent differences between s.c. and dietary administration of genistein for several responses, including uterine growth, that also correlated with free, unconjugated serum levels of genistein. The choice of route of administration will depend upon the stated regulatory policy needs, such as if the uterotrophic bioassay is used in a tiered, testing assessment scheme, and the characteristics of the individual test substances. The oral route is relevant to the dietary administration used in higher, long-term tests for adverse effects, as well as to the expected route of most exposures for humans and wildlife.

Table 17. Studies providing comparisons of subcutaneous and oral gavage routes of substance administration. [a]

Reference	Substances Assayed	Comments
Ashby and Tinwell (1998)	diethylstilbestrol; bisphenol A	High doses used (40 μg/kg/day DES; 400, 600, 800 mg/kg/day BPA). The maximum DES response appeared equivalent by both routes at these high doses. BPA response occurred at both doses, appearing to be equivalent at 800 mg/kg/day. S.c. route gave a slightly higher response at 400 and 600 mg/kg/day.
Ashby *et al.* (1999a)	diethylstilbestrol; resveratrol	High DES doses used (40 μg/kg/day). The resveratrol doses (30 μg - 120 mg/kg/day) did not elicit a uterine response by either route of administration.
Claussner *et al.* (1992)	RU 50667; RU 51625; RU 53637	A series of comparative experiments were run. Limited differences apparent between routes; s.c. was slightly more responsive (see **Figure 3**)
Dorfman and Kincl (1966)	50 structurally related chemicals compared to estrone and 17β-oestradiol	Comparisons of numerous substances with s.c. and oral gavage. Data are expressed as relative potency. Relative potencies did differ by route of administration.
Dukes *et al.* (1994)	17β-oestradiol benzoate; ZM 189,154; tamoxifen	Co-administration of oestradiol benzoate and ZM 189,154 show about one order of magnitude difference between s.c. and oral gavage doses in the ability of the anti-oestrogen to depress uterine weights.
Edgren (1958)	norethynodrel; 17α-ethynyl-19-nortestosterone	In Figure 1, two chemicals are compared using a log dose-response scale. The slopes are parallel for the two routes of administration for the same substance. The slopes are different between substances.
Everett *et al.* (1987)	17β-oestradiol; zeranol	A dose response curve with 5 doses by s.c. and 8 doses by oral gavage. All doses appear to generate a response. Oestradiol was more active by s.c. route by ~1 order of magnitude. Zeranol activity was equivalent (~3 fold increase) at 30 mg/kg/day by both routes of administration.
Gray *et al.* (1999)	methoxychlor; dibutylphthalate	Methoxychlor was more active by the oral route than subcutaneous. Even at 1000 mg/kg/day; the dibutylphthalate was inactive by both routes.
Jones *et al.* (1979)	estrone; [3,4-dihydro-2-(4-methoxyphenyl)-1-napthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phenyl]methanone, methanesulfonic acid salt	Antioestrogenic curves between s.c. and oral gavage appear similar in the percentage increases in uterine weight. See Table I of the paper where doses overlap 30-300 μg. However, no minimal effective dose was available to compare the different routes.
Jordan *et al.* (1977)	tamoxifen	No apparent route differences in a direct side-by-side comparison (see Figure 4 of that paper). May involve trade-off between more rapid systemic distribution via s.c. and activation of the compound in the liver via oral gavage.

Table 17. Studies providing comparisons of subcutaneous and oral gavage routes of substance administration.[a] (continued)

Reference	Substances Assayed	Comments
Lundeen *et al.* (1997)	7α-ethinyl oestradiol, 17β-oestradiol, 17α-oestradiol	Route of administration did affect the uterine weight increase, but the degree of difference varied amongst the compounds. Doses spaced at one order of magnitude.
Odum *et al.* (1997)	17β-oestradiol, 17β-oestradiol benzoate, ethinyl oestradiol, methoxychlor	The MED is lower for the s.c. route versus oral gavage. Methoxychlor is more active using oral gavage.
Routledge *et al.* (1998)	17β-oestradiol, methyl paraben, butylparaben	In expt. 1, the butyl paraben response was statistically significant ($P < 0.05$) at 200 mg/kg/d by s.c., but was inactive orally at 1200 mg/kg/d.
Van de Velde *et al.* (1994)	tamoxifen, RU 58668, ICI 182,780	In mice, a difference of about one order of magnitude in the minimal effective dose and other responses was observed between the s.c. and oral gavage routes. Compare Figure 5 (s.c) with Figure 6 (oral).
Wakeling and Bowler (1988)	ICI 163,964, ICI 164,275, ICI 164,384	Approximately one order of magnitude difference in doses for equivalent responses between s.c. and oral routes. See Figure 3 and Figure 4A of the publication.
Williams *et al.* (1997)	oestradiol benzoate	Figure 1 indicates very significant differences in both maximum uterine weight increase and minimal effective dose (0.05 µg/kg/d s.c. and 20 µg/kg oral gavage). This difference is very similar to that seen by Odum *et al.* (1997).

Vehicles Used in the Uterotrophic bioassay

127. Historically, a wide variety of vehicles have been used in the uterotrophic bioassay. Broadly, these may be divided into oils and solvent vehicles, and aqueous-based vehicles. Both groups of vehicles have been used with different routes of administration. As most oestrogens are hydrophobic with limited solubility, the predominant vehicles have been vegetable oils and solvents. Important issues include dispersion of test substances into the vehicle, and consequent bioavailability.

128. The major vegetable oil vehicles include arachis (peanut) oil, corn oil, and sesame oil. The major solvent has been dimethylsulfoxide (DMSO). For aqueous-based vehicles, substances have first been dissolved in a solvent, typically ethanol, and then diluted into aqueous solution, or suspended in aqueous solution using polymers such as hydroxypropyl or hydroxymethyl cellulose. The various vehicles used in the uterotrophic bioassay have been summarised from the literature for the immature rat version (**Table 18**).

129. Ideally, the selection of vehicles should be informed by the characteristics of the test substance. However, in the literature, the rationale for vehicle selection appears to have largely been based on the historical practice and preference of the particular investigating laboratory. Most oestrogens are hydrophobic with limited solubility, and the predominant vehicles used in the past have been vegetable oils and solvents. The important issues with substances of limited solubility are twofold; firstly, when preparing the substance for administration, a hydrophobic substance is dissolved or adequately dispersed in a oil or solvent vehicle and, secondly, the test substance must be rapidly released from the vehicle. The literature is largely silent on the issue.

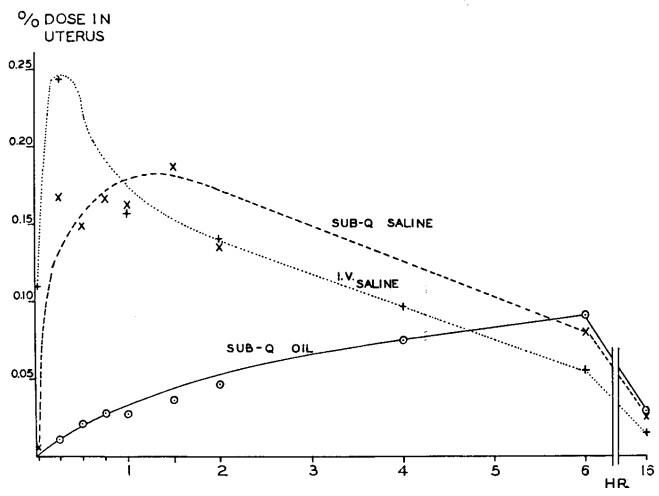
130. Jensen and Jacobson (1962) have shown that the vehicle can effect distribution and tissue doses. The distribution of oestradiol to the target uterine tissue and the uptake in the uterus are significantly faster using saline as the vehicle compared to the sesame oil (**Figure 9**).

131. Other reports that the vehicle influences the uterotrophic bioassay are limited. Booth *et al.* (1960) reported that vegetable oils have uterotrophic activity in mice (≥40% increase in uterine weight at dietary levels of 10%). Although the procedures appear valid, no other laboratory has reported that vehicles substantially increase the uterine weight. Although two reports from rats have reported data for untreated and vehicle controls side by side, neither used a plant-derived oil vehicle (see Bachmann *et al.*, 1998; Christian *et al.*, 1998). The first phase of the OECD standardization and validation program encountered no such difficulties with a variety of vehicles. No significant increases in uterine weights were observed with a variety of plant-derived oil vehicles (Kanno *et al.*, 2001).

Table 18. Vehicles reported to have been used in the uterotrophic bioassay in immature rats.[a]

Oil and solvent vehicles	
Arachis oil (see also Peanut oil)	Allen *et al.* (1980); Ashby & Tinwell (1998); Ashby *et al.* (1997a,b, 1999); Baker *et al.* (1999); Dukes *et al.* (1994); Edery *et al.* (1985); Jordan *et al.* (1977, 1978); Odum *et al.* (1997); Routledge *et al.* (1998)
Corn oil	Black & Goode (1980), Black *et al.* (1983), Gould *et al.* (1998), Jansen at al. (1993), Jones *et al.* (1984); Levin *et al.* (1967a,b); Li & Hansen (1995); Safe & Gaido (1998);
Cottonseed oil	Wenzel & Rosenberg (1956)
Ethyl oleate	Bicknell *et al.* (1995)
Olive oil	Qian & Abul-Haij (1990)
Peanut oil	Jordan (1976); Jordan and Gosden (1983)
Sesame oil	Christian *et al.* (1998); di Salle *et al.* (1990); Everett *et al.* (1987); Fail *et al.* (1998); Franks *et al.* (1982) (1:9 ethanol:sesame oil); Hammond *et al.* (1979); Hayes *et al.* (1981); Katzenellenbogen *et al.* (1980); Larner *et al.* (1985); Raynaud (1973); Schmidt & Katzenellenbogen (1979)
Propylene glycol	Cano *et al.* (1986); Whitten *et al.* (1992)
Dimethylsulfoxide	Galand *et al.* (1987) - 1:1 dimethylsulfoxide:propylene glycol; Ruh *et al.* (1995)
Aqueous-based vehicles	
Ethanol	Bachmann *et al.* (1998) - 25%; Bhavnani *et al.* (1998) - 10%,
5% HPC	Connor *et al.* (1996) HPC (Hydroxypropylcellulose)
0.25% MC	Duncan *et al.* (1963) Methylcellulose
0.15 M saline	Gazit *et al.* (1983); Katzenellenbogen & Ferguson (1980); Lan & Katzenellenbogen (1976) (2% ethanol); Lauson *et al.* (1939); Levin *et al.* (1967a,b)

Figure 9. Radioactivity in the uterus of 23-day-old rats after a single injection of approximately 0.1 μg. of 6,7-^{3}H oestradiol using saline and sesame oil as vehicles (from Jensen and Jacobsen, 1962).



Materials, Equipment, and Technical Skills Needed to Perform the Uterotrophic bioassay

132. There are no specialised, expensive equipment or special technical skills to restrict the practice of the assay. No other restrictions, e.g., patented organisms or reagents, apply. Therefore, the equipment, materials, and technical skills necessary to perform the uterotrophic bioassay should be widely available in most national regulatory jurisdictions.

133. Animal supply houses in most countries that can supply rats that are pathogen free and of a defined strain. Most suppliers can supply animals of the requested age. The ability to supply animals of a defined birth date is necessary for the intact, immature version, and the ability of the supplier to provide animals of a defined birth age should be confirmed. Most supply houses will perform ovariectomy upon request for an additional fee.

134. The equipment and facility needs for a laboratory to conduct the uterotrophic bioassay should pose no major restriction to conducting the assay. They are common and widely available:

- animal housing to receive, quarantine, and house the animals, including supplies of animal diet, bedding, and water;

- space to receive and store reagents, vehicles, and test substances;

- space to prepare doses, with balances, hoods, etc., for dispensing materials; magnetic stirring units for dose preparation, etc.;

- space to administer the test substance and simple equipment such as, syringes, gavage tubes, etc.,

- basic dissection areas and equipment such as scalpels, tweezers, and scissors for opening the abdominal cavity and for trimming fascia; balances capable of 0.1 mg weights; petri dishes to hold and weigh tissue, etc.;

- personnel with consistently good technical skills, e.g., animal husbandry, dose preparation, and dissection;

- ability to perform the experiments under Good Laboratory Practices (GLP) Guidelines;

- functional quality control unit to audit the recorded data.

Dissection and Tissue Preparation Techniques

135. The dissection and handling of the uterus, while not a complex procedure, does introduce several possible sources of variation to the assay. Removal of adhering fat and tissue, desiccation of the tissue, the amount of pressure during blotting, or the blotting paper used, etc., all combine to introduce the potential for variability amongst laboratories.

136. With proper care and practice, variables such as removal of adhering fat and tissue, desiccation of the tissue and blotting of the dissected uterus should not present a barrier to the qualitative detection of oestrogenic compounds. The extraction of the historical, published data show that most of the control, blotted uterine weights are in range of 20-35 mg for the immature version, and 80-110 mg for the ovariectomized version. These control uterine weights have historically been satisfactory to detect weak oestrogen agonists, where administered doses were sufficiently high.

137. In Phase one of the OECD validation program (Kanno *et al.*, 2001), the cervix was included when measuring the final weight of the uterus in order to preserve the luminal fluid. The cervical weight was nearly as responsive to oestrogen as was the uterus. Thus, no significant change in assay sensitivity or maximum response is expected with the inclusion of the cervix. The cervix weight should add approximately 5 to 10 mg to the blotted, vehicle control uterine weights.

138. The dissection of the uterus is not a complex procedure. The organ is easily identified and can be separated from surrounding organs and tissue with relative ease. Briefly, the procedure used in the OECD uterotrophic bioassay (Kanno *et al.*, 2001) requires a sequence of several steps after the animal has been humanely killed:

- the lower abdominal wall is opened;
- the female reproductive organs are identified;
- the uterus and, in the immature version, ovaries are detached from ligaments and mesentery;
- the uterus is severed from the vagina at the juncture with the cervix. This is intended to prevent the loss of the luminal fluid, as the wet weight of the uterus is a required endpoint;
- fat and adhering tissue are trimmed and removed from the body of the uterus without puncturing the wall and spilling the luminal contents;
- the wet weight of the uterus is taken and recorded;
- the wall of the uterus is pierced or split with a scalpel;
- the luminal fluid is removed by very gentle pressure with filter or blotting paper;
- the blotted weight of the uterus is taken and recorded; and
- the uterus and other tissues are placed in a fixative for preservation and possible histological examination.

139. A review of the methods from over 300 articles in the open literature reveals no such detailed instructions. The methodological descriptions are often vague so that the precise procedure cannot be verified. There is no evidence that a dissecting scope has been used for the trimming procedure. Thus, it is expected that there have been modest to considerable methodological variations in the dissection and tissue preparation procedures in the past. Even with a stricter standardization, some slight variation amongst laboratories and technicians may be expected.

140. Each step presents an opportunity to introduce variation. The degree to which these variations in laboratory technique are controlled would be expected to increase or decrease the standard deviation of the mean wet and blotted uterine weights. Such variation would likely be inconsequential for detecting potent agonists and antagonists, which is how the assay has been historically employed. However, increased variation might reduce the assay power for weak oestrogen agonists in the lower portion of the dose response curve. That is, a larger percentage increase in weight over the controls would be necessary to achieve statistical significance. This possible loss of assay responsiveness could be approached with improved standardization and laboratory training of dissection technicians, administration of higher doses of the test substance, or the use of larger group sizes. Checking the historical coefficient of variation for a dose response curve with the standard reference oestrogen, EE, may provide a useful periodic indicator for the need of a laboratory to reduce variability.

Desiccation as a Source of Variability

141. The dissection and removal of the uterus requires the exposure of a small, moist organ to the atmosphere. This raises the possibility that the tissue may dehydrate, decreasing the uterine weight. Thigpen *et al.* (1987a) conducted an experiment with mice to define the time course and extent of possible dehydration by recording weights at 30-second intervals. After examining uteri with mean initial blotted weights of 12mg, they found that the uterine weights decreased by nearly 12% in three minutes of exposure to the open atmosphere. These experiments suggest that some care is necessary to protect the uteri from dehydration during removal, processing, weighing, and blotting. The ability of laboratory personnel to work both accurately and rapidly, as well as the use of moistened chambers to hold and to weigh the uterine tissue, would be expected to reduce the time and opportunity for desiccation.

Inclusion of the Cervix in Dissection of the Uterus

142. Historically, many investigators have not only removed the vagina and the ovaries, but also the cervix. However, measurements have focused solely on blotted weights of the uterine body. The OECD validation exercise, which included both wet and blotted weight measurements of the uterus, also included the retention of the cervix to prevent loss of luminal fluid (Kanno *et al.*, 2001).

143. The literature indicates that including the cervix does not negatively affect the uterotrophic bioassay. The increases in the weight of cervix show a similar degree of responsiveness as the body of the uterus. Karkun and Mehrotra (1973) administered 0.1 μg of oestradiol dipropionate to adult OVX rats and observed a uterine mean weight increase from 66.5 to 333.6 mg, and cervical weigh increases from 13.9 to 49.8 mg. Ng *et al.* (1994) noted in control animals that uteri with fluid and cervix intact weighed about 17 mg, and about 13 mg without fluid or cervix. Datta *et al.* (1968a,b) recorded changes in both uterine and cervical weights during each stage of the estrous cycle in rats, and during administration of oestradiol to OVX rats. The tissue responses were concordant, and the relative percentage increases were qualitatively similar (**Table 19**).

144. Ashby and coworkers have produced two datasets on uterine weights with and without the cervix. The first dataset shows that the cervix responds in proportion to the uterus with a potent oestrogen, oestradiol benzoate (EB); a weak phytoestrogen, coumestrol (COM); and, in one publication, a

potent anti-oestrogen, faslodex (Ashby *et al.*, 1999b; Tinwell *et al.*, 2000a) (see **Table 20**). The uterus, vagina, and cervix in three uterotrophic versions (immature, intact; immature, OVX; young adult, OVX) responded in an equivalent fashion, although overall the immature version appeared to be more responsive at similar doses of COM and EB.

145. The second dataset was gathered in conjunction with the first phase of the OECD uterotrophic validation program using EE and are from an unpublished report (Odum *et al.*, personal communication, 2000). The data in **Table 21** are extracted from that report and compare the weights of: 1) the uterine body with cervix and imbibed fluid, 2) the blotted uterine body with cervix, 3) the cervix only, and 4) the blotted uterine body only. The responsiveness of the cervix to oestrogen and to anti-oestrogen is clear in both versions, and qualitatively similar to body of the uterus.

146. In making comparisons with the historical, control uterine weights extracted from the literature into **Tables 22** and **23**, and found in a later section of this docuement, the reader should recognise that these do not typically include the cervix. Inclusion of the cervix in the protocol as used by OECD would increase control weights ~5-7 mg in the immature version and ~12-18 mg in the OVX version. In addition, inclusion of intra-luminal fluid would be expected to add another 2-3 mg to the immature control rats and 4-5 mg for the OVX rats.

Table 19. Absolute and relative weights of the uterus and cervix in OVX rats (from Datta *et al.*, 1968a,b)

	uterus (mg)	**relative %**	**Cervix (mg)**	**relative %**
OVX				
Control	74.1		15.8	
Oestrogen[a]	248.1	335%	49.3	312%
Oestrogen[a] + progesterone[b]	213.8	289%	45.3	287%
Estrous cycle				
Dioestrus	111.9		15.9	
Proestrus	253.2	226%	42.8	269%
Oestrus	240.4	215%	39.6	249%
Metoestrus	211.6	189%	28.3	178%

[a] 1 µg per rat per day for 7 days via intramuscular injection;
[b] 1 mg per rat per day for 7 days via intramuscular injection

Table 20. Absolute and relative weights of the uterus and cervix from treated, immature rats (from Ashby *et al.*, 1999)

Daily doses	Uterus		Cervix	
	Blotted (mg) Mean ± SD	Relative %	Calculated (mg) Mean ± SD	Relative %
Vehicle	18.8 ± 2.6	100	7.4 ± 1.0	100
Oestradiol benzoate (400µg/kg)	99.6 ± 19.4	529.8	20.0 ± 4.2	270.3
Coumestrol (60mg/kg)	73.7 ± 4.8	392.0	19.8 ± 4.3	267.6
Faslodex (10mg/kg)	12.3 ± 2.4	65.4	5.6 ± 0.9	75.7
Coumestrol (60mg/kg) + Faslodex (10mg/kg)	12.8 ± 1.6	68.1	5.6 ± 0.6	75.7
Vehicle	22.1 ± 5.4	100	10.0 ± 1.4	100
Oestradiol benzoate (80µg/kg)	94.7 ± 8.4	428.5	27.2 ± 4.3	272.0
Oestradiol benzoate (400µg/kg)	91.2 ± 11.0	412.7	28.5 ± 2.7	285.0
Coumestrol (60mg/kg)	79.6 ± 8.0	360.2	28.8 ± 4.4	288.0

Table 21. Comparison of different procedures on the weights of the rat uterus and cervix (from Odum *et al.*, 2000b)

Group	Chemical/dose (mg/kg/day)	Uterus wet weight + cervix		Uterus blotted weight + cervix		Cervical weight		Uterus blotted weight less cervix	
		mg	relative [a]	mg	relative [a]	mg	relative [a]	mg	relative [a]
Immature rats with oral gavage									
1	None (untreated)	30.5		27.9		6.6		21.3	
2	Arachis oil 2 ml/kg	32.7		30.3		6.9		23.4	
3	EE 0.01 mg/kg/day	31.4	96.0	28.8	95.0	4.9	71.0	23.9	102.1
4	EE 0.03 mg/kg/day	34.4	105.2	31.8	105.0	8.3	120.3	23.5	100.4
5	EE 0.1 mg/kg/day	31.4	96.0	29.4	97.0	5.6	81.2	23.8	101.7
6	EE 0.3 mg/kg/day	38.3	117.1	35.5	117.2	7.4	107.2	28.1	120.1
7	EE 1.0 mg/kg/day	65.7	200.9	60.0	198.0	12.8	185.5	47.2	201.7
8	EE 3.0 mg/kg/day	110.2	337.0	94.8	312.9	19.9	288.4	74.9	320.1
9	EE 10.0 mg/kg/day	162.6	497.2	117.1	386.5	19.1	276.8	98.0	418.8
10	EE 3.0 mg/kg/day	121.5	371.6	99.9	329.7	22.5	326.1	77.4	330.8
	ZM 0.1mg/kg/day								

Table 21 (continued). Comparison of different procedures on the weights of the rat uterus and cervix (from Odum *et al.*, 2000b)

11	EE 3.0 mg/kg/day ZM 1.0 mg/kg/day	40.7	124.5	38.1	125.7	5.5	79.7	32.6	139.3
Immature rats with subcutaneous administration									
1	None (untreated)	25.7		23.6		4.5		19.1	
2	Arachis oil 2 ml/kg	29.8		27.2		5.8		21.4	
3	EE 0.01 mg/kg/day	28.5	95.6	26.4	97.1	5.6	96.6	20.8	97.2
4	EE 0.03 mg/kg/day	29.3	98.3	27.2	100.0	5.4	93.1	21.8	101.9
5	EE 0.1 mg/kg/day	37.9	127.2	34.6	127.2	6.5	112.1	28.1	131.3
6	EE 0.3 mg/kg/day	64.6	216.8	60.9	223.9	12.3	212.1	48.6	227.1
7	EE 1.0 mg/kg/day	40.7	472.1	112.5	413.6	18.8	324.1	93.7	437.9
8	EE 3.0 mg/kg/day	175.6	589.3	119.6	439.7	18.4	317.2	101.2	472.9
9	EE 10.0 mg/kg/day	203.1	681.5	125.1	459.9	21.1	363.8	104.0	486.0
10	EE 0.3 mg/kg/day ZM 0.1mg/kg/day	57.4	192.6	64.3	236.4	19.8	341.4	44.5	207.9
11	EE 0.3 mg/kg/day ZM 1.0 mg/kg/day	23.1	77.5	21.3	78.3	5.0	86.2	16.3	76.2
Mature ovariectomized rats with subcutaneous administration									
1	None (untreated)	88.6		84.0		16.4		67.6	
2	Arachis oil 2 ml/kg	83.7		79.2		14.5		64.7	
3	EE 0.01 mg/kg/day	92.2	110.2	87.7	110.7	16.6	114.5	71.1	109.9
4	EE 0.03 mg/kg/day	92.4	110.4	87.1	110.0	16.0	110.3	71.1	109.9
5	EE 0.1 mg/kg/day	110.2	131.7	105.1	132.7	20.0	137.9	85.1	131.5
6	EE 0.3 mg/kg/day	236.1	282.1	211.1	266.5	43.5	300.0	167.6	259.0
7	EE 1.0 mg/kg/day	406.4	485.5	287.7	363.3	49.2	339.3	238.5	368.6
8	EE 3.0 mg/kg/day	351.8	420.3	262.2	331.1	46.2	318.6	216.0	333.8
9	EE 10.0 mg/kg/day	391.3	467.5	273.7	345.6	51.7	356.6	222.0	343.1
10	EE 0.3 mg/kg/day ZM 0.1mg/kg/day	244.5	292.1	204.0	257.6	46.5	320.7	157.5	243.4
11	EE 0.3 mg/kg/day ZM 1.0 mg/kg/day	127.8	152.7	122.1	154.2	24.5	169.0	97.6	150.9

EE, Ethinyl oestradiol; ZM, ICI-182,780

[a] relative to vehicle control

Wet and Blotted Uterine Weights as Endpoints

147. Historically, the favoured endpoint for the uterotrophic bioassay has been the blotted uterine weight without the intra-luminal fluid. Early investigators noted that the inclusion of the intra-luminal fluid added variability to the recorded mean, and they questioned the interpretation and utility of this value. Although the accumulation of intra-luminal fluid is an oestrogen-mediated response, the value and interpretation of the occurrence, and magnitude a fluid imbibition response for weak oestrogen agonists, is currently unclear.

148. The uteri of rats and mice contain small amounts of intra-luminal fluid in the unstimulated state, and significant amounts following high doses of potent oestrogens (the latter is the basis for the Astwood assay). In the current discussion, a 'wet' weight is defined as including intra-luminal fluid and a 'blotted' weight is defined as having removed the intra-luminal fluid.

149. A comparison of background vehicle control uterine weights for immature and OVX adult rats has been complied in **Tables 22** and **23**, respectively. Literature reports of uterine control weights have been defined and divided into wet (containing intra-luminal fluid), blotted (without intra-luminal fluid), unspecified (procedures not clear or not given), and dry (oven dried). In citations where the methods used were unclear, comparisons of the uterine weights from controls and test groups in the papers cited leads to the judgment that many are actually blotted weights. This is because the published literature has not followed a standardised terminology. For example, Wakeling *et al.* (1983) use the term "wet" when referring to blotted weight: "Uteri were removed, dissected free of adhering fat, blotted dry after expulsion of uterine fluid, and the wet weight was recorded." In **Table 24,** detailed methods used to prepare the uteri where blotted weights were used are extracted from several manuscripts.

150. Historically, blotted uterine weights have been recorded in most reports. Early investigators noted that uterine weight (with intra-luminal fluid) was a more variable measure and required more care to prevent the loss of luminal fluid. They also questioned the usefulness of this measure. For example, Lausen *et al.* (1939) stated their conclusion: "Weights of the uteri before fluid was expressed and of the vaginas and ovaries were also recorded, but since they have been found to have no particular significance to this paper, they will not be included." Hisaw (1959) concluded that while some degree of fluid imbibition appeared to be common to the oestrogens they tested: "they differ widely in their ability to produce the effect." Phase one of the OECD uterotrophic validation program showed that the wet uterine weight with the luminal fluid included was indeed more variable, and did not appear to offer increased sensitivity (Kanno *et al.*, 2001).

151. Although, the accumulation of intra-luminal fluid in the uterus is clearly an oestrogen-mediated response, the value and interpretation of the occurrence, and magnitude of this imbibition particularly for weak oestrogens, is unclear. During the OECD standardisation phase of the uterotrophic bioassay, both wet and blotted weights were included to assist companions and decisions about future test guideline protocols. Keeping both endpoints preserves the ability to study the response patterns of weak oestrogen agonists with respect to fluid imbibition, as well as any possible association of this response with the appearance of adverse effects in other toxicological protocols.

Fixation of the Uterus Before Weighing

152. Some investigators have employed 24-hours fixation of the uterus in Bouin's solution before further processing and weighing. No comparisons of this, either following a standard procedure or comparing weights with and without fixation, have been found.

153. Some investigators, such as Bülbring and Burn (1935), have placed the uterus after trimming in Bouin's solution for approximately 24 hours. After fixation, the uterus was then blotted and weighed. Other examples using fixation are Brownlee (1938), Cheng *et al.* (1953a,b), Shiverick and Muther (1982), Berger *et al.* (1986), Hartmann and co-workers (1980, 1983, 1985, 1986), Jones and Pope (1960), and Kranzfelder *et al.* (1982). There are no reports of side-by-side comparisons of unfixed and fixed uterine weights, and variabilities in the means, to support the proported advantage of the fixation approach. Further, after fixation, it should be specified that the uterine wall be opened and the uterus pressed between blotting paper before weighing. In any proposed use of fixation it is important that wet weights are measured before fixation, and that standardised blotting procedures are followed afterwards.

Oven-Dried Uterine Weights

154. With regard to the use of oven-dried uterine weights as the endpoint for the uterotrophic bioassay, there are very limited data available to compare the merits of such an alternate procedure. Historically, some investigators have measured oven-dried uterine weights as an alternative endpoint for the uterotrophic bioassay. Most often this was done in conjunction with either a wet or blotted uterine weight. Examples: include Ashby and co-workers (1996a, 1997a; 1999b, Odum *et al.* 1999) and others (for example, Clement and Okey, 1972; Desombre *et al.*, 1988; Katzenellenbogen and Ferguson, 1980; Hisaw *et al.*, 1954; Markaverich *et al.*, 1995; Medlock *et al.*, 1995; and Omar *et al.*, 1994, 1996). In general, this has occurred when the oven-dried weights were included with other wet or blotted uterine weights and both values were either not statistically different from controls, or both values were significantly different from controls. In the case of Santell *et al.* (1997), a single case of statistical significance for the measured oven-dried uterine weight was found when the blotted uterine weight was not statistically significant. Overall, however, the data are too limited to show any advantages of measuring oven-dried uterine weights to improve the responsiveness of the assay.

Time of Necropsy

155. An increase in uterine weight is not dependent only on the dose of an oestrogen agonist. There is an expected temporal relationship between the last dose of administration and the timing of when the necropsy is performed and the uterus weighed. This is due to both a lower binding affinity, which might reduce the residence time of the receptor on the oestrogen response element and a faster rate of elimination from the body. This possibility of a temporal 'decay' in uterine weight increase was first raised by Levin and Tyndale (1937), who observed that extending the time of necropsy to ~48 hours resulted in a 60-70% reduction in the uterine weight increase otherwise observed at 20-24 hours.

Table 22. Published absolute uterine weights of immature rat experimental vehicle controls

Uterine wt.	References
Blotted uterine weight (luminal fluid removed)	
< 30 mg	Allen *et al.* (1980)[a], Ashby & Tinwell (1998)[a], Ashby *et al.* (1997a[a,b], 1997b, 1999)[a], Black & Goode (1980)[a,b], Black *et al.* (1983)[a,b,c], Duby *et al.* (1971)[a], Dukes *et al.* (1994)[a,b], Duncan *et al.* (1963), Hammond *et al.* (1979), Hayes *et al.* (1981)[b], Hossaini *et al.* (2000)[a], Jansen at al. (1993)[a,b], Jones *et al.* (1984), Jordan & Gosden (1983)[a], Katzenellenbogen & Ferguson (1980)[a,b], Lan & Katzenellenbogen (1976)[a,b], Lauson *et al.* (1939)[f], Levin *et al.* (1967a,b), Qian & Abul-Haij (1990)[b], Robertson *et al.* (1982)[b], Rosen *et al.* (1980)[e], Routledge *et al.* (1998)[a], Welch *et al.* (1969)[a,f]
30 - 40 mg	Allen *et al.* (1980)[a], Ashby & Tinwell (1998)[a], Ashby *et al.* (1997b[a,b], 1999)[a], Baker *et al.* (1999)[b], Bhavnani *et al.* (1998), Bicknell *et al.* (1995), Duby *et al.* (1971)[a], Ferguson & Katzenellenbogen (1975)[b], Franks *et al.* (1982)[b], Jordan & Gosden (1983)[a], Jordan *et al.* (1977, 1978)[a,b], Larner *et al.* (1985)[a,b], Odum *et al.* (1997), Pento *et al.* (1988)[a,b] Routledge *et al.* (1998)[a] Wenzel & Rosenberg (1956)
> 40 mg	Allen *et al.* (1980)[a,b], Ashby *et al.* (1999)[a], Connor *et al.* (1996), Edery *et al.* (1985) (40.2 mg), Larner *et al.* (1985)[a,b], Ruh *et al.* (1995)
Wet uterine weight (luminal fluid included)	
40 - 50 mg	None found
50 - 60 mg	Bachmann *et al.* (1998)[a], Jordan (1976),
> 60 mg	Bachmann *et al.* (1998)[a], Christian *et al.* (1998), Fail *et al.* (1998)
Uterus preparation unclear	
< 30 mg	Everett *et al.* (1987)[a], Gazit *et al.* (1983), Katzenellenbogen *et al.* (1979)[b], Mirocha *et al.* (1978)[d], Schmidt & Katzenellenbogen (1979)
30 - 40 mg	Gould *et al.* (1998), DeSombre *et al.* (1988)[a,b], di Salle *et al.* (1990)[a,b], Mirocha *et al.* (1978)[d], Raynaud (1973), Safe & Gaido (1998)
> 40 mg	DeSombre *et al.* (1988)[a,b], Cano *et al.* (1986), di Salle *et al.* (1990)[a,b], Mirocha *et al.* (1978)[d], Ostrovsky & Kitts (1962), Whitten *et al.* (1992)

[a] Multiple controls reported in paper, so more than one included; [b] Includes estimate from graph, not tabular data; [c] OVX immature animals; [d] Text states that control uteri ranged between 27 and 40 mg; [e] Text states that control mean of 89 rats from 6 experiments was 29.3 mg; [f] For Levin, controls were 19.6 mg; for Welch *et al.* one control set 18.2 mg and others 20.4 and 20.8, respectively (Tables 1-3 of Welsh *et al.*, 1969).

Notes: Acton *et al.* (1983) state that for 21 day old rats that control uterine weights were 56 ± 4 mg/100 g bw. This would lead to an absolute weight estimate of 25 mg; the vehicle used was Tween 80, and the reported weights appear to be blotted weights. Saeed *et al.* (1990), Sharma *et al.* (1990a,b) appear to be dry weights with uterus ~12 mg. Wakeling and co-workers use a blotting procedure, but report results relative to body weight. If 50-55 g body weights are assumed, then the uterine weights appear to be ~25-30 mg from Figures in these papers.

Table 23. Published absolute uterine weights of OVX young adult and adult rats experimental vehicle controls

Uterine wt.	Reference and Uterine Weight Reported			
	Recovery after OVX ≤ 10 days	**Recovery after OVX 11-18 days**	**Recovery after OVX ≥ 19 days**	**Recovery after OVX not given**
Blotted uterine weight (luminal fluid removed)				
< 100 mg	None found	Ashby *et al.* (1997a), 81.3 ± 9.2 Ashby *et al.* (2000), 4 controls; means 67-71 mg; Karkun & Methrotra (1973b), 66.5 mg Kono *et al.* (1981), ~80 mg from graph. Wakeling *et al.* (1991), ~75 mg from graph	Odum *et al.* (1999), 55-60 mg from graph.[b]	None found
Wet uterine weight (luminal fluid included)				
< 110 mg	None found	Santell *et al.* (1997), 76 ± 3.2 mg, non-OVX group, 386.6 ± 41.1 mg	Laws *et al.* (2000), 4 control groups 92-109 mg Gray *et al.* (1999), ~90-100 mg from graph	None found
> 110 mg	Velardo (1956), 129.4 ± 4.1 mg[a] Velardo (1959), 118 ± 7 mg Velardo & Sturgis (1959b), 115.5 ± 3.3 mg	None found	None found	None found
Uterus preparation unclear				
< 100 mg	None found	Welch *et al.* (1969) 89.4 ± 4.9 mg	None found	None found
> 100 mg	Hisaw *et al.* (1954), ~125 mg from graph	None found	Carthew *et al.* (1999b), 140-150 mg from graph	Gellert *et al.* (1972), 107 mg Jordan (1976), 125 ± 5 mg

[a] Velardo appears to have typically used 100 day old animals at OVX (9 wks); [b] OVX at 4-5 wks (45 days max); [c]; [d]; [e] Text states that control mean of 89 rats from 6 experiments was 29.3 mg; [f] For Levin, controls were 19.6 mg; for Welch *et al.* one control set 18.2 mg and others 20.4 and 20.8, respectively (Tables 1-3 of Welsh *et al.*, 1969).

Table 24. Recorded blotting instructions in experiments reporting blotted uterine weights

Citation	Instructions
Allen *et al.* (1980)	The uterus was pierced and pressed between sheets of blotting paper to remove intra-luminal fluid.
Ashby *et al.* (1997)	Uteri were excised, trimmed free of fat, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries.
Ashby & Tinwell (1998)	Uteri were excised, trimmed free of fat, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries.
Bachmann *et al.* (1998)	Care was taken to avoid loss of any fluid present in the uterus prior to weighing,
Black and Goode (1980)	Uteri were removed, dissected free of extraneous tissue, and fluid contents were expelled.
Brooks *et al.* (1971)	"… and uteri were removed, blotted, and weighed."
Duby *et al.* (1971)	At the termination of each experiment, the uteri were removed and weighed after expressing the luminal fluids and trimming all adnexa.
Grese *et al.* (1997)	"… uteri were removed and dissected free of extraneous tissue, and the fluid contents were expelled before determination of wet weight …"
Hisaw *et al.* (1954)	In those instances when expressed uterine weights were desired, the uteri were removed, weighted, and then nicked with scissors, and the luminal contents gently pressed out on moist paper towelling and reweighed.
Hossaini *et al.* (2000)	Uteri were excised, trimmed free of fat, pierced to remove excess fluid, and subsequently weighed.
Jordan & Gosden (1983)	"Uteri were dissected free of fat, expelled of intra-luminal fluid and weighed immediately …."
Jordan *et al.* (1977)	"… the uteri were dissected out, cleaned of adhering fat, blotted dry and weighed wet on torsion balance."
Lauson *et al.* (1939)	"Weights of the uteri before fluid was expressed and of the vaginas and ovaries were also recorded, but since they have been found to have no particular significance to this paper, they will not be included."

Table 24 (continued). Recorded blotting instructions in experiments reporting blotted uterine weights

Citation	Instructions
Larner *et al.* (1985)	The uteri were removed, dissected free of extraneous tissue, carefully blotted and weighed. Uteri containing fluid were slit longitudinally and the accumulated fluid was removed prior to weighing.
Lundeen *et al.* (1997)	The uteri were removed from the animals, drained of fluid, stripped of remaining fat and mesentery, and weighed.
Nephew *et al.* (2000)	Uteri were removed, luminal fluid was expressed, and the tissue was blotted dry prior to weighing.
Ng *et al.* (1994)	After being trimmed free of fat, each uterus was first weighed with uterine fluid sealed inside the uterine lumen. Then the cervical part was excised, the fluid was drained, and the uteri were blotted before weighing again.
Omar *et al.* (1994)	The animals were then killed and the uteri carefully dissected, blotted, weighed, and dried at 60°C for 24 h and weighed again.
Omar *et al.* (1996)	Animals were then sacrificed, their uteri were excised, cleaned from fats and other tissues and gently squeezed between filter paper. The uteri were weighed, dried at 60°C for 24 h and weighed again.
Ruentiz *et al.* (1983a,b)	The uteri were dissected, and fat and connective tissue were removed. After blotting lightly to remove intra-luminal fluid, the uteri were weighed.
Umberger at al. (1958)	The uteri were removed and weighed after pressing out the luminal fluid on moist filter paper.
Wakeling & Valcaccia (1983)	Uteri were removed, dissected free of adhering fat, blotted dry after the expulsion of uterine fluid and wet weight was recorded.

156. Although the vast majority of published experiments have used a 24-hour time for necropsy, there are a number of experiments that show that time to necropsy is a factor when determining the maximum uterine weight increase.

157. Time courses varying the time of necropsy after the last dose administration have been performed in several instances. The results show that the time of maximum uterine weight increase is test substance-specific. In the case of single doses, a comparison of several substances indicates a maximum response varies from 6 hours for estriol to 36 hours for certain estriol derivatives using the blotted or wet weight (Katzenellenbogen, 1984). Similar data demonstrating that the maximum uterine weight after a single dose is test substance-specific have been generated by Anderson *et al.* (1972) and Jordan *et al.* (1985). In the case of silastic implants with 17β-oestradiol, the maximum uterine weight was not achieved until after 7-10 days (Medlock *et al.*, 1991). Some work has indicated that the uterine weight increase from 'weak' oestrogens may decay at a somewhat faster rate, so that higher weights may be observed at times less than 24 hours.

158. Two recent publications are directly informative. The first is the work of Laws *et al.* (2000) where several chemicals were compared, with necropsies at 6 and 24 hours in the immature rat. Little difference was seen between the 6 and 24-hour time points for four chemicals: 17β-oestradiol, EE, methoxychlor, and nonylphenol. However, the 6 hour time point had higher uterine weights in the case of two other chemicals: octylphenol (OP) and BPA. Interestingly, the substances were administered by oral gavage and the doses in these cases appear to be at the lower portions of the dose response curves for these substances for this route (200 and 400 mg/kg/day for OP, and 100, 200, and 400 mg/kg/d for BPA). The findings of Laws *et al.* (2000) are supported by the recent work of Yamasaki *et al.* (2001). These investigators performed a detailed time course of 6, 12, 18, and 24 hours, using subcutaneous administration of three doses (8, 40, and 160 mg/kg/d). The results show that the responses were significant in all cases at the 40 and 160 mg/kg/d doses. However, the 6-hour response was somewhat higher with both doses, *e.g.*, 53.4 ± 12.3 mg blotted weight at 6 hours, and 39.4 ± 5.1 mg at 24 hours, for the 40 mg/kg/d dose (controls were 27.8 and 27.6 mg, respectively).

159. These data, in conjunction with data on binding affinity and elimination rates, suggest that very weak agonists such as BPA, which are also rapidly cleared, may have a somewhat lower response at 24-hours than at shorter time periods after the last administration.

Statistical Methods

160. Historically, the statistical methods used in the uterotrophic bioassay have not often been adequately described. The most common statistical approach appears to have been Student's t-test, but other methods have been used. No clear criteria have been elaborated in the literature for the use of one statistical approach over another, or the circumstances in which particular methods should be used.

161. In numerous papers extracted from the literature, the descriptions of the statistical methods used are limited or absent. In many cases, the statistical procedures are only briefly noted in table and figure legends. Often, only the criteria for significance are provided, *i.e.*, $P < 0.05$ or $P < 0.01$. Where described, the most common technique noted is Student's t-test, sometimes a one-sided t-test, and sometimes a two-sided t-test. In other literature, the methods are described as an analysis of variance, but without details. Clearly, more robust descriptions of the methods and the rationale for their employment would be useful.

In the first phase of the program (Kanno *et al.*, 2001), the statistics used are described here:

> *The ability of each individual laboratory to detect increased uterine weights at various doses of EE was evaluated by an Analysis of Variance approach, which included body weight as a covariable. As the variability in uterine weights tended to increase in direct proportion to the increase in mean uterus weight, a variance-stabilizing logarithmic transformation was carried out prior to the data analysis. The primary method of statistical analysis for making pairwise comparisons of a dosed group to vehicle controls was Dunnett's test. Dixon's outlier test was used to detect possible outliers in the data, and Bartlett's test was used to assess homogeneity of variances. If significant heterogeneity was detected, the nonparametric Mann-Whitney U test was used to compare dosed groups with the vehicle control group. This latter method of analysis makes no distributional assumptions, but does not readily allow for a quantitative comparison of uterine weight responses among laboratories or the adjustment for possible confounding factors such as body weight.*

Phytoestrogens, Laboratory Diets, and the Uterotrophic bioassay

162. Phytoestrogens are present in several components of laboratory animal diets, particularly in soy products. Phytoestrogens are ligands for the ER, and therefore would be expected to elicit positive responses in uterotrophic bioassays. They have, on occasion, elicited reproductive and developmental effects in livestock. The phytoestrogen content of natural foodstuffs and forage is highly variable. The chemicals present, and their levels, depend upon plant species, the growing season, and the preparation of plant or its derivative. This raises the possibility that such natural components in laboratory diets may influence the baseline weights of control uteri. Significantly elevated baseline weights could then reduce the responsiveness of the uterotrophic bioassay. The literature suggests that phytoestrogen levels ≥300-350 μg genistein, or its equivalent, per gram in the diet may increase baseline uterine weights in control animals.

163. Phytoestrogens and mycoestrogens have led to reproductive and developmental effects in livestock, including spontaneous abortion and both male and female infertility (Stob, 1983). The early history of the their discovery as causative agents of reproductive failure, and the revelation that these effects were oestrogen-mediated, is reviewed by Moule (1961). The toxicology and history of phytoestrogens has been reviewed on several occasions (Kaladas and Hughes, 1989; Livingston, 1978; Price and Fenwick, 1985; Stob, 1983; Whitten and Patisaul, 2001). As natural phytoestrogens and mycoestrogens are common constituents and contaminants of laboratory animal diets, the concern arises about their negative impact on the performance of the uterotrophic bioassay.

164. The uterotrophic bioassay has been used to isolate oestrogen fractions of forage plants and other foodstuffs, (*cf.* Cheng *et al.,* 1953a,b; Legg *et al.,* 1951). In these cases, extracts of soy, clover, and alfalfa gave clear, positive uterotrophic responses, often when the mouse was used as the test species. The active substances were later isolated and identified as genistein, coumestrol, daidzein, biochanin A, and other substances. A wide range of phytoestrogen levels were found in different plant species and strains, times during the growing season, treatment and storage time of the forage, and extraction and handling procedures (*cf.* Bartlett *et al.,* 1948; Bickoff *et al.,* 1959; Cheng *et al.,* 1953a; Ostrovsky and Kitts, 1963).

165. Independently, other investigators noticed sporadic changes in the control uterine weights of rodent colonies. These changes were attributed to new lots of the laboratory diet. Huggins *et al.* (1954) stated: "During preliminary experiments it was found that rations from two commercial sources induced

oestrus prematurely in adolescent rats, so that these foods could not be used." Zarrow *et al.* (1953) reported that OVX mice showed vaginal cornification and a number of mice out of a group of 100 showed uterine weights in excess of 40 mg. In contrast, typical blotted uterine control weights in that laboratory were 10-14 mg. This led them to suspect, and to experimentally test for, oestrogenic contamination of the laboratory diets. Their experiments confirmed that marked uterine weight increases were associated with extracts of the laboratory diets. Drane and co-workers (1975, 1980) provided evidence that particular dietary lots could nearly double the uterine weights of mice, and indicated that the source of the activity appeared to be the soy content of the diet.

166. Recent work on the influence of laboratory diets on the uterotrophic bioassay began with Thigpen *et al.* (1987b). During efforts to standardise a mouse uterotrophic bioassay, the impact of laboratory diets on uterine weights was noticed. Their data clearly indicated that different diets can influence the baseline uterine weights (**Table 25**).

167. With the application of the uterotrophic bioassay to weak oestrogen agonists and antagonists, the need for a high level of responsiveness for the assay has also re-emphasised concerns about dietary phytoestrogens. Boettger-Tong *et al.* (1998) reported a case where apparent dietary phytoestrogens led to increased uterine weights and changes in the uterine and vaginal histology of immature mice. **Table 26** summarises experiments with reported levels of genistein and daidzein.

168. Thigpen *et al.* (1999b) performed more detailed, analytical work of purified, open and closed, formula rodent diets. Their new data clearly show wide variations in analytical levels of phytoestrogens in laboratory diets (**Table 27)**.

169. In other work, Odum *et al.* (1997), after observing moderately elevated uterine weights in immature, intact rats (blotted uterine weights of 30-35 mg), experimented with several diets before choosing a specific diet for lactating and foster dams, and a second specific diet for pups after weaning. The new diets reduced blotted uterine weights of control animals to a 20-25 mg range with cervix excluded. Odum *et al.* (1997) concluded that "… the data underline that diet should be considered as an important variable … and that care should be taken to specify its source and constitution."

170. The outstanding question is whether there are dietary-induced changes in uterine weights that impair the responsiveness of the uterotrophic bioassay to detect weak oestrogen agonists. There are three recent papers relating uterine weight increases and the genistein content of specific synthetic of diets: Fritz *et al.* (1998), Casanova *et al.* (1999), and Santell *et al.* (1997). The data from these publications are combined in **Table 28**.

171. The work of Fritz *et al.* (1998) indicates that moderate levels of genistein do not increase the uterine weights of immature or young adult rats at 25 and 250 μg/g genistein in the diet. The same workers analyzed genistein in the serum and dam's milk. Modest changes in mammary terminal ductal structures were further investigated by measuring cell proliferation, which was negative at both doses of genistein. Reproductive success, anogenital distance in offspring, testes descent, and vaginal opening were also unaffected at both doses.

172. The work of Casanova *et al.* (1999) focused on moderate levels of genistein in the diet (200 μg/g diet). These levels did not increase the uterine weights of immature rats at pnd 21 when 1) the dams consumed the diet during pregnancy and lactation, 2) the diet was available to the pups before weaning, and 3) the same diet was provided to the pups after weaning. However, at 1000 μg/g (1000 ppm) of genistein, a definite increase in uterine weight was recorded; no other effects on either female or male pups were observed. The authors concluded that the levels of phytoestrogens in diets appeared to be in a borderline range just below the minimum effective level for increasing uterine weights. They did not

conclude that phytoestrogen-containing diets (NIH-07) should be avoided or replaced. Rather, they recommended, as did Thigpen *et al.* (1999), that the diets used be monitored for phytoestrogen levels and, if necessary, the levels be reduced.

173. The work of Santell *et al.* (1997) supports these data with similar observations. Clear statistical significance in the uterine weight increases was observed at dietary genistein levels of 375 and 750 μg/g. At the level of 150 μg/g, an absolute increase in the mean of the blotted uterine weight of about 21% was observed, but the increase was not statistically significant in these experiments. However, the oven-dry uterine weights from the 150 μg/g genistein diet were significantly different from the controls.

174. When these experiments are combined, the data indeed suggest that 250-300 μg/g dietary genistein is the region at which the rat uterus might first begin to respond (the cell proliferation of other tissues than the uterus, and other toxicological endpoints, were negative at these levels (Fritz *et al.*, 1998)). As is shown in **Tables 22** and **23**, there is little evidence for any major or consistent influence of laboratory diet on the uterine weights of rats and mice in the majority of well documented experiments. These observations are consistent with the dose-response data from the above experiments. Phytoestrogen levels in the diet of <250 ppm or μg/g as genistein or its equivalent should not affect the baseline uterine weight, and additional phytoestrogens increase this level.

175. In addition to the importance of knowing the phytoestrogen content, it is also important to know the specific laboratory diet used, and few investigators have recorded the laboratory diet that was used in their uterotrophic experiments (**Table 29**). Few researchers have either investigated or carefully selected their diets based on experimental data, as did Odum *et al.* (1997). No laboratory has reported that they periodically analyzed the diet used or that they received an analysis from the laboratory diet supplier.

176. In conclusion, infrequent lots of laboratory diet may contain sufficient phytoestrogen to raise the baseline weights of uterus in rats and mice. Such an increase in the baseline control weights would incrementally reduce the responsiveness of the test, primarily affecting detection in the lower portion of the substance's dose-response curve. There is, however, no evidence for a pervasive problem. With modest changes in laboratory diet, Ashby's laboratory reduced the immature control weights in rats about 10 mg and has successfully used the uterotrophic bioassay to detect very weak agonists such as BPA, nonylphenol, and others. This demonstrates an ability to detect weak oestrogen agonists even while using plant-derived diets. Limited data in the literature suggest that phytoestrogen levels equivalent to greater than 300-350 μg per gram in the diet may increase baseline uterine weights in control animals. Any such increase will be continuous and gradual. This suggests monitoring of control values versus historical data trends, and periodic analyses of the laboratory diet may be advisable.

177. In closing, the matter of increases in uterine weights from laboratory diets may not be solely a matter of phytoestrogen levels. A series of experiments with various diets based on various plant components (soy - RM3, RM1, 5001, and cereals - Global), synthetic diet (AIN-76A), and various infant formulas both with and without soy constituents have been conducted (Ashby *et al.*, 1999a, 2000; Odum *et al.*, 2001). Uterine, vaginal, and cervical weights, as well as developmental landmarks such as vaginal opening and day of first oestrus were measured. **Table 30** shows the effect of different diets on female tissue weights. The column on the left contains the results of the diet fed to the dams while nursing, and the results of feeding the diet after weaning is on the right. Changes in the weights of the uterus, vagina, or cervix, and the timing of developmental landmarks were observed, but did not correlate with phytoestrogen levels. Increased body weights were indicated as a factor, and, interestingly, Antarelix a GnRH inhibitor, effectively reduced the uterine weights in all cases. This supports a GnRH-related mechanism that would stimulate early puberty and oestrogen production by the ovary, rather than a direct oestrogenic action.

Table 25. Influence of diet on uterine weights of untreated, immature mice (from Thigpen *et al.*, 1987b)

	Added DES (ppb)	Uterine wt (mg) after days on diet		
		3	5	7
Certified Rodent Chow #5002	0	8.2 ± 2.7	11.7 ± 4.1	13.8 ± 6.8
Certified Rodent Chow #5002	6	15.0 ± 4.0	17.3 ± 6.4	22.1 ± 9.9
Rodent Laboratory Chow #5001	0	6.9 ± 1.0	8.2 ± 3.6	10.0 ± 2.9
Mouse Chow #5015	0	12.5 ± 3.1	16.4 ± 4.4	22.2 ± 5.7

Table 26. Dietary analyses for genistein and daidzein content

Diet	Genistein (ppm or μg/g)	Daidzein (ppm or μg/g)	Reference
Artificial - undosed	non-detect [a]	non-detect [a]	Casanova *et al.* (1999)
Artificial – dosed	200 & 1000		Casanova *et al.* (1999)
NIH-07	160	144	Casanova *et al.* (1999)
Unknown	210	140	Boettger-Tong *et al.* (1998)
AIN-76 or AIN-93G	150, 375, 750[b]	Not applicable	Santell *et al.* (1997)
Purina Lab Chow #5001	214	277	Thigpen *et al.* (1999a)
Purina Mouse Chow #5015	97	130	Thigpen *et al.* (1999a)
NIH-07	104	124	Thigpen *et al.* (1999a)
NIH 31	30	20	Holder *et al.* (1999)

[a] Detection limits were stated as approximately 1 ppm in diet in the paper's methods.
[b] Animals were also fed 17β-oestradiol in the diet at 0.5, 1 and 1.5 ppm.

Table 27. Phytoestrogen content of purified, open, and closed-formula rodent diets (from Thigpen *et al.*, 1999b)

Rodent Diet	No. of batches	Soybean meal or soy-protein (%)	Daidzein (µg/g)	Genistein (µg/g)	Total daidzein & genistein[1] (µg/g)	DES Equival.[2] (µg/g)	Estrone Equival.[3] (µg/g)	Potential daily intake of daidzein and genistein	
								Mouse[4] (mg)	Rat[5] (mg)
Closed-formula, natural-ingredient									
PMI No. 5001	1	*	277	214	491	4.3	62.3	2.5	7.4
PMI No. 5002	2	*	86	73	159	1.4	20.3	0.8	2.4
PMI No. 5015	1	*	130	97	227	2.0	28.7	1.1	3.4
PMI No. 5058	4	*	80	71	151	1.3	19.4	0.8	2.3
HSDTeklad 7012 LM485	1	*	126	134	260	2.3	33.8	1.3	3.9
Open-formula, natural-ingredient									
NIH31	4	5.0	48	46	94	0.8	12.1	0.5	1.4
NIH07	4	12.0	89	77	166	1.5	21.3	0.8	2.5
NTP88	1	5.0	38	31	69	0.6	8.8	0.4	1.0
NTP2000	4	5.0	53	58	111	1.0	14.5	0.6	1.7
Open-formula purified									
AIN76A (casein)	2		ND	ND	ND	ND	ND	ND	ND
AIN76A (soy)	1	20[6]	74	137	211	2.0	28.6	1.1	3.2
AIN93M	1		ND	ND	ND	ND	ND	ND	ND
Soy flakes (control)	1		697	785	1,482	13.4	193.8	7.4	22.2

*Closed-formula diet: soybeans present, percentage not reported. ND = not detectable (limit of detection, 5 µg/g).

[1] Total genistein and daidzein (ppm of whole diet); 11 mg of daidzein is equivalent to 8 mg of genistein. [2] 18 mg of genistein is equivalent to 0.083 µg of diethylstilbestrol (DES). [3] 8 mg ofgenistein is equivalent to 1.20 µg of estrone activity. [4] Assumes an adult mouse consumes 5.0 g of diet/day. [5] Assumes an adult rat consumes 15.0 g of diet/day. [6] Pure isolated soy protein.

Table 28. Uterine weight data compared with the dietary levels of genistein and specific synthetic formulated diets.

Investigator and Experimental Group	Dietary content of genistein	Uterine weights (mg)
Fritz *et al.* (1998)		
AIN-76A	Non-detectable	22 ± 2
AIN-76A 25 ppm genistein	25 μg/g genistein	20 ± 1
AIN-76A 250 ppm genistein	250 μg/g genistein	25 ± 1
Casanova *et al.* (1999)		
Soy and alfalfa free diet (SAFD)	Non-detectable (~1 μg/g of diet det. limit)	26.9 ± 1.3
SAFD 200 ppm genistein	200 μg/g genistein	24.2 ± 60.6
SAFD 1000 ppm genistein	1000 μg/g genistein	60.6 ± 5.2
NIH-07	160 μg/g genistein & 144 μg/g diadzein [a]	27.4 ± 0.7
Santell *et al.* (1997)		
Control	Non-detectable	76.5 ± 3.2
Genistein Diet 1	150 μg/g genistein	92.4 ± 2.6
Genistein Diet 2	375 μg/g genistein	135.6 ± 9.8
Genistein Diet 3	750 μg/g genistein	189.3 ± 26.6

[a] Other phytoestrogens were not analyzed

Table 29. Types of diets reported in the literature for the uterotropic assay

Diet not specified	Allen *et al.* (1980), Bhavnani *et al.* (1998), Bicknell *et al.* (1995), Black & Goode (1980), Black *et al.* (1983), Cano *et al.* (1986), Connor *et al.* (1996), di Salle *et al.* (1990), Dukes *et al.* (1994), Edery *et al.* (1985), Everett *et al.* (1987), Franks *et al.* (1982), Gazit *et al.* (1983), Gould *et al.* (1998), Hammond *et al.* (1979), Jansen at al. (1993), Jones *et al.* (1984), Jordan (1976), Jordan & Gosden (1983), Jordan *et al.* (1977, 1978); Katzenellenbogen & Ferguson (1980), Katzenellenbogen *et al.* (1979), Lan & Katzenellenbogen (1976), Larner *et al.* (1985), Lauson *et al.* (1939), Li & Hansen (1995), Qian and Abul-Haij (1990), Raynaud (1973), Ruh *et al.* (1995), Safe & Gaido (1998), Schmidt & Katzenellenbogen (1979), Wenzel & Rosenberg (1956)
R&M No. 3 weaning (18-21 pnd), R&M No. 3 after	Ashby & Tinwell (1998), Ashby *et al.* (1997 a,b [b], 1999), Odum *et al.* (1997) [b], Routledge *et al.* (1998)
PCD or Harlan Tekald TRM	Ashby *et al.* (1997b) [b], Baker *et al.* (1999); Odum *et al.* (1997) [A]
Kilba maintenance diet 24-343-3	Bachmann *et al.* (1998), Christian *et al.* (1998)
Purina laboratory diet or chow	Duncan *et al.* (1963), Hayes *et al.* (1981), Levin *et al.* (1967, 1968b)
Purina #5002	Fail *et al.* (1998),
AO-4 chow	Galand *et al.* (1987)
Rockland animal diet	Levin *et al.* (1968a)
AIN diet and CHW chow	Whitten *et al.* (1992)

[b] May be more than one diet used in study.

Table 30. Effects of different diets on female tissue weights and development (Ashby *et al*., 2001)

Parameter	**RM3**	**RM1**	**AIN-76A**	**AIN-76A**	**RM3**	**AIN-76A**	**Global**	**Global**	**5001**	**5001**
Approximate Dietary analysis (μg phytoestrogen/g diet)										
Diadzein	65	30	Nd		65	nd	nd		110	
Genistein	105	45	Nd		105	nd	nd		170	
Pnd 26 (weights in mg)										
Blotted uterus	21.8 ± 3.86		30.4 ± 3.8**		43.2 ± 5.6**		24.9 ± 3.8		42.4 ± 4.2**	
Vagina	25.2 ± 4.0		30.0 ± 2.3		38.9 ± 3.5**		25.8 ± 5.6		35.6 ± 3.2**	
Cervix	6.7 ± 2.53		7.7 ± 0.4		10.1 ± 1.3**		5.9 ± 0.6		9.2 ± 1.3**	
Ovaries	31.4 ± 2.4		33.4 ± 2.9		36.4 ± 2.4**		29.2 ± 2.0		35.6 ± 3.8*	
Developmental landmarks										
Age at onset of VO (days)	34.9 ± 1.5		32.3 ± 0.7**		31.3 ± 0.5**		34.5 ± 1.8		33.8 ± 0.8	
Body wt at VO Completion (g)	111.2 ± 10.5		104.2 ± 6.8		97.4 ± 5.3**		99.0 ± 6.9*		110.8 ± 5.8	
Day of 1st oestrus	39.2 ± 2.6		37.5 ± 2.4		34.7 ± 2.2**		38.2 ± 2.0		36.1 ± 1.3*	

* $P < 0.05$, ** $P < 0.01$

CHAPTER 5: PERFORMANCE CHARACTERISTICS OF THE UTEROTROPHIC BIOASSAY

178. This section addresses issues regarding the performance characteristics of the uterotrophic bioassay. In validation terms, this involves the consideration of the relevance of a protocol, in terms of 1) the reproducibility of assay results over time and among laboratories when using the same protocol and test substance, and 2) the assay specificity, *i.e.*, whether assay will correctly identify a substance as positive or a negative (OECD, 1996, 1998a; ICCVAM, 1997). For the uterotrophic bioassay, this means the correct identification of oestrogen agonists and antagonists. The following points are addressed:

- The evidence to support the reproducibility of the uterotrophic bioassay within and among laboratories;
- The evidence to support the specificity of the uterotrophic bioassay;
- How the use of the animals can be reduced in the uterotrophic bioassay itself and in the use of the assay in an overall chemical testing and assessment strategy;
- The possible limitations in the use of the uterotrophic bioassay to test substances; and
- The techniques currently under investigation that could improve the procedures for the assay.

Reproducibility of the Uterotrophic bioassay Within and Among Laboratories

179. Although there are conflicting reports that some test whether substances are false negative or false positive in the uterotrophic bioassay, in most cases, these occurrences involve assay results 1) where the uterine weight increases were small (15-40%), or 2) at the very lower end of the dose response curve where the uncertainty and lack of reproducibility would be expected to be the greatest. In one exception, the control weights of the animals in the original positive report were well outside the typical range for mice.

180. These reports indicate several important points: 1) a standardised protocol is necessary, 2) a low frequency of false positives will be more likely to occur when there is a relatively small increase in uterine weights (15-40% uterine weight increases), and 3) results in the lower portion of the dose response curve may be susceptible to greater variation.

181. The evidence of a test method's reliability involves establishing the reproducibility of the results from the test among laboratories over time (Balls *et al.*, 1990, 1995; OECD, 1998; ICCVAM, 1997). Reproducibility is defined as the variability between single test results obtained in a single laboratory (intra-laboratory reproducibility) or in different laboratories (inter-laboratory reproducibility) when using the same protocol and test samples (ICCVAM, 1997).

182. Where repeat experiments have been reported in the literature, the overwhelming majority of the experiments have indicated that the results of the uterotrophic bioassay can be replicated both within and among laboratories. The most extensive use of the uterotrophic bioassay has been by the US Public Health Service in the 1950s and 1960s, when 745 steroidal and 360 non-steroidal substances were analyzed. The reference oestrogen was estrone. Over a period of eight years, 319 dose-response replicates were run via the subcutaneous route, and 215 dose-response replicates were run via the oral gavage route, all with estrone. These data are reported in Hilgar and Palmore (1968). The combined data

for estrone for this study are reproduced in **Figure 10** (subcutaneous) and **Figure 11** (oral gavage) respectively.

183. In addition, control data for estrone, including vehicle controls for the mice, were reported at all subcontracting laboratories for both dose routes. These data show that interlaboratory variability was comparable. The variability in the vehicle control data suggests that a low frequency of false positives will occur. That is, a comparison of sets with the highest values of control uterine weight means can yield a statistically significant difference from the control means with the lowest values. Both vehicle and estrone dose data that was considered high or low are marked in these tables and are not infrequent (see pages 5-9 of Hilgar and Palmore, 1968). The differences between the highest and the lowest value data sets for the vehicle controls would yield an apparent difference of 15-40% over the low values. In addition to the variability in the control baseline, it is also worthwhile to note that the dose-response data shown in **Figures 10 and 11** begin with doses that are 50-100% greater than the typical range of the vehicle control weights. Variability at the lowest region of the dose response curve was not characterised. Also, statistical significance is not an absolute guarantee that the observations are different. For example, at a 95% confidence level, there remains a 5% chance (beta risk) that the results are not actually different.

184. There also have been some recent reports questioning the reproducibility of the results from the uterotrophic bioassay with weak ER agonists. An example is the case of resveratrol, a constituent of red wine (Flynn *et al.*, 2000). First, Ashby *et al.* (1999a) observed that resveratrol was a weak oestrogen agonist for the ER ($IC_{50} \sim 10^{-4}$ M), a weak positive in a yeast transactivation assay ($>10^{-4}$ M), but did not consistently result in a significant increase in uterine weight by s.c. or p.o. administration in a series of uterotrophic experiments with doses up to 120 mg/kg/d. Second, Freyberger *et al.* (2001) tested resveratrol at higher doses of 18, 58, and 575 mg/kg/d using s.c. administration. While modest decreases in the levels of ER were observed in uterine tissues, no treatment related effects were observed in uterine histology or uterine weight. In fact, the trend was towards reduced, not increased, uterine weights.

185. There are also reported difficulties in reproducing data within a single laboratory. Tinwell *et al.* (2000) performed a total of eight individual experiments with bisphenol A administered by subcutaneous injection in the mouse. Four experiments were negative at 200 mg/kg/d (one adding a dose at 300 mg/kg/d was also negative); another experiment was positive at a level of $p<0.05$ at 200 mg/kg/d; and three experiments were positive at 0.01 at 200 mg/kg/d. The relative uterine weight increases in those three instances were 24, 25, and 37%. There also were two instances of isolated positives in a dose-response series, one at 200 µg/kg/d and one at 5 mg/kg/d (with uterine weight increases of 27 and 34%), which were negative in other experiments, indicating that 1) the doses of Tinwell *et al.* may have been in the very lower part of the dose response curve, and 2) that clear, robust, and consistent responses can be achieved in the ascending and upper portions of the dose response curve. Similarly, Matthews *et al.* (2001) observed in the rat a 21% increase at 100 mg/kg/d, which was not statistically significant, and a 117% increase at 800 mg/kg/d which was significant.

186. The experiments of Lemini *et al.* (1995, 1997) reported uterotrophic activity for benzoic acid (BA) and *p*-hydroxybenzoic acid (HBA) in immature mice. These data are unique in two respects, the percentage increase over controls was 60-70% for both chemicals, and the mean weights of blotted uteri for the vehicle controls were ~30 mg (BA) and 38 ± 2.2 mg (HBA). In regards to the control uterine weights, the literature mean is 10-12 mg for the blotted uterus in mice (Annex -**Table D**), and no other laboratory has reported so high a blotted uterine weight in immature mice. The positive results for these chemicals have not been reproduced in two other independent laboratories. Both BA and HBA were negative in *in vitro* reporter assays (Ashby *et al.*, 1997b; Routledge *et al.*, 1998). Also, the uterotrophic results were negative for BA (Ashby *et al.*, 1997b) and HBA (Hossaini *et al.*, 2000) in both rats and mice at doses equal to, or higher than, used in the original experiment.

187. In conclusion, as with all bioassays, the uterotrophic data has inherent variability, resulting in difficulty in reproducing results in several situations. First, there may be occasional false positives, which are characterised by weak responses of 15-40% due to group-to-group variability in the mean uterine weights. Several authors have reported instances of isolated doses that were significantly different, such as those of Tinwell *et al.* (2000b), who saw isolated instances of positives at 200 µg/kg/d and at 5 mg/kg/d BPA in a total of eight dose-response experiments. Second, weak uterine responses that would be expected in the lower portion of the dose response curve may be more likely to be non-reproducible. This suggests that that preliminary range-finding experiments, or other procedures to select suitable test doses, and procedures for addressing suspect data, are necessary. Third, the data of Lemini *et al.* (1995, 1997) also suggest the need to consider and to define data acceptance criteria, such as a maximum uterine control weight of 45-50 mg in the immature rat, and 16-18 mg in the immature mouse.

Specificity of the Uterotrophic bioassay

188. Positive results with uterotrophic bioassay are not absolutely definitive that a substance is an oestrogen agonist or antagonist. Positive uterotrophic results consistently occur with some non-oestrogens, *e.g.*, androgens, progestins, and other growth factors have induced uterine weight increases. At the molecular level, the plausibility of non-oestrogen pathways is supported by the presence of several promoter elements in addition to ER elements upstream of 'oestrogen-responsive' genes. In order to improve the assay's overall specificity, three strategies have been suggested: 1) screen candidate substances by performing precursor assays, such as ER binding; 2) provide for complementary and confirmatory metrics that can be concurrently acquired, such as histological changes in the vaginal or uterine epithelium; or 3) use both strategies.

189. *Specificity* is defined as the proportion of inactive substances that are correctly identified as inactive (ICCVAM, 1997). Those incorrectly identified are false positives. For the uterotrophic bioassay, the question of specificity becomes: Do substances other than direct oestrogen agonists and antagonists increase uterine weight? If so, can these substances be identified in advance? Are there complementary endpoints that can be used concurrently to identify these false positives without duplication of the experiment?

Figure 10. Data from a large study by the U.S. Public Health Service showing mean uterine weights in mice from vehicle control groups over a four year period, measured following s.c. administration.

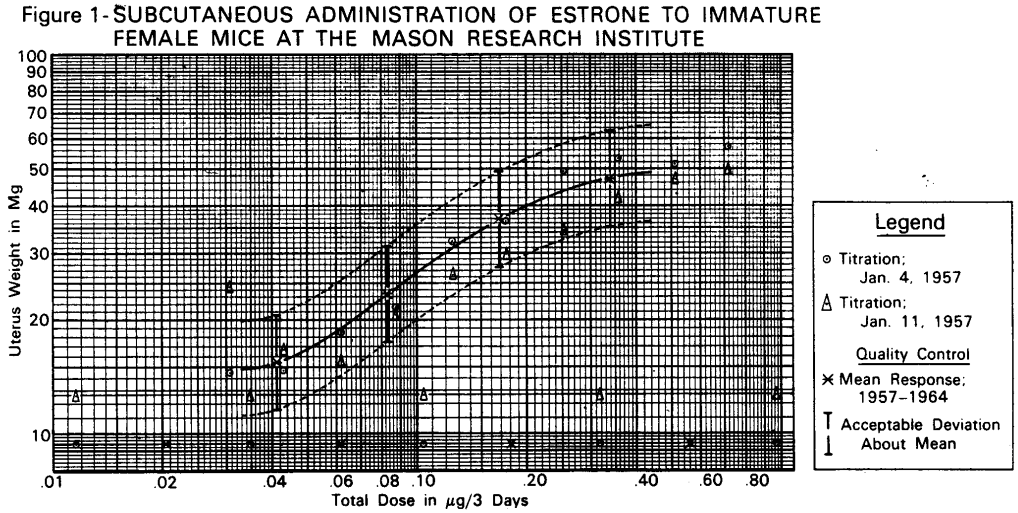
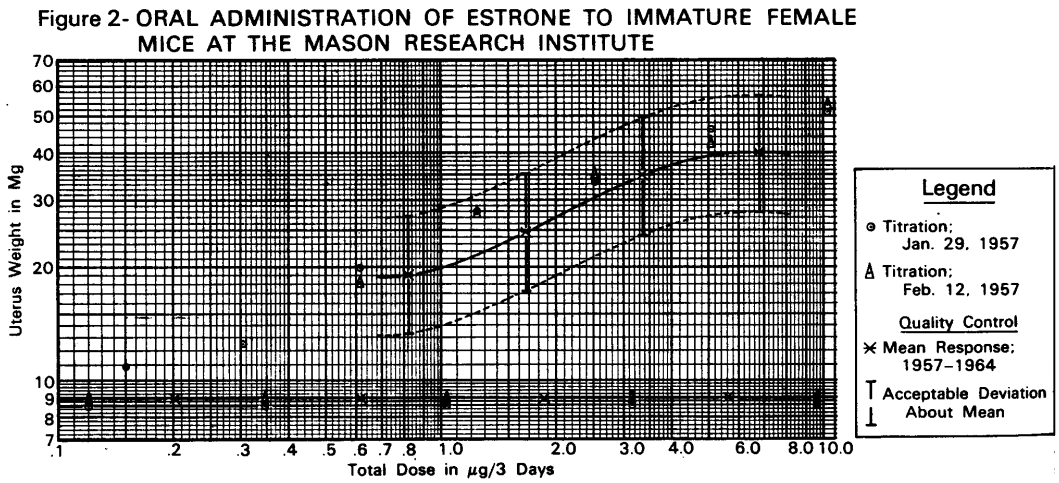


Figure 11. Data from a large study by the U.S. Public Health Service showing mean uterine weights in mice from vehicle control groups measured over a four-year period following administration of vehicle by oral gavage.



190. The uterotrophic bioassay is not always definitive for oestrogen agonists and antagonists, and may reflect hypertrophy (or suppression of hypertrophy in the case of antagonists) not associated with the stimulation of cell division via the ER. In a review of the uterotrophic bioassay, Kupfer (1988) quoted Lerner *et al.* (1958) to emphasise caution on this subject: "Uterotrophic and estrogenic should not be used interchangeably." Reel *et al.* (1996) and Gray *et al.* (1997), in their reviews, have likewise emphasised the fact that the uterotrophic bioassay is not absolutely definitive for oestrogen agonists and antagonists.

Androgens and progestins as well as oestrogens increase uterine weight. MER 25[12] is anti-oestrogenic when 17β-oestradiol is used as the stimulatory hormone, but not when testosterone is used." Androgens and progestins at high concentrations have been found to increase uterine weights in several laboratories over time (*cf.* Edgren and Calhoun, 1961; Jones and Edgren, 1973; Velardo, 1959). The evidence that epidermal growth factor has also found to increase uterine weight (Mukku and Stancel, 1985; Gardner *et al.,* 1989; Nelson *et al.,* 1991), is discussed above, together with the fact that multiple signalling pathways are able to modulate the oestrogen-mediated mitotic stimulus to the uterus.

191. Given the evolutionary similarity among the steroid nuclear receptors, it is not surprising that ligands of one receptor would possibly activate another receptor at high doses. In fact, the quantities of androgens and progestins that elicit a positive response far exceed physiological concentrations. A parallel issue for other substances would be the administration of excessive doses without consideration of exposure. The plausibility for other non-oestrogen pathways leading to a positive uterotrophic response is supported by the presence of several promoter elements, in addition to ER elements, upstream of 'oestrogen-responsive' genes (*cf.* lactoferrin; Liu and Teng, 1992; Shi and Teng, 1994).

192. In case of a positive uterotrophic result, Kufper (1988), Gray *et al.* (1997), and Reel *et al.* (1996) have suggested strategies to address the non-specific nature of the uterotrophic bioassay. The first strategy involves a hierarchical approach, where candidate substances are pre-screened based on structure-activity relationships, and precursor *in vitro* assays are performed in an initial tier prior to the uterotrophic bioassay. ER-binding assays and transcription of reporter genes mediated by the ER were suggested as *in vitro* assays. Such an approach would be expected to significantly reduce the number of substances entering the uterotrophic bioassay and should, by pre-screening substances, effectively reduce the number of false positives in the assay. The substantial reduction in animal use is also apparent from this strategy.

193. The second strategy involves substantiation or confirmation of oestrogen-mediated activity by the addition of additional endpoints to the uterotrophic bioassay that are more specific for oestrogens, but somewhat less sensitive, *e.g.,* vaginal cornification was suggested by Reel *et al.* (1996). The additional endpoint(s) would verify that the observed uterine weight increase is an oestrogenic response to the test substance. Other possible confirmatory endpoints that could be investigated with preserved tissue include oestrogen molecular markers such as lactoferrin, examination of the morphology of the uterine epithelia, measurement of the age of vaginal opening, and length of estrous cycle. Laws *et al.* (2000) compared the measurement of increased uterine weight with several additional measures of possible oestrogenic activity: acceleration of the age of vaginal opening, induction of cornified vaginal epithelial cells, and extension of the time to complete the estrous cycle. As noted previously, this work included several weak oestrogen agonists. Overall, the measurement of uterine weight increase (uterotrophic bioassay) was the most consistent and sensitive method in their laboratory, and the vaginal cornification assay suggested by Reel *et al.* (1996) was relatively insensitive for the weak oestrogen agonists.

194. A third strategy may be to have a separate assay that could address questionable results. One example is the system of Cotroneo and Lamartiniere (2001) who conducted experiments using Sprague-Dawley rats, and used a set of oestrogenic responses based on endometriosis implants. Their data, while promising, was insufficient at this stage to recommend the assay. Its relative complexity compared to the uterotrophic bioassay would, however, suggest that it might be used to confirm or to refute the results of equivocal uterotrophic data.

[12] MER 25 was one of the original anti-estrogenic chemicals to be discovered. Its inability to inhibit the action of androgen was interpreted to indicate that a pathway other than involving 17β-estradiol was active (this was also before the discovery of the ER).

195. These data suggest that results of the uterotrophic bioassay, particularly modest increases in uterine weight, should be interpreted carefully using a weight-of-the-evidence approach. In any testing strategy for detecting potential endocrine disrupters, guidance of when to use additional tests and/or endpoints will be needed.

Numbers of Animals Needed for the Assay

196. The uterotrophic bioassay appears to be efficient in the use of animals and can be successfully conducted with as few as six animals per dose group. The use of animals would be substantially reduced by assessing the structures of unknown substances as possible ligands for the ER or conducting *in vitro* screens, *e.g.*, receptor-binding affinity or *in vitro* assays responsive to oestrogen ligands. In addition to assisting the interpretation of results, as suggested above, this would also help reduce the number of animals used in any overall testing strategy that includes the uterotrophic bioassay.

Possible Limitations in the Use of the Uterotrophic bioassay

197. The uterotrophic bioassay requires that test substances be relatively stable in aqueous and solvent environments that are used in vehicles, and within the test animal. Results for reactive or unstable substances may be either of limited usefulness or validity. No reports in the literature were retrieved addressing this issue.

Possible Future Improvements in the Assay

198. Biological assays can always be improved over time as more experience is gained using a standardised protocol. In the case of the uterotrophic bioassay, the application of specific pituitary and gonadal antagonists are under investigation. These techniques present the possibility to suppress the limited background production of endogenous oestrogens and the surge in oestrogen production as individuals enter into early puberty. The techniques would, therefore, reduce baseline uterotrophic weights in immature and possibly OVX young adults, increasing the responsiveness of the assay.

199. Ashby *et al.* (2000) used three selective antagonists to challenge the uterotrophic activities of several dietary formulas: 1) the peptide antarelix which inhibits GnRH secretion from the hypothalamus; 2) anastrazole, which inhibits the conversion of the precursor androgens to oestradiol; and 3) faslodex, which is a receptor antagonist of oestradiol. The first two substances inhibit ovarian production of endogenous oestrogens. Both the GnRH inhibitor and the aromatase inhibitor successfully reduced the baseline uterotrophic weights of immature control animals on several dietary formulas, both with and without plant constituents. Although biologically plausible, these techniques have not yet been applied to animals receiving test doses of weak oestrogens. Therefore, the utility and benefit of these techniques to increase the responsiveness of the assay in conjunction with test substances has not yet been demonstrated.

CHAPTER 6: PERFORMANCE OF THE ASSAY

200. This chapter describes the pharmacodynamics and the pharmacokinetics that are likely to affect the activity of oestrogens and weak oestrogen agonists administered in the uterotrophic bioassay, and to present the available data on selected substances. It also makes some initial proposals for the use of the uterotrophic bioassay for the evaluation of chemicals. The following issues are discussed:

- The basis for the pharmacodynamics and the pharmacokinetics of weak oestrogen agonists. How these would be expected to affect the uterotrophic bioassay procedures and results.

- Whether the available is *in vitro* data are in agreement with previously generated uterotrophic data in the literature, and whether they support the ability of these *in vitro* tests to be useful before running the uterotrophic bioassay.

- What available toxicological data from longer term bioassays that can be used to evaluate whether additional reproductive and/or developmental toxicity testing is needed.

- The possible use of the uterotrophic bioassay in the evaluation of chemicals.

Pharmacodynamics and Pharmacokinetics

201. A variety of pharmacodynamic and pharmacokinetic factors need to be considered when evaluating the likely results from testing in the uterotrophic bioassay. This subchapter summarises the range of possible metabolic and other events that affect the free concentration of the ligand in the serum and, thus, the potential activity of that ligand in the uterotrophic bioassay.

202. Pharmacokinetics includes an understanding of how an external, administered dose is transformed into the substance's concentration that will give a biological response or toxicological effect at the internal target site. In the case of the uterotrophic bioassay, the target site is uterine tissue, and the concentration of interest is the intracellular ER ligand concentration. This is consistent with previous sections of this report which have described the roles of binding and activation of the ER in initiating a cascade of events leading an increase in uterine weight. An important determinant of how a given substance responds in the uterotrophic bioassay should then involve its pharmacokinetic and pharmacodynamic characteristics. The physiological functions of absorption, metabolism, distribution, and excretion (ADME) will modify the extent of free ligand, the strength of binding, and consequently the potency of a substance.

203. There are many important pharmacodynamic and pharmacokinetic factors that affect the activity of weak oestrogen agonists in the intact animal. Rozman and Klaassen (1995) provide an overview of the specific factors that influence the free concentration of the ER ligand, such as:

- the mechanism of intestinal uptake,

- intestinal microbial metabolism,

- diffusion from the vehicle at the subcutaneous injection site,

- non-specific binding in serum of the parent test substance and metabolites (these often are hydrophobic molecules),

- specific binding by serum hormone-binding proteins,
- metabolism by the liver and at non-hepatic sites which may either activate or deactivate a chemical,
- possible conjugation in the intestinal wall and the liver,
- rates of excretion in the bile and urine, etc.

204. Collectively, these factors would be expected to affect the results obtained when a test substance is administered by different routes of administration (*e.g.*, oral, intraperitoneal, and subcutaneous route differences in the factors are apparent) as could some doses as some metabolic pathways may become saturated. The critical assumption is that a trans-membrane equilibrium exists between the free concentration of a ligand in the serum and the intracellular concentration of the ligand, which is available to bind the ER. The free concentration in the serum controls the biological activity of the administered substance. These factors illustrate the importance of appropriate vehicle selection, route of administration, selection of doses, limit doses, etc., in ensuring that the uterotrophic bioassay yields relevant and meaningful information for testing decisions.

Routes of Administration

205. Two primary options to administer test substances, subcutaneous injection and oral gavage, can be used in the uterotrophic bioassay. Subcutaneous administration is presumed to allow the substance direct entry into the general circulation, thereby avoiding physical barriers and metabolism in the gut or intestinal wall, and reducing the initial extent and rate of hepatic metabolism. An inherent assumption, as noted above, is that substances diffuse freely and rapidly from the injecting vehicle. This assumption may not be valid in cases of unfavorable partition coefficients, when the entry into the general circulation would occur at lower rates. The work of Jensen and Jacobson (1962) with oestradiol indicates that partitioning is a relevant concern (**Figure 9**). Highly hydrophobic compounds with high octanol-water partition coefficients, e.g., PCB and DDT structures, may also be expected to enter the general circulation more slowly.

206. Oral gavage requires uptake of administered substances through the intestinal tract. The degree and rate of uptake depends upon bioavailability of the test substance from the intestinal contents, including uptake mechanism(s) through the intestinal wall, microbial metabolism, the potential for competition/inhibition during uptake, and ,as noted below, direct conjugation by enzymes present in the intestine. Substances crossing the intestinal wall enter the portal circulation and are transferred to the liver.

Oral administration

207. The liver has the ability to detoxify or to bioactivate substances due to its high metabolic activity and capabilities. This includes Phase I metabolism, metabolism by other pathways, and Phase II conjugation reactions (see description by Parkinson, 1995, and the review by King *et al.*, 2000). Conjugation typically enhances biliary excretion and clearance (see Temellini *et al.* 1991). The liver is the primary site for the metabolism of endogenous oestrogens (Diczfalusy and Levitz, 1970; Musey *et al.*, 1979; Mulder *et al.*, 1990). For exogenous exposures, early experiments by Nielson *et al.* (1946) found significant differences in the results of the vaginal cornification assay depending upon the routes of administration (subcutaneous, oral gavage, i.p., and intravenous) of six different test oestrogens. After

administration of hepatotoxic doses of carbon tetrachloride prior to oestrogen administration, the ratio of the oral gavage ED_{50} to the subcutaneous ED_{50} was altered in a chemical-specific manner from 0 to 200-fold. This clearly suggests significant and differential liver first-pass metabolism of some substances and not others.

208. The liver is also capable of converting inactive substances to active metabolites. The oestrogen agonist, methoxychlor, and the partial agonist/antagonist, tamoxifen, are activated in the liver (Bulger *et al.,* 1978; Fromson *et al.,* 1973; Jordan *et al.,* 1977). Methoxychlor is demethylated, revealing hydroxyl groups that significantly increased binding affinities and, hence, possible activity up to two orders of magnitude over the parent compounds (previous references and Blair *et al.,* 2000*).* In contrast, tamoxifen appears to undergo hydroxylation of one of its aryl rings (Fromson *et al.,* 1973; Jordan *et al.,* 1977). Other substances, such as PCBs and PAHs, may be 'activated' to very weak agonists by direct oxidation, which introduces hydroxyl compounds onto the rings of the parent chemicals. This apparently confers ER-binding affinity on the metabolite (see *e.g.,* Nishira *et al.,* 2000; Charles *et al.,* 2000; Blair *et al.* 2000). However, in the case of the polyaromatic benzo[a]pyrene, the parent chemical, which does not bind to the ER, appears to undergo hydroxylation via the P450 system, which is expressed in the liver, so that certain daughter metabolites that are hydroxylated at the 3, 9, or 10 carbons, bind the ER, and are active in *in vitro* assays at low levels (Blair *et al.,* 2000; Charles *et al.,* 2000; Fertuck *et al.,* 2001). However, these same metabolites are inactive in the uterotrophic bioassay at doses up to 10 mg/kg/d orally and 20 mg/kg/d subcutaneously (Fertuck *et al.,* 2001).

209. The induction of liver enzymes by pre-exposure to inducing substances enhances the rate of 17β-oestradiol metabolism and reduces the increases in uterine weight (Welch *et al.,* 1968, 1969, 1971; Levin *et al.,* 1967, 1968a,b). This may also occur in other specific cases, and monitoring for increased liver weight is one way to investigate this effect.

Conjugation in the intestinal wall and liver

210. In the intestinal wall, liver, and other tissues, conjugation with glucuronide and sulfate occurs readily for substances having an accessible hydroxyl group, such as the vast majority of oestrogen agonists and antagonists (see review of glucuronyl transferase enzyme family substrate activity and tissue distribution, by King *et al.,* 2000). The capacity of the glucuronide conjugation pathway is greater than the sulfate pathway. Notably, both pathways are saturable.

211. Glucuronide and sulfate conjugation are directly applicable to substances with accessible hydroxyl groups, such as the vast majority of oestrogen agonists and antagonists, as well as those that may be converted to hydroxylates via Phase I P450 oxidation. Conjugation increases a substance's water solubility and accelerates biliary and urinary excretion. Importantly, conjugation should also abolish the binding affinity of the parent compound for the ER. A glucuronide or sulfate residue on the active hydroxyl group should disrupt the coordination with the essential amino acid residues, as well as the confined ER ligand pocket itself. Further, as charged anions, the ability of conjugates to freely diffuse from the serum across the cell membrane to the intracellular ER is expected to be inhibited. The data with octylphenol and nonylphenol glucuronides (Moffat *et al.,* 2001) and with BPA glucuronide, (Matthews *et al.,* 2001; Snyder *et al.,* 2000) and with biological responses to genistein occurring at apparently equivalent serum levels of unconjugated genistein (Cortroneo and Lamartiniere, 2001) support this hypothesis. Detailed pharmacokinetic physiological-based versions incorporating such features as hepatic and intestinal glucuronidation, enterohepatic circulation, and receptor binding at the target tissue have not yet been developed in sufficient detail to provide further supporting data . In addition to assisting clearance, conversion to glucuronide or sulfate conjugates would be expected to reduce the concentration of the physiologically active form of the test substance.

Body compartments

212. Partitioning into body compartments such as adipose tissue is a further possibility for hydrophobic test substances, but some hydrophobicity is also necessary for ligand binding at the ER. In this way partitioning can compete with the ER for the ligand and can reduce the free concentration in serum. Evidence also exists that binding to proteins other than the ER may occur in certain tissues, *e.g.*, liver in female rats and thyroid in both sexes, in the case of genistein (Chang *et al.* 2000).

Binding of ligands in the serum

213. In the general circulation, two means exist to further reduce the circulating concentration of free oestrogenic compounds. First, as many oestrogen agonists are relatively hydrophobic, non-specific binding to serum proteins, such as albumin, can occur. Second, specific binding may occur in some cases to sex hormone binding proteins (see Hammond, 1995, 1997, for reviews).[13] In both circumstances, the degree to which the circulating free concentration of a substance is reduced in competition with the receptor would vary in a substance-specific manner.

214. Due to the variety and apparent importance of the pharmacokinetic factors, the *in vitro* potency of oestrogen agonists may be difficult to extrapolate to *in vivo* conditions. While the potency of many substances should be significantly reduced by conjugation and rapid elimination, the potency of other substances may be increased, *e.g.*, hydroxylation of aryl rings or demethylation of methoxy groups on aryl rings. Coldham *et al.* (1997) and several other laboratories have noted the lack of strict concordance between *in vitro* and *in vivo* results. For example, the data of Elsby *et al.* (2000) for 6-hydroxytetralin showed that the substance had features considered to be structural alerts (unhindered hydroxyl group on an aryl ring with an adjacent second hydrophobic ring structure), was weakly positive in a receptor-binding assay, and was also weakly positive in *in vitro* reporter gene assays. However, the 6-hydroxytetralin was negative in the uterotrophic bioassay at four doses from 50 mg/kg/day to above the limit dose of 1000 mg/kg/day. As with the previously noted hydroxylated benzo[a]pyrene, this experiment suggests that many weak agonists that may have structural alerts or be positive *in vitro* assays, may be rapidly inactivated or cleared from the body so that they are negative *in vivo*.

215. One generalization that can be formulated is that, for most agonists with available, unhindered hydroxyl groups (a structural prerequisite for effective agonist binding activity), oral administration will lead to significant conjugation in the intestine and liver. This will result in lower circulating levels of free ligand when compared to subcutaneous administration at similar administered dose levels. This will reduce the uterine weight increase observed for the oral route versus the subcutaneous route at equivalent administered doses. The exceptions are likely to be substances that are activated by processes such as demethylation of a methoxy group, de-esterification, or hydroxylation of an aryl ring.

Review of Receptor Binding Affinity and *In Vitro* Transcriptional Assay Data

216. The classical receptor binding affinity assay utilises the ERα present in the uterine cytosol from rats, although data exist in a variety of species and also for the ERβ. As there is no single standardised protocol at this time, even for from the rat uterine cytosol ERα, the existing data may not be quantitatively comparable from one laboratory to another. ERα binding data for selected and related substances that are drawn from a single body of work (Blair *et al.* 2000) are shown in **Table 31**. Other investigators have generated similar binding data on one or more of these substances, and the data are at

[13] The binding domain of sex hormone binding globulin has recently been isolated and the structure determined by x-ray crystallography. The hormone-binding domain is totally unrelated to the nuclear receptor-binding domain found in the ER (Grishkovskaya *et al.*, 2000).

least qualitatively similar (see, *e.g.*, Gould *et al.*, 1998; Ireland *et al.*, 1980; Nelson, 1973; Santell *et al.*, 1996; Shelby *et al.*, 1996).

217. Despite their diversity and technical difficulties, *in vitro* transcriptional assays for the selected compounds are in qualitative concordance with the ER binding affinity data. However, the precise rank order and potency relative to 17β-oestradiol may vary somewhat depending upon assay and the performing laboratory. For the selected agonists, the following results from transcriptional assays can be consulted as examples: for BPA (Coldham *et al.*, 1997; Gaido *et al.*, 1997; Gould *et al.*, 1998); for genistein (Coldham *et al.*, 1997); for octylphenol and nonylphenol (Beresford *et al.* 2000; Coldham *et al.* 1997; Gaido *et al.* 1997; Odum *et al.* 1996; Shelby *et al.* 1996); methoxychlor and sometimes the demethylated methoxychlor metabolite (Beresford *et al.*, 2000; Coldham *et al.*, 1997; Gaido *et al.*, 1997; Odum *et al.*, 1997; Shelby *et al.*, 1996); and *o,p'*-DDT (Coldham *et al.*, 1997; Gaido *et al.*, 1997; Shelby *et al.*, 1996). In many cases, various potent reference oestrogens (17β-oestradiol, EE, and DES) are included in the experiments.

218. Characteristically, the reference oestrogen response occurs at very low concentrations, has a high slope, and has a maximum response that few weaker agonists achieve except at very high doses. The selected weak agonists respond at concentrations from several to many fold higher than the oestrogens, may have modest or shallow slopes, and their maxima are often substantially lower than for the various potent oestrogens.

Table 31. Rat uterine cytosol ERα receptor-binding data from a single laboratory protocol (Blair *et al.*, 2000; Branham *et al.*, 2001)

Chemical Name (Abbreviation)	Mean IC_{50} (M) ± S.E.M.	RBA (%)	Log RBA
17β-Oestradiol (E1)	$8.99 \times 10^{-10} \pm 0.27 \times 10^{-10}$	100.000	2.00
Diethylstilbestrol (DES)	$2.25 \times 10^{-10} \pm 0.05 \times 10^{-10}$	399.556	2.60
Ethinyl oestradiol (EE)	$4.73 \times 10^{-10} \pm 0.60 \times 10^{-10}$	190.063	2.28
4-Hydroxytamoxifen (4-OHTAM)	$5.13 \times 10^{-10} \pm 1.12 \times 10^{-10}$	175.244	2.24
Tamoxifen (TAM)	$5.55 \times 10^{-8} \pm 0.05 \times 10^{-8}$	1.620	0.21
Genistein (GN)	$2.00 \times 10^{-7} \pm 0.21 \times 10^{-7}$	0.443	-0.35
Dihydroxymethoxychlor (HPTE)	$3.55 \times 10^{-7} \pm 0.15 \times 10^{-7}$	0.253	-0.60
Methoxychlor (MX)	$1.44 \times 10^{-4} \pm 0.66 \times 10^{-4}$	0.001	-3.20
4-Nonylphenol (NP)	$3.05 \times 10^{-6} \pm 0.15 \times 10^{-6}$	0.029	-1.53
Bisphenol A (BPA)	$1.17 \times 10^{-5} \pm 0.64 \times 10^{-5}$	0.008	-2.11
4-Octylphenol (OP)	$1.95 \times 10^{-5} \pm 0.15 \times 10^{-5}$	0.005	-2.34
o,p'-DDT	$6.43 \times 10^{-5} \pm 0.89 \times 10^{-5}$	0.001	-2.85

CHAPTER 7: BACKGROUND PHARMACODYNAMIC AND TOXICITY INFORMATION FOR SELECTED WEAK OESTROGEN AGONISTS

219. The concentration of free ligand in the serum is affected by the route of administration, distribution, metabolism, and excretion. The key difference with oral gavage relates to differences between intestinal glucuronidation, first-pass liver metabolism, and biliary excretion. Oral administration would tend to lead to reduced levels of serum ligand. However the extent of the difference is expected to be test substance specific. Exceptions to this rule may be expected as a result of metabolic activation.

220. This section reviews the available pharmacodynamic and toxicity data for the test substances and related substances recommended for use in Phase 2 of the OECD validation program:

- Methoxychlor
- Bisphenol A
- Octylphenol and Nonylphenol
- Genistein
- *o,p'*-DDT

221. The importance of these data lies in the observation that the relative potency of substances to 17β-oestradiol based on receptor binding assays and other *in vitro* assays has not predicted the relative potency observed with the uterotrophic bioassay. In a number of cases, the *in vivo* activity differed several orders of magnitude from that observed with *in vitro* assays (see for example the data of Coldham *et al.*, 1997 and Shelby *et al.*, 1996). Some substances such as nonylphenol and BPA exhibit far less potency *in vivo* while others, such as methoxychlor, are greater. This gives further support that metabolic and other processes in the intact animal have a major influence on the uterotrophic response.

222. **Table 32** summarises how the differences in conjugation and biliary excretion will be influenced by different routes of administration.

Methoxychlor

223. The work of Bulger *et al.* (1978) shows that hepatic demethylation converts methoxychlor to the more active dihydroxymethoxychlor. Therefore, oral gavage should lead to a uterine response at lower administered doses than s.c. due to liver first-pass effects. However, the data of Kapoor *et al.* (1970) indicate that rapid glucuronidation of the methoxychlor metabolites also occurs after oral administration. Chapin *et al.* (1997) analyzed the sera from dams and pups for methoxychlor and its metabolites after oral gavage and lactational intake, respectively. However, the levels reflect a 3-6 hours delay for dams after their dosing and a 27-30 hour delay in analysis of the serum from pups after the dams were dosed. Pup plasma levels had levels of methoxychlor that were typically non-detectable (detection limit, <5 ng/ml serum). Given the rapid rate of clearance, these data do not capture the peak levels.

Genistein

224. There are significant data available in the rat on the absorption, metabolism, and excretion of genistein. Sfakaianos *et al.* (1997), using bile cannulas, clearly demonstrated the biliary excretion of genistein glucuronide conjugates, supporting the suspected enterohepatic circulation of genistein. Coldham *et al.* (1999) quantitated the metabolism in rats of a 4 mg/kg b.w. dose to the genistein glucuronide and sulfate conjugates. Coldham and Sauer (2000) demonstrated a 8-12 hr genistein half-life with apparently significant enterohepatic circulation. In plasma, genistein was found primarily as the genistein glucuronide, with lower quantities of the 4-hydroxyphenyl-2-proprionic acid metabolite, and

followed by free genistein (less than ~5% of the genistein glucuronide concentration, which would include both protein-bound and unbound quantities). Some of the strongest evidence that the free genistein is the physiologically active species is the concordant responses of several oestrogen sensitive tissues and molecular markers when equivalent free genistein levels in serum were reached by s.c. administration and by dietary intake (Cotorneo and Lamartiniere, 2001). Frtiz *et al.* (1998) also analyzed free and total genistein after dietary administration and observed substantial conjugation in the sera of both dams and offspring. Chang *et al.* (2000) and Holder *et al.* (1999) analyzed genistein after dietary administration in P_1 and F_1 animals as well as offspring. Serum genistein levels were approximately 94% glucuronide conjugate, with the 7-glucuronide genistein being the major form and the 4'-glucuronide the minor, 3% the sulfate conjugate, and 3% the physiologically active free genistein. The serum levels of all forms appeared to increase linearly up to 100 mg/kg/d, indicating that conjugation pathways were not saturated at the highest dietary dose. Chang *et al.* (2000) also suggested that several tissues may potentially accumulate genistein, although the tissues were not perfused to remove serum genistein. Doerge *et al.* (2001) studied the maternal transfer of genistein to the foetus and neonate, and demonstrated that nursing neonates had serum levels over an order of magnitude lower than the dam. However, foetal levels of free genistein were similar to those in the dam due to probable placental transfer.

225. Additional data are available in the mouse and humans, and with other substances. Supko and Malspeis (1995) studied the levels of free, unconjugated genistein in the mouse using tail vein injection, i.p. injection, and p.o. administration. The oral route resulted in the lowest levels of free genistein (~1 µg/mL peak at 15-30 minutes for the maximum 180 mg/kg dose), which compares favourably to the ~0.5 µg/mL in male rats via dietary levels of 100 mg/kg/d (Chang *et al.* 2000). Human data confirming the high ratio of conjugated genistein to free serum genistein has been generated, although the urinary route of excretion predominates in humans (*cf.* Aldercreutz *et al.* 1993,1995). For substances related to genistein, Bayer *et al.* (2001) have demonstrated that administration of 100 mg/kg of the related isoflavone daidzein also results in significant faecal excretion (86% of the dose in both sexes), consistent with enterohepatic circulation and the appearance of daidzein glucuronide and sulfate conjugates in the urine. Serum levels were not analyzed in these experiments.

Octylphenol (OP)

226. Certa *et al.* (1996) provide data on OP, which is structurally related to nonylphenol. *In vitro* glucuronidation and sulfation of OP were demonstrated, and blood concentrations of unconjugated OP were analyzed after both i.v. (5 mg/kg bw) and p.o. (50 and 200 mg/kg bw) administration. Using the area under the curve, it was calculated that approximately 2 - 10% of the p.o. doses, respectively, reached the bloodstream as free OP. While 50 mg OP/kg/d for 14 days did not lead to significant tissue accumulation (adipose tissue, liver, and others), detectable levels were found in adipose tissue, liver, and other tissues with the 200 mg OP/kg/d dose. These data suggest that the 200 mg/kg/d dose may begin to saturate clearance pathways and, thereby, increase the effective internal dose. Upmeier *et al.* (1999) also demonstrated the rapid conjugation and excretion of OP. The route of administration significantly affected the serum concentrations in their experiments, *i.e.,* i.v. administration led to far higher serum levels than p.o. administration.

Nonylphenol (NP)

227. Knaak *et al.* (1966), using oral and i.p. administration of [^{14}C]-NP and its ethoxylates, demonstrated the hydrolysis of the ethoxylate groups to reveal the parent NP. High proportions of NP circulated in the serum as glucuronide or sulfate conjugates. Gardner *et al.* (1980) investigated an oral dose of 100 mg/kg ethoxylated nonylphenol and found that ~80% of the dose was rapidly cleared into the cannulated bile duct, and identified a number of NP metabolites and conjugates, supporting enterohepatic circulation. Müller *et al.* (1998) exposed human volunteers to [^{13}C]-NP by both i.v. and oral routes. The

circulating level of unconjugated NP was significantly reduced by oral administration (< 1% of the glucuronide and sulfate conjugates) compared to i.v. administration (30-70% of the dose was unconjugated, depending upon time after administration) (**Table 33**). The 'free' fraction included NP which may have been bound to lipid and/or serum proteins. These data also show kinetics consistent with the expected enterohepatic circulation.

228. Additional experiments on NP in rats have been conducted by Fennell and MacNeela (1997) and Fennell *et al.* (1998). Using i.v. and oral gavage administration, NP levels in the circulation were consistent with the presence of a significant level of conjugates and metabolites, and relatively low levels of 'free' NP. I.v. administration led to a substantially greater level of 'free' NP than oral gavage administration. The concentration of NP identified as "free" would possibly include nonylphenol that was bound to serum proteins and lipids.

Bisphenol A (BPA)

229. The turnover and elimination of BPA is rapid. Long *et al.* (2000) reported a 90-minute serum half-life after s.c. administration. Miyakoda *et al.* (1999, 2000) reported a peak BPA concentration at one hour in maternal plasma, foetal tissues, and male plasma, after p.o. administration of 10 mg/kg. Snyder *et al.* (2000) reported on the disposition of labelled BPA in F344 and CD rats, and showed that significant quantities of the administered dose were retained in the intestine (83% at 1 hr, 75% at 8 hr, and 26% at 24 hours) suggesting extensive enterohepatic circulation. The primary components in the serum and urine were the glucuronide conjugate. Small quantities were found in milk, and Miyakoda *et al.* (1999, 2000) indicate that free BPA can cross the placenta to the foetus, and that foetal levels appear to be slightly below those of the dam. Miyakoda *et al* (1999, 2000) reported that less than 0.01% of the dose administered to dams was found in pups, and no conjugate was detected in the foetus, due to the possible absence of glucuronidation. The authors, however, did not look at the possibility of there being sulfate conjugates.

230. Pottenger *et al.* (2000), in the most detailed set of experiments, reported T_{max} values ranging from less than 30 minutes for p.o. administration to approximately one hour for s.c. administration, at doses of 10 and 100 mg/kg. Pottenger *et al.* (2000) also clearly showed that free BPA serum levels following p.o. administration at the C_{max} were ~10% of the level by either i.p. or s.c. administration. **Table 34** provides detailed values comparing i.v., s.c., and p.o. routes of administration). Their more detailed experiments also showed that modest quantities of BPA circulated as a sulfate conjugate, and that a secondary serum peak appeared later in time, suggesting enterohepatic circulation. Both Miyakoda *et al.* (2000) and Pottenger *et al.* (2000) demonstrated that the BPA glucuronide comprised >90% of the circulating BPA, followed by the sulfated and free BPA. Comparison with the 800 mg/kg dose used by Knaak and Sullivan (1966) suggests that saturation of the glucuronidation pathway leads to the appearance of new BPA metabolites.

***o,p'*-DDT**

231. No detailed studies of *o,p'*-DDT metabolism were found. The chemical would be expected to bind to serum proteins due to its hydrophobicity, to partition to adipose tissue for the same reason, and to bypass intestinal and hepatic conjugation due to the absence of an available hydroxyl groups. Combined with possible slow release from oily subcutaneous vehicles, *o,p'*-DDT would be expected to behave differently than most other weak agonists.

Table 32. Route of administration differences in conjugation and biliary excretion

	Oral gavage	**Intraperitoneal**	**Subcutaneous**
Intestinal UDP-glucuronide transferase	Substances subject to significant level of intestinal glucuronide conjugation (assuming that free hydroxyl groups are available). Same is true in the case of enterohepatic circulation.	Absent	Absent
Liver UDP-glucuronide transferase	Increased level of administered dose subject to liver glucuronide and sulfate conjugation due to first pass effect and enterohepatic circulation.	Increased level of administered dose subject to liver conjugation due to first pass effect for that portion of the dose entering the portal circulation.	Subject to liver conjugation dependent solely upon general circulation delivery or in the case of enterohepatic circulation.
Biliary excretion	Quantity dependent on liver levels of conjugate, which are increased from intestinal conjugation and liver first pass. Process may be saturable.	Quantity dependent on liver levels of conjugate, which are increased from liver first pass (but not intestinal conjugation).	Quantity dependent on liver levels of conjugate, which depends solely on delivery from general circulation.

Table 33. Nonylphenol (NP) levels and conjugation in humans by route of administration (from Müller *et al.* 1998).

Oral administration (66 µg/kg bw)				i.v. administration (14 µg/kg bw)			
Time after dosing (min)	Parent NP[a] (pg/g blood)	Conjugate NP (pg/g blood)	Percent 'free'[b] NP	Time after dosing (min)	Parent NP (pg/g blood)	Conjugate NP (pg/g blood)	Percent 'free'[b] NP
15	326	8476	3.7%	35	626	117	84.3%
32	646	86040	0.7%	--			
63	320	64603	0.5%	--			
93	251	40237	0.6%	--			
122	181	25897	0.7%	106	172	60	74.1%
182	130	23400	0.6%	216	94	49	65.7%
301	90	5223	1.7%	--			
424	56	1617	3.3%	448	21	47	30.9%
522	33	825	3.8%	558	10[c]	27	27.0%

[a] Using MS fragments for NP indicative of unmetabolised parent after serum extraction.
[b] After cyclohexane extraction, hence, any NP bound to lipid or protein would be included in the analysis.
[c] The analytical detection limit was 10 pg/g, so this value is a 'non-detect.'

Table 34. Pharmacokinetic parameters of bisphenol A by different routes of administration (from Pottenger *et al.* 2000)

Route of Administration	**Oral gavage**				**Intraperitoneal**				**Subcutaneous**			
Sex	**Male**		**Female**		**Male**		**Female**		**Male**		**Female**	
Dose (mg/kg)	10	100	10	100	10	100	10	100	10	100	10	100
Free Bisphenol A												
Tmax (hr)	NA	0.083	0.25	0.25	0.5	0.25	0.25	0.25	0.75	0.5	4	0.75
Cmax (µg-eq g)	NQ (0.07)	0.22	0.04	2.29	0.69	9.7	0.87	13.13	0.39	5.19	0.34	3.97
Time to NQ (hr)	0.083	0.75	1	ND	8	12	24	72	18	24	48	72
AUC (µg-eq hr/g)	NA	0.1	0.42	4.4	1.1	16.4	1.4	26.2	2.6	24.5	3.1	31.5
Total Bisphenol A (free + conjugates + metabolites)												
Tmax (hr)	0.25	0.25	0.083	0.25	0.5	0.25	0.25	0.5	1	0.75	0.75	0.75
Cmax (µg-eq g)	0.73	3.92	1.82	28.33	1.26	29.3	2.27	67.81	0.61	6.33	0.52	5.66
Time to NQ (hr)	72	72	72	72	96	96	72	120	96	144	120	168
AUC (µg-eq hr/g)	8.1	66.5	9.54	94.9	16.9	170	15.3	247	15.5	218	21.6	297

NQ, time in hours, when no longer quantitatable

CHAPTER 8: BIOASSAY DATA ON PROPOSED OECD TEST SUBSTANCES

Reference Toxicants for an Oestrogen Mode of Action

232. In order to evaluate the toxicological profile of weak oestrogen agonists, benchmark data for a reference toxicant are necessary. For example, a recent one-generation reproductive and developmental study has been conducted with 17β-oestradiol. The protocol used a thorough battery of 17β-oestradiol sensitive endpoints, including those already incorporated or recently added by the USEPA (1996, 1998), e.g., developmental benchmarks such as the day of vaginal opening and first oestrous, a diverse set of female (ovaries, uterus, vagina, and mammary glands) and male (testes, epididymis, prostate, and seminal vesicles) tissue weights and histopathology, and female (mating and fertility indices, number of implantation sites, estrous cyclicity) and male (sperm number and analyses) parameters, as well other exploratory endpoints, *e.g.*, serum hormone levels (Biegel *et al.* 1998a,b; Cook *et al.* 1998). This study provides a benchmark to assess whether weak oestrogen agonists elicit one or more of these oestrogen-mediated effects. Comparisons of the number, severity, and types of effects, as well as NOAELs and LOAELs for the weak oestrogen agonists can then be made against the 17β-oestradiol benchmark. In order to conclusively identify the profile for an oestrogenic mode of action, additional studies with other reference oestrogens, such as DES and EE, are necessary. However, studies with these substances using a complete battery of oestrogen-sensitive endpoints or enhanced endpoints for the detection of oestrogen-mediated effects, *e.g.*, pubertal development and reproductive parameters for F_2 animals, are not currently available.

233. In a one-generation assay of oestradiol by Biegel *et al.* (1998), the expected family of responses were observed over a range of doses. At 50 ppm 17β-oestradiol in the diet (3.2-4.1 mg/kg/d), the reproductive effects expected of potent oestrogens were observed. The mating and fertility indices were zero in all pairs of the F_0 generation. At 10 ppm in the diet (0.53-0.69 mg/kg/d), no pregnancies were observed after mating. At this dose, the weights and histology of most male and female reproductive tissues were affected, as were sperm numbers, motility, and morphology. Other endpoints were affected with the 10 and 50 ppm diets; these included decreases in liver and spleen weights, centrilobular hepatocellular hypertrophy, hyperplasia of the pituitary, feminization of the male mammary glands, cystic follicles in the ovaries, mild anaemia, reduced serum cholesterol, and altered splenic lymphocyte subtypes.

234. Reproductive effects were not observed with the 0.05 and 2.5 ppm oestradiol diets. At 2.5 ppm in the diet (140-170 μg/kg/d) and above, adult and pup body weights were decreased in both sexes. Adult body weights were unaffected by the 0.05 ppm diet (~3 μg/kg/d), but pup weights at birth were decreased by the 0.05 ppm and higher diets. In the F_1 generation, the 2.5 ppm diet resulted in statistically significant differences in liver weights (decreased absolute weights in males and increased relative weights in females), increased absolute and relative adrenal weights in both sexes, decreased weights of testes, epididymis, and sex accessory tissues in the male, and increased ovarian weights in the females. Histopathological changes were observed in several tissues of both sexes, including the reproductive tract. The 0.05 ppm diet did not elicit any of these observations. Anogenital distances were unchanged in both sexes. For developmental benchmarks, the 2.5 ppm diet led to an average delay of 8.2 days in preputial separation and accelerated vaginal opening by 8.8 days. The 0.05 ppm diet accelerated vaginal opening by 1.6 days, which was statistically significant.

235. Biegel *et al.* (1998b) Cook *et al.* (1998) assessed hormonal changes in both sexes as well as oestrus cyclicity and testicular and sperm parameters. In females, a dose-related decrease in progesterone receptor (PR) was observed at 90 days in the P_1 generation with the 0.05 ppm diet and above. In the F_1

generation, PR was decreased by the 2.5 ppm diet, but not the 0.05 ppm diet. At 90 days in the P_1 generation, lutenizing hormone (LH) was decreased by the 10 ppm diet, and prolactin was increased by the 50 ppm diet. In the F_1 generation, no changes in LH or prolactin were observed (no pups were produced at 10 and 50 ppm). It is noteworthy that the detection of these hormonal changes required careful correlation with the exact stage of the estrous cycle and group sizes. The estrous cycle was affected by the 2.5 ppm diet and above in the P_1 generation and by 0.05 ppm diet and above in the F_1 generation (Biegel *et al.*, 1998b). In males of the P_1 generation, testicular and epididymal sperm numbers were decreased, and sperm motility decreased with the 10 and 50 ppm diets after 90 days, although sperm morphology was normal. After a 109-day recovery period, tissue and sperm parameters returned to normal. In the F_1 generation, a slight decrease in epididymal sperm number was observed with the 2.5 ppm diet, and no recovery was observed for this parameter after 109 days. The investigators also tested the hypothesis that *in utero* exposure to oestradiol would reduced the testicular Sertroli cell number, but found no effect. Hormonal analyses indicated the tissue and sperm changes correlated with decreased testosterone, LH, and FSH levels, and increased prolactin levels. At 90 days in the P_1 generation, a testosterone decrease was the most sensitive change and occurred with the 0.05 ppm diet. In the F_1 generation, decreased testosterone and LH, and increased prolactin, occurred with the 2.5 ppm diet, but not the 0.05 ppm diet (Cook *et al.*, 1998).

236. Reproductive and developmental data for 17β-oestradiol, BPA, genistein, NP, methoxychlor, and *o,p'*-DDT are summarised in **Table 35** and support the concept of different effects having different dose-response curves which appear with increasing severity for potent oestrogens. The data also suggest the possibility of a pattern of effects. The first changes to appear may be the developmental benchmarks, particularly, vaginal opening. This response may be followed by decreases in the weights of male reproductive and sex accessory tissues and characteristic changes in female reproductive tissues. Other parameters that may be affected include estrous cyclicity and sperm numbers. The apparent pattern would culminate in reproductive effects at high doses. Together, the pattern would indicate an oestrogen mode of action that could be elicited by weak oestrogen agonists. There were also effects on body weight, which can also occur through systemic toxicity and might confound several of the endpoints of interest, particularly developmental benchmarks. These decreased body weights may overlap to varying degrees with several possible oestrogen effects. It is important to not over-interpret decreased body weight as 'oestrogenic' in itself. Further, this possible profile or pattern needs to be confirmed with multiple generation tests with oestradiol using a more refined selection of doses, and in other potent oestrogens such as DES, EE, and oestradiol benzoate.

Weak Oestrogen Agonists Compared With Oestradiol

237. This section reviews the reproductive and developmental toxicity data for ~~the~~ selected weak oestrogen agonists, methoxychlor, genistein, OP, NP, BPA, and *o,p'*-DDT. The aim is to identify representative studies that include multi-generation designs and an array of oestrogen-sensitive endpoints. The outcomes of these studies will then be compared to the apparent toxicological profile of oestradiol that was described in the previous section. As each of the selected compounds is positive in *in vitro* assays and has been at least weakly active in the uterotrophic bioassay via oral administration, this could provide some insight into the possible predictive capability of the uterotrophic bioassay.

238. A review of the literature has identified multiple generation tests for NP (Chapin *et al.*, 1999) and the related OP (Tyl *et al.*, 1999). Multi-generation tests have recently been completed for BPA (Tyl *et al.*, 2000; Ema, 2000). Reproductive and developmental tests for methoxychlor have been conducted (Chapin *et al.*, 1997; Chapin, 1999; Gray *et al.*, 1989). Limited reproductive and developmental tests incorporating oestrogen-sensitive reproductive and developmental endpoints have been reported for genistein (Casanova *et al.*, 1999; Fritz *et al.*, 1998; Levy *et al.*, 1995; Santell *et al.*, 1997) and for *o,p'*-DDT (Gellert *et al.*, 1972, 1974, 1975; Wrenn *et al.*, 1970, 1971).

239. These test data are not equivalent. The tests for OP (Tyl *et al.,* 1999) and BPA (Tyl *et al.,* 2000) were multi-generation reproductive protocols with a battery of oestrogen-sensitive endpoints. These endpoints include the updated USEPA endpoints and that were conducted under good laboratory practices (GLP) guidelines. The 3-generation test with NP (Chapin *et al.,* 1999) included a set of oestrogen-sensitive endpoints that resemble those for updated 2-generation reproductive protocols. This test was not conducted under GLP guidelines. The studies of methoxychlor (Chapin *et al.,* 1997; Chapin, 1999; Gray *et al.,* 1989) did include a set of oestrogen sensitive endpoints. These were *in utero* and developmental exposures with an assessment of sexual development and/or adult reproductive capacity, but were not strict regulatory guideline tests and were not conducted under GLP guidelines. The genistein studies (Casanova *et al.,* 1999; Fritz *et al.,* 1998; Levy *et al.,* 1995) also included *in utero* and developmental exposures, but the battery of endpoints was less broad and thorough, and they were not conducted under GLP guidelines. The *o,p'*-DDT studies (Gellert *et al.,* 1972, 1974, 1975; Wrenn *et al.,* 1970, 1971) were more limited in scope, with fewer general and oestrogen-sensitive endpoints, and were conducted prior to development of GLP guidelines.

Methoxychlor

240. Several studies were identified for methoxychlor, but none are multi-generational reproductive and toxicity studies. Oestrogenicity was suggested by very early methoxychlor studies in rats that found severe testicular atrophy or significant decreases in testicular and sex accessory tissue weights, as well as histopathological findings in the same tissues after ingesting 10 mg/g methoxychlor in the diet (Hodge *et al.,* 1950; Tullner and Edgcomb, 1962), as well as the demonstration of uterotrophic activity in the same time period (Tullner, 1961).

241. More recent developmental methoxychlor studies incorporated developmental or *in utero* exposure as well as a battery of potentially oestrogen-sensitive endpoints (Gray *et al.* 1989; Chapin *et al.* 1997). Gray *et al.* (1989) began oral gavage dosing of rats at pnd 21 with 25, 50, 100, and 200 mg/kg/d methoxychlor using different groups to cover pubertal maturity and adult reproduction. Chapin *et al.* (1997) administered methoxychlor by gavage at 5, 50, and 150 mg/kg/d starting at gestation day (gd) 14 and continuing until pnd 21 for one cohort and until pnd 42 for another cohort.

242. In both the Gray and Chapin studies, methoxychlor led to overt reproductive effects, *e.g.,* no pregnant females at 200 mg/kg/d (Gray *et al.* 1989) and 150 mg/kg/d (Chapin *et al.* 1997), and a reduction in the number of live pups per litter at doses of 100 mg/kg/d and 50 mg/kg/d, respectively. No effects on male fertility were observed at any dose. In the Gray dataset, decreased body weights were observed in both sexes at 100 and 200 mg/kg/d doses at early ages and in 9-10 week old males at the 25 and 50 mg/kg/d doses. In males, seminal vesicle and epididymal weights and caudal epididymal sperm counts were decreased (50 mg/kg/d); age at preputial separation was increased and testicular weights were decreased (100 mg/kg/d). In females, age at vaginal opening, first oestrus, and first oestrus cycle were decreased at 25 mg/kg/d, and ovarian weights were decreased and histopathology affected at 100 mg/kg/d and higher in a dose-related manner. Changes in liver, kidney, and adrenal weights were elicited at 100 mg/kg/d and higher in both sexes. In the Chapin dataset, decreased body weights were observed at 150 mg/kg/d, but not at lower doses, in both sexes at several time points. At the 50 and 150 mg/kg/d doses, effects included acceleration of vaginal opening, delayed preputial separation, irregular or absent oestrus cycle, decreased weights in ovaries and male sex accessory tissues and testes, increased uterine weights, and histological changes in ovary, uterus, and testes. Vaginal opening was accelerated over controls by 2 days at the lowest dose in the study (5 mg/kg/d; LOEL) where other effects were not observed. Other endpoints, such as anogenital distance, were not affected at any dose.

243. In conclusion, methoxychlor elicits a broad range of potentially oestrogenic effects in a dose-related manner, including overt reproductive effects at high doses. Those doses that are first positive in the uterotrophic bioassay are similar to the doses where the acceleration of vaginal opening occurred in the Chapin *et al.* (1997) study. The results for selected oestrogen-sensitive endpoints from these studies are summarised and compared with 17β-oestradiol in **Table 35**.

Genistein

244. Three recent studies were reviewed for genistein, but none are multigenerational reproductive toxicity studies. This gap is a concern given that genistein is found in the human diet, including baby formulas and the recent finding that *in utero* administration can lead to latent carcinogenic effects. Casanova *et al.* (1999) began to dose pregnant dams at gd 1 on dietary levels of 200 and 1000 ppm genistein (~15 and 75 mg/kg/d). Dosing continued with one group of both sexes sacrificed at pnd 1, one group of females through lactation until all had completed vaginal opening, and one group of males through sexual maturation until pnd 56. Fritz *et al.* (1998) also began pregnant dams at gd 1 on dietary levels of genistein at 25 and 250 ppm (~2 and 20 mg/kg/d). Flynn *et al.* (2000) began pregnant dams at gd 7 on diets containing 25, 250, and 1250 ppm genistein (~2, 20, and 100 mg/kg/d) and continued both sexes of the offspring on the diets through pnd 77. The potentially oestrogen-sensitive endpoints in these studies were limited and were largely concentrated on developmental markers, and reproductive and sex accessory tissues. Other than measures such as litter size, none of the studies adequately measured the reproductive capacity of the P_1 animals, and no reproductive measures of the F_1 animals were addressed.

245. In the study of Casanova *et al.* (1999), vaginal opening was accelerated and increased uterine weight was seen in female offspring at 75 mg/kg/d (LOEL,) but not 15 mg/kg/d (NOEL). In males, no changes were seen in the available oestrogen sensitive endpoints (testes and prostate weights, and age at preputial separation). In the study of Fritz *et al.* (1998), no effects were observed on litter size, anogenital distance, vaginal opening, testes descent, or oestrus cyclicity at dietary levels up to ~20 mg/kg/d (NOEL). The study of Flynn *et al.* (2000) focused on a behavioural battery, but observed decreased body weights in both males and females starting on pnd 42 with the 1250 μg/g genistein diet.

246. The limited studies using genistein that were reviewed suggest oestrogen sensitive endpoints (acceleration of vaginal opening) in the 15-75 mg/kg/d genistein range. Given the apparent potency of genistein *in vitro* and in the uterotrophic bioassay relative to other weak oestrogen agonists, the substantial quantities in the human diet, particularly baby formulas, and the recent finding that genistein administration *in utero* leads to latent carcinogenic effects in the adult (Newbold *et al.*, 2001b), a reliable multigeneration study covering a full battery of reproductive and developmental endpoints is needed. Importantly, the results of Newbold *et al.* (2001b) were positive for the genistein dose of 50 mg/kg/d, generally concordant with doses from both the uterotrophic results and those of the above experiments. The in-life phase of such a multiple generation study with genistein and other substances (EE, methoxychlor, and NP) have been completed at the US FDA National Center for Toxicological Research (Delclos, personal communication; Jefferson and Newbold, 2000b), but the full analysis and reporting of the results has not yet been completed.

Octylphenol(OP) and Nonylphenol (NP)

247. Octylphenol and nonylphenol have a close structural relationship, are both weak ER agonists (**Table 31**), have been positive in *in vitro* assays and, when sufficient doses were administered, have been positive in the uterotrophic bioassay. OP has consistently been one to two orders of magnitude less potent than NP in the uterotrophic bioassay (Diel *et al.*, 1999; Laws *et al.*, 2000; Odum *et al.*, 1997, 1999a,b). Comprehensive multiple generation studies are available for both chemicals. The OP study was conducted using the updated USEPA guidelines with a full battery of oestrogen-sensitive endpoints,

as well as a set of enhancements that included extended dosing of the F_2 generation until females achieved vaginal opening and sperm parameters could be determined for males (Tyl *et al.* 1999). One NP study (Nagao *et al.*, 2001) included some oestrogen-sensitive endpoints and also analyzed circulating serum hormone levels, but these endpoints were not examined and recorded in all generations. Another NP study (Chapin *et al.*, 1999) encompassed data for four generations (P_1, F_1, F_2, and F_3) and included enhancements for potential oestrogen endpoints such as vaginal opening, oestrus cyclicity, testes descent, tissue weights, histopathology for a set of female and male reproductive tissues, and extended dosing of the F_3 generation to observe developmental landmarks and reproductive tissues after puberty.

248. OP at dietary levels of 0.2, 20, 200, and 2000 ppm (from 11-33 μg/kg/d to 111-369 mg/kg/d) did not result in any overt reproductive effects (Tyl *et al.* 1999). At the highest OP dose, ~300 mg/kg/d, significant decreases in the body weights of all three generations were observed. No effects were observed on any female or male reproductive tissue; female oestrous cyclicity, male anogenital distance, and male sperm parameters were unchanged. Slight changes in female anogenital distance (0.03 to 0.09 mm) were not dose responsive, and this parameter is under androgenic control. Vaginal opening was delayed 1.3 days in the F_1 and 0.7 days in the F_2 generation at the highest dose, and preputial separation was delayed 1.6 days in the F_1 and 0.7 days in the F_2 generation. As these benchmarks are sensitive to body weight and were reduced in parallel with decreased body weights, these changes were not attributed to a hormonally-mediated effect. Thus, the study for octylphenol elicited an adverse systemic effect, decreased body weight, at ~300 mg/kg/d, but did not elicit any apparent oestrogen-related effect.

249. In the study of Nagao *et al.* (2001), NP was administered by oral gavage at doses of 2, 10, and 50 mg/kg/d, which covers the lower portion of the dose-response region of the Chapin *et al.* (1999) study. The only change in the battery of potentially oestrogen-sensitive endpoints was an acceleration of vaginal opening at 50 mg/kg/d. A small study at a higher NP dose level of 250 mg/kg/d elicited nephrotoxicity and decreases in body weight similar to those observed by Chapin *et al.* (1999). A decrease in serum testosterone was observed, but oestrous cyclicity was not recorded.

250. In a more comprehensive study (Chapin *et al.* 1999), NP was administered at dietary levels of 200, 650, and 2000 ppm (from 9-35 mg/kg/d to 100-350 mg/kg/d), no overt reproductive effects were seen. At the highest NP dose (~300 mg/kg/d), significantly decreased body weights were observed in both sexes from the F_1, F_2, and F_3 generations. At the next lower dose level (~55 mg/kg/d), pnd 21 and terminal body weights were sometimes decreased, but not simultaneously, in both sexes. NP elicited increased kidney weights in males at the intermediate and high doses, and kidney histopathology characteristic of hyaline droplet pathology starting at the lowest dose of 200 ppm nonylphenol (12-18 mg/kg/d). Increased kidney weights were also observed in female offspring in the highest dose groups. For potentially oestrogen-sensitive endpoints, significant changes included increases in oestrous cycle length at the top dose, a decrease in absolute ovarian weight at the top dose in all three offspring generations (but relative ovarian weights decreased only in the F_2 generation), and vaginal opening was accelerated at the top and intermediate doses in all offspring generations. Male sperm counts were decreased at the high and intermediate doses in the F_2 generation, but were not affected the F_1 generation. Other changes in oestrogen sensitive endpoints were inconsistent and not dose-related.

251. With OP, a positive response in the uterotrophic bioassay was not predictive because this chemical does not elicit any of the adverse effects that would expected from an oestrogenic mode of action in a robust reproductive and developmental testing protocol. With NP, the primary effect observed in the Chapin *et al.* (1999) study was histopathological evidence of nephrotoxicity at the lowest (200 μg/g) dietary level. The most sensitive change in potentially oestrogen sensitive endpoints was an acceleration of vaginal opening at the 650 μg/g NP dietary level (~55 mg/kg/d). The authors expressed uncertainty if this modest increase can be considered an adverse effect. However, the changes in the oestrous cycle at the highest dose level were judged to be an adverse effect. The Nagao *et al.* (2001)

study repeated the observation on vaginal opening at 50 mg/kg/d by oral gavage, but did not find evidence of adult nephrotoxicity at a similar dose level.

Bisphenol A

252. Three studies are identified for BPA. The earliest was a continuous breeding study in mice with dietary levels of 2,500, 5,000, and 10,000 ppm BPA (estimated intakes of 437, 875, and 1,750 mg/kg/d) (Reel *et al.*, 1985). This study observed evidence of systemic and organ toxicity as well as reproductive toxicity. Although relative epididymis weights decreased, changes in testes and sex accessory gland weights were not dose-related, and no effects were observed on epididymal sperm number or morphology. Overall, while the endpoints in the study are insufficient to define an oestrogenic mode of action, the study suggests that other toxicities may be elicited confounding interpretation.

253. Ema (2000) conducted a two-generation study using gavage administration at doses from 0.2 to 200 μg/kg/d BPA. This study was intended to address the so-called low dose hypothesis, and no adverse substance related effects were observed. This study is not included in **Table 35** as no adverse substance related effects were seen.

254. Tyl *et al.* (2001) conducted a multiple-generation study in rats employing 6 dietary levels to produce BPA doses from 1 μg/kg/d to 500 mg/kg/d. The study was based on the recently revised USEPA guidelines and included several enhancements for detecting oestrogen related effects including extended dosing of the F_2 generation to better determine developmental benchmarks and some reproductive parameters. Significant body weight reductions (>25%) were observed at 50 and 500 mg/kg/d. Reduced litter sizes, several changes in non-oestrogen target organs, and female renal and hepatic histopathology, were also observed at 500 mg/kg/d. After adjusting for body weights, no effects were observed in males on sperm parameters, testes weights, weights of other accessory reproductive tissues, or histopathology of these same tissues. After adjusting for body weights, the only effect observed in the female reproductive tract was decreased ovarian weights at 500 mg/kg/d. Other female parameters, including oestrous cyclicity, were not affected. Both vaginal opening and preputial separation were delayed at 500 mg/kg/d. Given that these effects are correlated with body weight, these changes were not attributed to any hormonally-mediated effect. Further, the delay in vaginal opening is opposite to the expected acceleration for an oestrogenic mode of action.

255. BPA did not elicit adverse effects expected from an oestrogen mode of action in a robust and well conducted multiple generation study in rats. The Tyl *et al.* (2001) study demonstrates the occurrence of systemic and organ toxicities, but there was a lack of any expected oestrogen-mediated effects at BPA doses up to 500 mg/kg/d. Adverse effects associated with other toxicities (*e.g.*, systemic body weight losses) were observed. The Ema (2000) study is consistent with that of Tyl *et al.* (2001) and supports the lack of any oestrogen mediated effects where the uterotrophic doses were negative.

o,p'-DDT

256. For *o,p'*-DDT, the reported studies are less comparable and examine fewer oestrogen-sensitive endpoints. Several studies were reviewed for *o,p'*-DDT, none of which are multi-generational reproductive assays, and all are older toxicity studies with a very restricted number of oestrogen sensitive endpoints. Ovarian effects possibly related to an oestrogen mode of action were observed by Gellert *et al.* (1972, 1974, 1975) in a series of experiments, and an acceleration in vaginal opening at 2.5 mg/kg/d were observed by Wrenn *et al.* (1970, 1971). Clement and Okey (1972) observed accelerated vaginal opening with all doses of *o,p'*-DDT in the diet, including the lowest dose of ~50 mg/kg/d. Their rats were exposed to the test substance diet beginning on pnd 23 and vaginal opening occurred as early as pnd 27,

suggesting that even lower doses could be effective. Collectively, the evidence is highly suggestive, but not confirmatory, of oestrogen mediated effects. These studies are also summarised in **Table 35**.

Comparison With LOELs

257. To expand this comparison, the data on positive uterotrophic doses and various low observable effect levels (LOELs) are summarised in **Table 36**. As the effect most often recorded at the lowest dose with weak oestrogen agonists was acceleration of vaginal opening, other data on vaginal opening from Laws *et al.* (2000) have also been included in **Table 36** for an additional consistency check. First, the data shows that a positive result in the uterotrophic bioassay is not always a predictor of adverse effects in a definitive testing assay. Second, the data support that a negative dose in the uterotrophic bioassay remains negative in a definitive testing assay. Third, no evidence has been found to date for the uterotrophic bioassay giving a false negative prediction.

Table 35. Comparison of bioassay results for oestrogen-sensitive endpoints*

	17β-stradiol	**Bisphenol A**	**Genistein**	**Octylphenol**	**Nonylphenol**	**Methoxychlor**			***o,p'*-DDT**	
Endpoint	Biegel *et al.* (1998)	Tyl *et al.* (2000)	Casanova *et al.* (1999)	Tyl *et al.* (1999)	Chapin *et al.* (1999)	Chapin (1999)	Chapin *et al.* (1997)	Gray *et al.* (1989)	Gellert *et al.* (1972, 1974, 1975)	Wrenn *et al.* (1970, 1971)
Parental generation										
ovary	0.17 mg/kg/d	[2]	Not done [3]	300 mg/kg/d	No effect [4]	Not applicable [5]	Not applicable [6]	Not applicable [7]	Not applicable [8]	Not applicable [9]
uterus	0.55 mg/kg/d	No effect	Not done	No effect1[10]	No effect	Exposure begun	Exposure begun	Exposure begun	Various models	Exposure begun
testes	0.55 mg/kg/d	No effect	Not done	No effect	No effect	on gestation	on gestation	on postnatal	or postnatal	on postnatal
epididymis	0.55 mg/kg/d	No effect	Not done	No effect	No effect	day 14	day 14	day 21	exposure	day 18
other male tissues	0.55 mg/kg/d		Not done	No effect	No effect	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
sperm parameters	0.55 mg/kg/d	No effect	Not done	No effect	No effect	Not applicable	ot applicable	Not applicable	Not applicable	Not applicable
F_1 generation in repro studies or treated offspring in developmental studies										
	Pnd 21 analysis	**Adult analysis included**	**Adult analysis included**	**Adult analysis included**	**Adult analysis included**	**Adult analysis included**	**Adult analysis included**	**Adult analysis included**	**See above**	**See above**
Ovary	0.17 mg/kg/d[11]		No done	No effect	Abs 212 mg/kg/d[12]	Not done	50 mg/kg/d	100 mg/kg/d [13]	10 mg/kg/d	No effect
Uterus	0.17 mg/kg/d[11]	No effect	15 mg/kg/d	No effect	No effect	Not done	50 mg/kg/d	Not done	No effect	No effect
Testes	0.14 mg/kg/d[11]	No effect	No effect	No effect	Abs 171 mg/kg/d[12]	50 mg/kg/d Abs[14]	50 mg/kg/d	100 mg/kg/d [13]	No effect	Not done
Epididymis	0.14 mg/kg/d[11]	No effect	Not done	No effect	No effect	50 mg/kg/d Abs[14]	50 mg/kg/d	50 mg/kg/d [13]	No effect	
other male tissues	0.14 mg/kg/d[11]	No effect	No effect on ven. Prostate	No effect	Ven. Prostate Abs 171 mg/kg/d[12]	150 mg/kg/d Abs[14]	50 mg/kg/d	50 mg/kg/d [13]	No effect	No effect

sperm parameters	0.003 mg/kg/d	No effect	Not done	No effect	No effect	150 mg/kg/d	150 mg/kg/d	50 mg/kg/d	Not done	Not done

Table 35 (continued). Comparison of bioassay results for oestrogen-sensitive endpoints*

	17β-oestradiol	**Bisphenol A**	**Genistein**	**Octylphenol**	**Nonylphenol**	**Methoxychlor**			***o,p'*-DDT**	
vaginal opening	0.003 mg/kg/d	Delayed – 500 mg/kg/d	15 mg/kg/d	No effect	68 mg/kg/d	Not done	5 mg/kg/d	25 mg/kg/d	Not done	2.5 mg/kg/d
preputial separation	0.17 mg/kg/d[11]	Delayed – 500 mg/kg/d	No effect	No effect	171 mg/kg/d	Not done	50 mg/kg/d	100 mg/kg/d	Not done	Not done
estrous cycle	Not done	Not reported	Not done	No effect	212 mg/kg/d	Not done	50 mg/kg/d	Not done	Not done	Not done
Other parameters		16-26% body wt decreases at 500 mg/kg/d	No effect on AGD	Paired ovarian follicle count – no effect		No effect on prostate androgen receptor levels	No effect on male AGD. Female mating trials 50 mg/kg/d and males 150 mg/kg/d	None measured	Not done	Not done
Endpoint	Biegel *et al.* (1998)	Tyl *et al.* (2000)	Casanova *et al.* (2000)	Tyl *et al.* (1999)	Chapin *et al.* (1999)	Chapin (1999)	Chapin *et al.* (1997)	Gray *et al.* (1989)	Gellert *et al.* (1972, 1974, 1975)	Wrenn *et al.* (1970, 1971)
F_2 generation										
	Not done	**Pnd 21 analysis**	**Pnd 21 analysis**	**Pnd 21 analysis**	**Adult analysis included**	**Not done**	**Some adult**	**Some adult**	**Not done**	**Not done**
Ovary	Not done		Not done	No effect	72 mg/kg/d	Not done	Not done	No effect [1]	Not done	Not done
Uterus	Not done	No effect	Not done	No effect	No effect	Not done	5 mg/kg/d	Not done	Not done	Not done
Testes	Not done	No effect	Not done	No effect	188 mg/kg/d	Not done	50 mg/kg/d	No effect[1]	Not done	Not done
Epididymis	Not done	No effect	Not done	No effect	Not done	Not done	50 mg/kg/d	No effect[1]	Not done	Not done
other male tissues	Not done	No effect	Not done	No effect		Not done	150 mg/kg/d	No effect[1]	Not done	Not done
sperm parameters	Not done	No effect	Not done	No effect	58 mg/kg/d	Not done	Not done	No effect[1]	Not done	Not done
vaginal opening	Not done	Delayed – 500 mg/kg/d	Not done	No effect	72 mg/kg/d	Not done	Not done	50 mg/kg/d[1]	Not done	Not done
preputial separation	Not done	Delayed – 500 mg/kg/d	Not done	No effect	188 mg/kg/d	Not done	Not done	No effect[1]	Not done	Not done

Table 35 (continued). Comparison of bioassay results for oestrogen-sensitive endpoints*

	17β-oestradiol	Bisphenol A	Genistein	Octylphenol	Nonylphenol	Methoxychlor			*o,p'*-DDT	
estrous cycle	Not done	Not reported	Not done	Not done	216 mg/kg/d	Not done	Not done	50 mg/kg/d[1]	Not done	Not done
F_3 generation (pnd 21 analysis in 3-gen nonylphenol study only)										
Ovary	Not done	Not done	Not done	Not done	Abs 320 mg/kg/d	Not done	Not done	Not done	Not done	Not done
Uterus	Not done	Not done	Not done	Not done	No effect	Not done	Not done	Not done	Not done	Not done
Testes	Not done	Not done	Not done	Not done	Abs 320 mg/kg/d _	Not done	Not done	Not done	Not done	Not done
Epididymis	Not done	Not done	Not done	Not done	No effect	Not done	Not done	Not done	Not done	Not done
other male tissues	Not done	Not done	Not done	Not done	No effect	Not done	Not done	Not done	Not done	Not done

* Highest dose for which measurements were conducted was 50 mg/kg/d.
[1] Endpoints are susceptible to a number of modes of action; an oestrogen mode of action is only one of several possibilities.
[2] Maximum dose of bisphenol A for no effect is 500 mg/kg/d. [3] Maximum dose of genistein for no effect is 15 mg/kg/d. [4] Maximum dose of nonylphenol for no effect is ~200 mg/kg/d, depending upon sex and group. [5] Maximum dose of methoxychlor for no effect is 150 mg/kg/d. [6] Maximum dose of methoxychlor for no effect is 150 mg/kg/d. [7] Maximum dose of methoxychlor for no effect is 200 mg/kg/d. [8] Maximum dose of *o,p'*-DDT for no effect is 100 mg/kg/d. [9] Maximum dose of *o,p'*-DDT for no effect is 3 mg/kg/d. [10] Maximum dose of octylphenol for no effect is 300 mg/kg/d
[11] Highest dose available with F1 progeny; in higher doses no progeny were produced. [12] Relative weights were not significant. [13] Absolute weights significant, but relative weights not calculated even though there was a significant difference in terminal body weights. [14] Relative weights were statistically significant for testis only at 150 mg/kg/d with no effects on epididymis or other male accessory tissues (terminal body weights at these doses were significantly lower than controls).

Table 36. Comparison of vaginal opening, uterotrophic positive doses, and reported NOELs and LOELs

Chemical	Dose accelerating vaginal opening in mg/kg/day (days accelerated) Laws *et al.* (2000)	Uterotrophic bioassay – Positive Dose Literature oral gavage (po) dose in (mg/kg/day)	LOEL from toxicity studies	
			Dose (mg/kg/day)	Endpoint
Genistein	ND	15-50 (Breinholt *et al.*, 2000; Cheng *et al.*, 1954; Farmakalidis and Murphy, 1984b; Farmakalidis *et al.*, 1985; Perel and Linder, 1970)	50-75	Vaginal opening accelerated (Casanova *et al.*, 1999) Latent carcinogenesis (Newbold *et al.*, 2001b)
Methoxychlor	50 (8.4 d)	16-30 (Gray *et al.*, 1999; Laws *et al.*, 2000; Odum *et al.*, 1997)	5	Vaginal opening accelerated (Chapin *et al.*, 1997)
o,p'-DDT	ND	< 100 (Diel *et al.*, 2000)	2.5	Vaginal opening accelerated (Wrenn *et al.* 1970, 1971; Clement and Okey, 1972)
Octylphenol	200 (3.18)	≥ 200 (Diel *et al.*, 2000; Gray *et al.*, 1999; Laws *et al.*, 2000)	300	Based on systemic and other organ toxicities, no estrogen mediated effects observed at the highest dose. (Tyl *et al.*, 2000)
Nonylphenol	25 (1.5 d) 50 (5.3 d)	45-60 (Laws *et al.*, 2000; Odum *et al.*, 1997, 1999a, 1999b)	68	Vaginal opening accelerated (Chapin *et al.*, 1999)
Bisphenol A	400 – negative (top dose)	400-600 (Ashby and Tinwell, 1998; Diel *et al.*, 2000; Laws *et al.*, 2000; Matthews *et al.*, 2001; Tinwell *et al.*, 2000b; Yamasaki *et al.*, 2000)	50	Based on systemic and other organ toxicities, no estrogen mediated effects observed up to 500 mg BPA/kg/day. (Tyl *et al.*, 2001)

CHAPTER 9: POSSIBLE USE OF THE UTEROTROPHIC BIOASSAY IN THE TESTING AND ASSESSMENT OF CHEMICALS

258. A hierarchical or tiered assessment for a chemical having a possible oestrogenic mode of action has been suggested by previous expert workshops (Carney *et al.*, 1997; EDSTAC, 1998; EU, 1997; Gray *et al.*, 1997; Kupfer, 1988; OECD, 1988b; Reel *et al.*, 1997; SETAC-Europe, 1997). These experts envisioned evaluation of all existing data, a structure-activity assessment, and that several possible *in vitro* assays would be used initially to screen substances for further investigation. These relatively rapid and inexpensive steps would be used to identify candidate substances and to eliminate other chemicals from further study.

259. The uterotrophic bioassay is intended as a short term assay that fits between consideration of structure-activity relationships and longer-term bioassays. The available evidence summarised in this background document supports the judgement of these experts. Firstly, the uterotrophic response is indicative of an oestrogenic mode of action *in vivo*. Secondly, there is a need for the assay due to an extensive number of pharmacodynamic and pharmacokinetic factors which come into play in the intact animal that makes the extrapolation of *in vitro* data uncertain without confirmatory *in vivo* data. Thirdly, there is support for the uterotrophic bioassay to properly identify candidates for further in-depth testing, such as for reproductive and developmental effects.

260. In previous workshops and deliberations, the consensus of expert opinion has envisioned the regulatory use of the uterotrophic bioassay within a hierarchical array or series of tiers (Carney *et al.*, 1997; EDSTAC, 1998; EU, 1997; Gray *et al.*, 1997; Kupfer, 1988; OECD, 1998b; Reel *et al.*, 1997; SETAC-Europe, 1997). This includes the Weybridge workshop in the EU that was co-sponsored by the OECD, as well as the parallel EDSTAC activity in the US. The initial tiers preceding the uterotrophic bioassay would be:

- The compilation and evaluation of available toxicological data, particularly for reproductive and developmental tests, to determine if there were already adequate data on a substance.
- An assessment of the likelihood that the test substance is an ER ligand or can be metabolised to an ER ligand based on quantitative structure-activity relationships.
- The application of *in vitro* assays to test the structural prediction after the substances have been prioritised. Examples are ER-binding assays and either yeast or cultured cell reporter-gene systems.

261. Collectively, these procedures and assays should adequately prioritise candidates for which additional information should be gathered. From an animal welfare perspective, these steps would substantially reduce the use of animals by removing negative substances from being tested in the uterotrophic bioassay. The uterotrophic bioassay could then would then be used in a regulatory context to assess the possible biological activity of the identified candidate chemicals *in vivo.* A positive result would suggest the need for substances to advance to further in-depth reproductive and developmental testing.

262. The results of the uterotrophic bioassay could be used in an number of ways in regulatory testing strategies. For example, in the instance of a positive result, the dose-response characteristics of the uterotrophic bioassay would be one factor in the decision to proceed, and in the priority assigned to a particular substance. Alternatively, the uterotrophic bioassay or an *in vitro* assay could also be used to seek the mode of action for a substance already found to have adverse effects and whose pattern of effects suggested a possible oestrogenic mode of action. In the case of a negative

uterotrophic result with a valid protocol, a relevant route of administration and a sufficiently high dose, such as a limit dose of 1000 mg/kg/d, the testing for an oestrogen mode of action would not appear to be warranted.

263. The data summarised in this document provide broad support for the validation and regulatory use of the uterotrophic bioassay as an *in vivo* screen for possible oestrogen agonists and antagonists:

- Structure-activity relationships and *in vitro* assays appear able to identify substances with an oestrogenic mode of action as candidates for the uterotrophic bioassay, thereby minimizing the use of resources and animals.

- Clear evidence supports an oestrogen mode of action that begins with the binding of a ligand to the ER. This binding initiates a cascade of molecular, biochemical, and physiological events that culminate in uterine growth, which is measured gravimetrically in the uterotrophic bioassay.

- The extensive history of the uterotrophic bioassay supports the ability of the assay to evaluate the oestrogenic potential of substances, even weak oestrogen agonists with log RBAs <1 and >3.

- The two major versions for the uterotrophic bioassay, the intact sexually immature rat and the OVX sexually mature rat, appear to be technically equivalent.

- The major procedural variables for the uterotrophic protocol are known. The OECD protocol used for its validation studies is justified.

- A sufficient number of laboratories have the technical skill, equipment, and facilities to conduct the uterotrophic bioassay.

- The overall reproducibility and specificity of the uterotrophic bioassay is adequate, and the limits of its application to different classes of chemicals are evident. In regards to specificity, modest increases in uterine weight (20-40%) at high doses present the possibility that a false positive result may have occurred. Therefore, clear criteria for data acceptance, *e.g.*, maxima for acceptable vehicle control uterine weights, and clear criteria for interpretation, must be defined.

- The data clearly demonstrate that pharmacodynamics and pharmacokinetic factors in the intact animal can modify the activity of a test substance. This supports 1) the need to use a relevant route of administration for the individual test substance, and 2) the benefits of a hierarchical testing strategy when selecting the most appropriate tests.

- There is a general correspondence between the uterotrophic bioassay and the testing outcomes for adverse effects.

- Several substances that are positive in the uterotrophic bioassay have elicited oestrogen-mediated effects in reproductive and developmental assays.

- Other substances that are positive in the uterotrophic have not elicited oestrogen-mediated effects in reproductive and developmental assays, *i.e.*, false positives occur.

- No evidence for a false negative prediction by the uterotrophic bioassay has been found when the original result was sound and reproducible.

- At doses where no evidence for adverse effects has been found in robust reproductive and developmental assays, the uterotrophic bioassay has been negative by a similar route of administration.

264. In summary, the available data support the fitness of the uterotrophic bioassay for identifying those substances that may act though an oestrogen mode of action and warrant consideration of further testing for adverse effects.

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ANNEX:

EXTRACTION OF PUBLISHED LITERATURE FOR ASSAYS USING THE INCREASE IN UTERINE WEIGHT AS A METRIC FOR OESTROGENIC ACTIVITY.

This Annex is a basic abstraction of various published papers since the 1930s for the uterotrophic (or uterotropic, as the spelling varies) assay. The actual protocol has varied widely (e.g., species, strain, diet, vehicle, routes of administration (intravenous, intramuscular, dietary, oral gavage, subcutaneous, and intraperitoneal), group size, uterine preparation (wet with intraluminal fluid, blotted with the fluid removed, or dried under heat to remove all fluid, etc.). The basic metric is an increase in uterine weight. However, the exact measurement reported has varied with absolute weight used in most cases and the increase in the uterine weight expressed as a percentage or ratio relative to the body weight in other cases. The vaginal cornification responses (Allen-Dosiy test) are considered a separate screening assay for estrogen, and are not included in this summary. Were described in the paper material and methods, these parameters have been abstracted along with notes on uterine control weights, other assays performed in conjunction with the uterotrophic assay, and notes about significant findings or apparent anomalies. Other assays conducted include a number of the early molecular, cellular, and tissue events of the uterotrophic response that culminates in mitotic events and uterine tissue weight gain. The annex is organised into a series of tables as follows:

1. At least two days of consecutive test substance administration to the rat:

    - Table 1A–1: focusing on 3 days of consecutive s.c. or oral administrations with necropsy on the 4th day to the immature, intact animals (the 'standard' assay) which covers the basic OECD protocols A and B as different routes of administration are included in the table.
    - Table 1A-2: focusing on 3 days of consecutive s.c. or oral administrations with necropsy on the 4th day to the adult OVX animals (a second form of the 'standard' assay) which covers the basic OECD protocol C, again different routes of administration are included in the table.
    - Table 1A-3: using variations of the administration time, route (i.p., i.v., dietary, dermal, dietary, etc.) or other procedures with 1) immature; intact 2) immature, OVX animals; and 3) older OVX animals of varying ages. In a few cases there are 4 or more days of administration or administration of a test substance (possibly covering OECD protocol C prime).

2. At least two days of consecutive administration to the mouse:

    - Table 1B: This includes all of the variations noted for the rat, but due to the fewer number of reports using the mouse, these have not been separated into sub-tables.

3. A single administration, typically followed by necropsy within 24 hours. This is typically called the Astwood assay:

    - Table 2A: compilation of rat data
    - Table 2B: compilation of mouse data

The tables then reflect the diversity of historical practice for the uterotrophic protocol. A number include work with antiestrogens, receptor binding or competition assays, other molecular and cellular assay performed in parallel with uterine weight measures, and even other tissues such as the vagina. It should also be apparent that, until recently, use for very weak partial agonists in the 10-1000 mg/kg/dose range was relatively rare until the recent surge in interest on this class of substances.

Note: In all tables, there may be a variation in the uterine preparation (wet, blotted, or dried), in metric used (absolute mg uterine weight or relative to 100 g body weight), etc., and some effort is made to capture these.

Citations for the extracted literature are listed in the attachment to this Annex.

Table 1A -1. Uterotrophic assays in immature, intact rats. Assays involving 3 daily consecutive administration of test compounds using either subcutaneous or oral gavage routes.

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
RAT					
Immature, intact animals and 3 days administration ('standard' uterotrophic assay)					
Acton *et al*. (1983)	Rat – no strain given	Immature, intact/ 21 days	Oral gavage/ 3 days	26 synthesised estrogenic derivatives (dibenz[b,f]oxepins, dibenzo[b,f]-thiepins, dibenzo[a,e]cyclooctenes, and dibenzo[b,f]thiocins)/ receptor binding and antiimplantation activity.	5 rats/group; Tween 80 vehicle. Not specified if wet or blotted uterine wt. Results reported as relative to estradiol group (agonist) or inhibition of coadministered oestradiol (antagonist)
Allen *et al*. (1980)	Rat - Alderly Park	Immature, intact/ No age given (35 to 50 g bw[14])	Subcutan.[15]/ 3 days	Ethinyl oestradiol, 17β-oestradiol, mestran-ol, oestradiol benzoate (EB), tamoxifen, fluorotamoxifen, chlorotamoxifen, methyltamoxifen, monohydroxyltam-oxifen, methoxytamoxifen/ Relative receptor binding and vaginal cornification when instilled into vagina.	Minimum 7 rats/group; arachis oil vehicle. Blotted uterine weights 'pressed between sheets of blotting paper to remove intraluminal fluid.' Vehicle control uteri varied ~20-48 mg among 4 experiments; sd[16] estimate ± 4 mg. 5X weight increase using 0.5 µg daily EB. Agonist and antagonist actions in the uterotrophic assay.
Ashby and Tinwell (1998)	Rat - Alderly Park (Alpk:AP)	Immature, intact/ 21-22 days, 38-48 g bw	Subcutan. and oral gavage/ 3 days	Diethylstilbestrol, bisphenol A/ dry uterine weights, premature vaginal opening	5 rats/group for DES, 7/group for BPA, and 10/group for controls. Vehicle control uteri 25.04 ± 4.05 mg, 27.7 ± 7 mg, 29.1 ± 5.2 mg, and 31.9 ± 5.6 mg. Arachis oil vehicle. 'Uteri … pierced, and blotted to remove excess fluid.' 3.5-fold DES increase at 40µg/kg/day, max BPA increase 1 fold at 800 mg/kg/day.
Ashby *et al*. (1997a)	Rat - Alderly Park (Alpk:AP)	Immature, intact and adult OVX / 21-22 days, 38-48 g bw	Oral gavage/ 3 days	17β-oestradiol, raloxifene, ICI182,780/ Vaginal opening in all treatment groups, vaginal cytology (includes endometrial height, number of glands, and mitotic number in some groups).	5-7 rats/group. Immature appears slightly more sensitive than OVX. Vaginal opening in some 17β-oestradiol immature rats; vaginal cornification not observed in raloxifene individuals with increased uterine weights; ICI 182, 780 did inhibit uterine weight increase, cell mitotic figures were generally observed where uterine weights were increased. 'Blotted to remove excess fluid' and oven dry uterine weights. Control immature uteri ~25 mg; sd estimate ± 5 mg. OVX ~80 mg.

[14] bw – body weight
[15] Subcutan. - subcutaneous
[16] sd – standard deviation of the mean

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds.

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Ashby *et al.* (1997b)	Rat - Alderly Park (Alpk:AP); mouse Alpk:AP	Immature, intact & OVX/ 21-22 days & 7 wk ovx used 2 wk later	Oral gavage/ 3 days, clofibrate 2X per day	17β-oestradiol, benzoic acid, clofibrate/ vaginal opening, vaginal cornification, and dry as well as wet uterine weights.	5-10 rats/group; vehicle arachis oil. Immature control uterine weights 25.5 to 37.5 mg. OVX 81.3 mg. Neither test compound indicated response, i.e., both failed to reproduce previously reported results in other labs.
Ashby *et al.* (1999a)	Rat - Alderly Park (Alpk:AP); mouse Alpk:AP	Immature, intact/ 21-22 days, 38-48 g bw	Subcutan. and oral gavage/ 3 days,	17β-oestradiol, diethylstilbestrol, resveratrol/ estrogen receptor binding, transfected cell assays with both α and β estrogen receptor, vaginal opening, vaginal cornification, cell mitotic index included, dry as well as wet uterine weights.	5 –10 rats or mice/group; rat vehicle arachis oil. Eight controls in repeat experiments ranging from 25.5 to 43 mg (seven ≤ 32 mg). Blotted uterine weights. Resveratrol was positive in dose responsive manner in only one of eight experiments despite being a weak agonist in *in vitro* experiments.
Ashby *et al.* (1999b)	Rat - Alderly Park (Alpk:AP)	Immature, intact/ 21-22 days, 38-48 g bw	Oral gavage/ 3 days,	oestradiol benzoate, coumestrol, faslodex or ICI 182,780/ vaginal opening, vaginal and cervical weights, DNA content, endometrium and luminal epithelium height (morphometry), BrdU labeling	6-7 rats/group; arachis oil vehicle. Both wet and blotted uterine weights recorded as well as oven dry. Coumestrol was active when administered with oestradiol benzoate. Other markers of activity were relatively consistent with wt increase. The coumestrol activity was inhibited by the faslodex antiestrogen.
Baker *et al.* (1999)	Rat - Wistar	Immature, intact/ 22-23 days 37-53 g bw	Oral gavage/ 3 days	17β-oestradiol, diethylstilbestrol, coumestrol, ß-sitosterol, phytosterol mixture (47.% sitosterol, 28.8% campesterol, and 23.3.% stigmasterol)/ estrogen receptor competitive binding, yeast reporter gene assays	10 rats/group; arachis oil as vehicle. Control uteri appear slightly higher than average in most reports (~40 mg). Phytosterols and phytosterol esters were negative in receptor, yeast reporter, and uterotrophic bioassays.
Bicknell *et al.* (1995)	Rat - Wistar	Immature, intact/ 23 day	Subcutan./ 3 days	diethylstilbestrol, octylphenol (OP) or (4-*tert*-octyl)-phenol/ Uterine histologic cross section and brain preoptic area histology.	5 rats/group; ethyl oleate as vehicle. 10 mg OP/day yielded 100% uterine weight increase (2X). Control uteri 39 mg ± 4 mg; 56 g bw.
Bhavnani and Woolever (1991)	Rat - Sprague Dawley	Immature, intact/ 21 days	Subcutan./ 3 days	17β-oestradiol, equilin, equilenin, 17β-dihydroequilin, 17β-dihydroxy-equilenin, 17α-dihydroequilin, 17α-dihydroequilenin, β, estrone, 17α-oestradiol/ receptor binding and ligand competitive assays (rat and human tissues)	10 rats/group; sesame oil vehicle. Not specified whether wet or blotted weights used. Results expressed as absolute weight (mg), uterine wt/ bw ratio, and as percentage increase over controls.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Black and Goode (1980)	Rat - Holtzman	Immature, intact/ 19-20 days, 40-45 g bw	Subcutan./ 3 days	17β-oestradiol, tamoxifen, trioxifene and LY-117018/	6 rats/group; corn oil vehicle. Control uteri were ~25 mg. Uteri were blotted.
Brooks *et al.* (1971)	Rat - Carworth	Immature, intact/ 21 days	Oral gavage/ 3 days	diethylstilbestrol, zearalane, 7'-formyl zearalane, A isomer and B isomer 7'-formyl zearalane, 7'-carboxy zearalane, A isomer and B isomer 7'-carboxy zearalane	6 rats/group; sesame oil vehicle. Uteri were blotted. Control uteri in 23 rats 28 mg (not to 0.1 mg measurement).
Cano *et al.* (1986)	Rat - Wistar	Immature, intact/ 21 days	Subcutan./ two times 3 days and 17 days	danazol and testosterone/ vaginal smears, binding affinity	5-9 rats/group; propylene glycol vehicle. Not specified whether wet or blotted weights used. Control uteri at 3 days were 62 ± 16 mg.
Chandra *et al.* (1982)	Rat - no strain given	Immature, intact/ 19-21 days 15-25 g bw	Oral gavage/ 2X daily	clofibrate (CF), phenylbutazone (PHB), ethinyl oestradiol (EE)/ std. uterotrophic and some groups followed by EE administration in a modified Astwood design.	Body weights unusually low. Results expressed as g uterine wt/g bw. 6-17 rats/group; arachis oil vehicle. CF ~50% over controls at 2mg/kg/d, PHB ~20% at 50 mg/kg/d. Ashby et. (1997b) could not reproduce results.
Christian *et al.* (1998)	Rat - Wistar and Sprague Dawley	Immature, intact/ 21-22 days	Oral gavage/ 2X daily for 4 days	Statistically analysis of untreated, vehicle controls, and positive reference controls (DES) 218 rats total	10 rats/group; sesame oil vehicle. Wet, non-blotted weights (care taken to avoid fluid loss). 4-day administration, 2X daily. Control uteri 52-80 mg A certain proportion of rats when administration begun on day 22 apparently begin to enter puberty prior to the end of the study, generating high outliers. Table 8 contains statistical summaries of the various strains, blocks, outliers, etc. Shown in even Figures from 6-16 for various groups.
Connor *et al.* (1996)	Rat - Sprague Dawley	Immature, intact	Oral gavage and i.p./ 3 days	17β-oestradiol (E), atrazine, simazine	5 rats control and 4 rats treated/group; 5% hydroxypropyl cellulose vehicle. Wet uterine weights used; controls ~50 mg. 4-6 fold increase using 10 µg oestradiol daily.
DeSombre *et al.* (1988)	Rat - Sprague Dawley	Immature, intact/ 22 days	Subcutan./ 3 days	17β-oestradiol and 12 triphenylhalo-ethylene derivatives/ estrogen receptor binding	3-10 rats/group; sesame oil-ethanol and saline-ethanol vehicles used. Not specified whether wet or blotted weights used (termed wet, but oven dry weights also reported). Controls appear to be in 35-45 mg range from Figures.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
di Salle *et al.* (1990)	Rat - Sprague-Dawley	Immature, intact/ 21 days	Oral gavage/ 3 days	oestradiol benzoate (EB), tamoxifen, toremifene/ uterine receptor-binding assays and DMBA-induced mammary tumor response	6 rats/group; 0.5% methylcellulose vehicle for test compounds, sesame oil for EB by s.c. Control uteri in several expts–appear to be ~30, 45, and 48 mg. Stated that uteri were wet, but not specific if contents retained.
Dorfman and Dorfman (1954)	Rat - albino	Immature, intact/ 22-23 days	Subcutan. and oral gavage/ 3-4 days	series of sulfates: estrone sulfate, equilin sulfate, reduced estrone (17β-oestradiol) sulfate, dihydroequilin sulfate	7-32 rats/group. Data expressed as uterine/body weight ratio.
Duby *et al.* (1971)	Rat - Sprague Dawley	Immature, intact and adult OVX 21 day old immature	Subcutan. and dietary/ 1,2,3 day time series	estrone, *p,p'*-DDT, *o,p'*-DDT	Unknown group size; corn oil vehicle. Blotted uterine weights used. ~27-32 mg control uteri. OVX rats were fed compounds for 175 days.
Dukes *et al.* (1994)	Rat - Alderly Park; mice	Immature, intact and adult OVX/ no age or body weights, ref Wakeling *et al.* (1983)	Subcutan. and oral gavage/ Immature 3 day assays. OVX 7, 14, and 28 day treatment regimens.	17β-oestradiol benzoate, ZM189,154, tamoxifen	5 rats/group, 2 replicates; arachis oil vehicle. Control uteri ~25 mg in immature; 85 and 173 mg in two sets of adult animals of different ages. Comparative ZM 189,154 data: ED_{50} 0.09 mg/kg s.c. and 0.7 mg/kg oral in immature rat ED_{50} using oral administration was 0.7 mg/kg in immature rats, 1.3 mg/kg in OVX rats, and 6.2 mg/kg in OVX mice.
Edery *et al.* (1985)	Rat - Sprague Dawley	Immature, intact/ 20 days, 25-35 g bw	Subcutan./ 3 days	17β-oestradiol, tamoxifen, cis-broparoestrol, trans-broparoestrol/nucleic acid and protein assays of uterus	10-14 rats/group; peanut oil vehicle. Not specified whether wet or blotted weights were used, but reference Rubin procedure (used blotted wts). Control uteri 40.2 ± 9.8 mg.
Everett *et al.* (1987)	Rat - Sprague Dawley	Immature, intact/ ~19 days	Subcutan. and oral gavage/ 3 days	17β-oestradiol, 7α-zearalenol, zeranol, zearalanone, taleranol, zearalenone, 7β-zearalenol	9-10 rats/group; sesame oil vehicle. Dose response curves for compounds. Oestradiol and zeranol both oral and s.c. curves. Not specified wet or blotted weights. Control uteri (3 groups): 23.1, 22.8, and 27.0 mg.
Fail *et al.* (1998)	Rat - Sprague Dawley	Immature, intact/ 21 days, 35 - 64 g bw	Oral gavage/ 3 days	Diethylstilbestrol (DES), 17β-oestradiol and 7 polystyrene extracts/yeast reporter gene assays in parallel	10 rats/group; sesame oil vehicle. Wet uterine weights, included luminal fluid. Control uteri were 57.5 ± 3.1 mg. DES given both s.c. and oral, but dosage insufficient to make route comparison.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Ferguson and Katzenellen-bogen (1975)	Rat - supply lab Holtzman, WI, strain not named	Immature, intact/ 20-21 day (no body weights)	Subcutan./ 3 days	17β-oestradiol; Upjohn antiestrogens UA, U-23, and 94X; Parke-Davis antiestrogens CI-628 and CI-680 (all structures in Fig. 1)/ Receptor binding competition, sucrose gradient receptor binding, temporal course of estrogen receptor levels in cytosol and nucleus of uterus (time course), protein synthesis induction and inhibiting estrogen uterine weight increase with single injection at 36 and 72 hours	10 rats/group; 0.15M NaCl (saline) vehicle. Control uteri ~35 mg. Estrogen at 5 μg levels increases uterine weights 3.6 fold or 363% ± 34%. Injections of two materials at separate sites. Results suggest antagonistic potency involves molecular structure, ability to translocate receptor from cytosol to nucleus, and temporal duration of occupancy of uterine receptors in cell nuclei.
Franks *et al.* (1982)	Rat - Sprague Dawley	Immature, intact/ 23 days	Subcutan./ 3 days, 2x day	oestradiol, 2-hydroxyoestradiol, 4-hydroxyoestradiol/ histopathology of uterus, uterine induced protein	4-6 rats/group; vehicle of 1:9 ethanol:sesame oil with 0.01% ascorbic acid. Not specified whether wet or blotted uterine wts used. Fig. 1 control uteri ~35 mg; Table 1 vehicle control uteri 77.5 ± 3.3 mg.
Gabbard and Segaloff (1983a)	Rat - Fischer	Immature, intact/ age not given	Subcutan./ 3 days, 2X day	thirty estrogen steroid derivatives (14-dehydrogenation and axial methyl groups at C-7, C-9 and C-11)/ receptor-binding assay	Group size not specified; cottonseed oil vehicle. Uteri were blotted. Results are reported relative to estrogen (100) so effectively the percentage increase relative to estrogen.
Gazit *et al.* (1983)	Rat - Sabra	Immature, intact/ 21 days	Subcutan./ 3 days	oestradiol, hexestrol, amino-clomiphene, fluoro-clomiphene/ receptor binding studies	6-8 rats/group; propylene glycol for most compounds as vehicle. Not specified whether wet or blotted uterine wts used. Amino-clomiphene prepared by several schemes, and each scheme tested. Control wts. 29.7 ± 2.2 mg
Gould *et al.* (1998)	Rat - Sprague Dawley	Immature, intact/ 21 days	Oral gavage/ 3 days	17β-oestradiol, bisphenol A/ estrogen receptor competitive binding, cell transfection assays with reporter genes, inhibition of activity by ICI 182,780, uterine peroxidase, and progesterone receptor induction	5 rats/group; corn oil vehicle. Not specified whether wet or blotted uterine wts used. Control uteri 40 ± 8 mg. Bisphenol A did not increase uterine weight at doses up to 150 mg/kg per day. Peroxidase and progesterone receptor induction were modest, but statistically significant. Compares with a 80% increase at 500 mg/kg per day given i.p. (see Cook *et al.*, 1997).

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Hammond *et al.* (1979)	Rat - Sprague Dawley	Immature, intact/ 21 days	Subcutan./ 3 days	17β-oestradiol, *o,p'*-DDT, mirex chlordecone (kepone)/ competitive binding, time course of estrogen receptor nuclear accumulation, progesterone receptor	5-7 rats/group. Sesame oil vehicle. Control uteri 27.7 ± 1.8 mg. Note: see Fig. 2 compares direct uterine weight and indirect uterine/body weight ratio. At high does, toxicity and loss of body weight increase values artificially.
Harper (1969)	Rat - Alderly Park	Immature, intact and OVX (also ADX) 36-68 g bw	Subcutan./ 3 days	17β-oestradiol, dehydroepiandro-sterone (DHA) and DHA sulfate/ implantation studies	10 rats/group; arachis oil vehicle. Uteri blotted. Reported as relative to body weight (however, see Fig. 1 and 4, 50-60 at 50 g bw would be 25 to 30 mg for controls).
Harper and Walpole (1967)	Rat - Alderly Park	Immature, intact 35-50 g bw	Subcutan. and oral gavage/ 3 days	17β-oestradiol, ICI 46,474, ICI 47,699/ implantation studies, antiestrogen (coadministration), vaginal cornification in both rats and mice, effect on intact males	10 rats/group; arachis oil vehicle. Uteri blotted. Reported as relative to body weight.
Hayes *et al.* (1981)	Rat - Sprague Dawley	Immature, intact/ 20-24 days (20 days specifically for uterine assays)	Subcutan./ 3 days	17β-oestradiol, CI 628, CI 628M (presumed metabolite), U 23,469, U 23,469M, (presumed metabolite)/ ER binding affinity and competition, cytosolic depletion and accumulation of receptor, time of nuclear retention of receptor, inhibition of labeled estrogen nuclear uptake, uterine peroxidase. Some are time courses	80 rats/group; sesame oil vehicle. Control uteri ~25-26 mg. ER binding assays. Agonist and antagonist assays; not 17-beta compounds are agonists when administered alone, but are strong antagonists when coadministred with estrogen.
Hossaini *et al.* (2000)	Rat – Wistar (Wist/han)	Immature, intact/ 18-20 days, 32 ± 2 g bw	Subcutan./ 3 days	Oestradiol benzoate, *p*-hydroxybenzoic acid, butyl parabens/	10 rats /group; peanut oil vehicle. Results reported as both absolute and relative uterine weights. Control uteri were 21.4 ± 3.2 and 22.7 ± 4.0. Butyl parabens uteri were slightly increased (statistically significant) at 600 mg/kg/day.
Hostetler *et al.* (1996)	Rat - Sprague Dawley	Immature, intact/ 18-19 days	Subcutan./ 3 days	17β-oestradiol , diethylstilbestrol, 4,4'-diamino-2,2'-stilbene disulfonic acid/ both wet and blotted weights were recorded, competitive receptor assays, MCF-7 cell growth stimulation	5 rats per group; sunflower oil vehicle. Results reported as % increase over control (no control weights given). Test compound was negative at 230 and 750 mg/kg doses.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Jones *et al.* (1984)	Rat - Holtzman	Immature, intact,/ 19 -20 days, 40 - 45 g bw	Subcutan./ 3 days	17β-oestradiol, tamoxifen, six related thiophene structures some receptor-binding assays and some assays of effects on mammary tumor growth	6-18 rats/group; corn oil vehicle. No description of uterine dissection or weighing. Control uteri 26.1 ± 1.0 mg. Dose response at order of magnitude from 1 to 1000 μg (rat/day).
Jordan (1976)	Rat - no strain given	Immature, intact/ age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen relative binding inhibition, cytosol and nuclear receptor ratios, and mammary tumor assays	8 rats/group; peanut oil vehicle. Vehicle control uteri ~40 - 50 mg (figure legend indicates 'wet' weights).
Jordan and Gosden (1983)	Rat - Sprague Dawley	Immature, intact/ 22 days, 40-50 g bw	Subcutan./ 3 days	17β-oestradiol, tamoxifen, monohydroxy-tamoxifen, LY117018/ receptor-binding assays, luminal cell height, progesterone receptor and other molecular/biochemical markers	8 rats/group; peanut oil vehicle. Uteri 'expelled of intraluminal fluid.' Control uteri were 31.8 ± 1.2 mg and 27.5 ± 1 mg.
Jordan *et al.* (1977)	Rat - Sprague Dawley	Immature, intact/ age not given, 35 - 50 g bw	Subcutan. and oral gavage/ 3 days	17β-oestradiol, tamoxifen, monohydroxy-tamoxifen, dihydroxytamoxifen/ inhibitory binding curves and sedimentation assays	8 rats/group; arachis oil vehicle. Route of administration comparisons. Uteri 'cleared of adhering fat, blotted, and weighed'. Control uteri ~38-42 mg; 50 mg in dihydroxy-tamoxifen experiments p. 311. Oral and s.c. comparison in Figure 4 and 5
Jordan *et al.* (1978)	Rat - Alderly Park	Immature, intact/ age not given, 35-45 g bw	Subcutan./ 3 days	tamoxifen, monohydroxytamoxifen, dihydroxytamoxifen, ICI 3188/ cytoplasmic receptor binding, tritiated oestradiol binding to tissues *in vivo*.	8-12 rats/group; peanut or arachis oil vehicle. Primarily a summary and review of previous work. Compare Figure 4 and 7 for different responses between immature rat and OVX mouse, respectively. Tamoxifen is partial agonist and partial antagonist in rat, appears to be full agonist and lack antagonism in mouse (species difference). Agonist dose sensitivity appears approximately the same between species.
Kallio *et al.* (1986)	Rat - Sprague Dawley	Immature, intact/ 18-20 days	Refers to Terenius, indicates subcutan. for 3 days	17β-oestradiol, Fc-1157a / receptor affinity binding, nuclear receptor translocation	Route and time not clearly specified - mentions 'injection.' Group size not specified; sesame oil vehicle. Reported as mg uterine weight per 100 g bw. Not specified whether wet or blotted uterine weights were used.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Katzenellen-bogen and Ferguson (1980)	Rat - from supply lab Holtzman, WI, but no strain noted	Immature, intact/ 21 - 25 days	Subcutan./ 3 days	17ß-oestradiol, three antiestrogens: CI-628 Parke-Davis, U-11,100A or nafoxidine Upjohn, and MER-25 Wm. Merrell Both wet and oven dry uterine weights. Receptor binding competition, sucrose gradient receptor binding, and temporal effects of administration on estrogen receptor levels in cytosol and nucleus of uterus (time course) and protein synthesis induction.	5-6 rats/group; saline control vehicle with 1-5% ethanol. Not specified whether the uterine wet or blotted uterine weights were used. Control uteri 30.5 ± 2.3 mg, 28.8 ± 2.9 mg wet and 6.1 ± 0.5 mg oven dry.
Katzenellen-bogen *et al.* (1979)	Rat - from supply lab Holtzman, WI, but no strain noted	Immature, intact/ 20 - 24 days; 21 days noted for uterotro-phic assays.	Subcutan./ 3 days	17ß-oestradiol, zearalenone, and 2 hydroxy derivatives (epimers) zearalanols/ Receptor-binding assays (direct and competitive), cytosolic and nuclear receptor levels, protein synthesis induction and sucrose gradient receptor binding.	4 rats/group; sesame oil vehicle. Not specified whether wet or blotted uterine weights were used. Control uteri ~22 mg. 2 µg (rat/day) oestradiol gives ~4.7 fold increase in uterine weight. One epimer ~100-1000 fold less potent than 17ß-oestradiol in uterotrophic and ~10 fold less for receptor binding potency.
Kitts *et al.* (1983)	Rat - Wistar	Immature, intact/ 21-24 d	Dietary, subcutan. and i.v./ dietary 3 days, inj. 2 days	17β-oestradiol, coumestrol, zearalanol/ distribution of estrogen receptor between cytosol and nucleus, including time course; uterine receptor levels, and total cytosolic protein.	10 rats/group; vehicle not specified. Not specified whether wet or blotted uterine weights were used. 2 days injection or 3 days dietary. Uterine weights recorded as mg/gm body weight. Coadministration of coumestrol could reduce oestradiol response (i.e., antiandrogen)
Lan and Katzenellen-bogen (1976)	Rat - supply lab Holtzman, WI, strain not named	Immature, intact/ 20 - 24 days, uterotrophic 21 days	Subcutan./ 3 days	17ß-oestradiol, estriol, 17α-ethinylestriol, 17α-ethinylestriol-3-cyclopentyl ether, estriol-3-cyclopentyl ether/ Receptor-binding assays, cytosolic and nuclear receptor distribution, 2-deoxy-*D*-glucose phosphorylation and tritium labeled thymidine incorporation into DNA	Min. 5 rats/group; 2% ethanol in 0.9% saline. Not specified whether wet or blotted uterine weights were used. Control uteri ~35 mg. Multiple dosing for 3 days appears to increase activity of weaker compounds (see 24 hr Astwood assay subchapter). Note: both the cyclopentyl ethers did not bind receptor, appear to be metabolically activated.
Larner *et al.* (1985)	Rat - Sprague Dawley	Immature, intact/ acquired at 22 days, 35-40 g bw (age at use not specified)	Subcutan. and i.v. (tail vein)/ 3 days	Oestradiol and oestradiol-17-stearate/ competition against labeled ligand in uterine tissue (displacement)	6 rats/group; sesame oil vehicle. Blotted uterine weights were used. Several experiments with control uterine weights 30-45 mg.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Lauson *et al.* (1939)	Rat - Sprague Dawley	Immature, intact/ 22 - 23 days, 34 - 39 g bw	Subcutan./ 2X daily for 3 days	17β-oestradiol, estrone, estriol/ Vaginal opening	4-15 rats/group; aqueous vehicle. Vaginal opening not as sensitive. Blotted weights. Ovaries weighed 'no particular significance found' 482 observations; control uteri 19.6 ± 2.6 mg (n=55). ~5X induction @ 0.4 µg oestradiol (rat/day). Oestradiol and estrone dose response curves (eight and seven doses, respectively)
Laws *et al.* (2000)	Rat - Long Evans	Immature, intact/ 21 d	Subcutan. and oral gavage/ 3 days	Octylphenol, nonylphenol, bisphenol A, methoxychlor, ethinyl oestradiol, and 17β-oestradiol/ Advancement of vaginal opening with continuous administration starting at 21 d.	6 rats/group. Corn oil vehicle. Sacrifice 6 hours after last dose Fig. 2 sc and Fig. 3 oral. Significant numbers of controls given in Figure legends. Apparent wet weights (methods say wet and blotted, data reported as wet wts). Results expressed relative to controls. Vaginal opening complemented uterotrophic wt (see Table 1 versus Fig. 3)
Levin *et al.* (1967)	Rat - Sprague Dawley	Immature, intact/ 19-20 days, 28 - 32 g bw	I.p. and subcutan./ 3 days pretreated, 3 days treat	17β-oestradiol in response to pretreatment/ Tritiated oestradiol uptake in uterus; oestradiol metabolism by liver microsomes.	6-8 rats/group; saline and corn oil vehicles. 3 day pretreatment with phenobarbital decreased oestradiol response by ~65% versus saline; also reduced tritiated oestradiol uptake by uterus. Not specified whether wet or blotted uterine weights were used. Control uteri 18-20 ± 0.6-0.9 mg. Note: HPX controls similar, but ADRX control uteri were 25-35 mg. Both 20-22 days old
Markaver ich *et al.* (1988)	Rat - strain not given, only supplier source	Immature, intact / 21 days	Subcutan./ 3 days	17β-oestradiol, luteolin, quercetin/ Wet uterine weights; receptor and DNA competitive binding assays; MCF-7 cell proliferation	Group size not reported; DMSO vehicle. Results reported as % of controls (no weights). Test compounds appear to be antagonistic when co-administered with oestradiol. Unclear if they have minor agonist activity when administered alone.
Odum *et al.* (1997)	Rat - Alpk:AP; mice - Alderly Park	Immature, intact/ 21-22 days, 38-48 g bw	Subcutan. and oral gavage/ 3 days	17β-oestradiol, 17β-oestradiol benzoate, ethinyl oestradiol, cyproterone acetate, ICI 182,780, coumestrol, methoxychlor, nonylphenol structures (n-nonyl and 2 commercial branched nonyl), nonyl-phenol benzoate, 17β-desoxyoestradiol, 17β-desoxyoestradiol benzoate	5-15 rats/group; arachis oil vehicle. Vaginal opening recorded; less sensitive than appearance of uterotrophic effect. Blotted uterine weights used; ~35 mg immature rat control uteri. Fig. 9 contains dietary data and their effect on uterine weights.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Odum *et al.* (1999b)	Rat - Alpk:AP; and Sprague Dawley	Immature, intact/ 21-22 days, 38-55 g bw; ovx at 28-35 days, used 42-59 days	Oral gavage and implanted mini-pump./ 3 and 11 days	diethylstilbestrol, branched nonylphenol / histology of vagina and also mammary gland proliferation	5-12 rats/group; arachis oil vehicle. 29-30 mg immature rat control (3 days, 25 day age) and 44-45 mg (11 days, 32-33 day age) uteri. Surprisingly, the s.c. mini-pump was negative at 27 mg/kg/day while oral experiments positive at 75 and 100.
Ostrovsky and Kitts (1962)	Rat - Wistar	Immature, intact/ no age given, 38 - 45 g bw.	Dietary, i.p., and oral gavage/ 3 days	Diethylstilbestrol and extracted red clover forage	6 rats/group. Controls had mean uteri wts of 41.46 mg at mean body wt of 40.5 g. Details of uterine preparation not given. Dietary route slightly greater response than gavage, which was 50% greater than i.p. administration.
Pento *et al.* (1988)	Rat - Sprague Dawley	Immature, intact/ 19-20 days, 40-50 g bw	Subcutan./ 3 days	Diethylstilbestrol, and five compounds (3 inactive)/ uterine histology, receptor-binding assay	5-6 rats/group; corn oil vehicle. Blotted weights used. Control groups appear to be 35-40 mg in Figures.
Qian and Abul-Haij (1990)	Rat - Sprague Dawley	Immature, intact/ 22 days	Subcutan./ 3 days	17β-oestradiol, 11β-[2-(N,N-dimethylamino0ethoxyestra-1,3,5(10)-triene-3,17β-diol, 11β-[3-(N,N-dimethylamino0ethoxyestra-1,3,5(10)-triene-3,17β-diol	10 rats/group: ethanol:olive oil vehicle at 1:9. Uteri were 'slit longitudinally, blotted.' Control uteri ~21 mg from Figure. Dose response curves.
Raynaud (1973)	Rat - Sprague Dawley	Immature, intact/ 3 weeks old	Subcutan./ 3 days	17β-oestradiol and R 2858 / α-fetoprotein levels and binding constants for oestradiol	10 rats/group. Studies impact of α-fetoprotein on dosage necessary to induce uterine growth. Tab 2 and Fig. 1 shows a rapid decline in α-fetoprotein levels from gestational d 20 thru postnatal d 29. Responsiveness of R 2858 (α-fetoprotein non-binding) compared to 17β-oestradiol (uterine wt increase) over time.
Robertson *et al.* (1982)	Rat - Holtzman supply lab, specific strain not given	Immature, intact/ 20-22 days	Subcutan./ 3 days	Tamoxifen and 10 analogues modified at the nitrogen moiety/ Relative binding affinities with oestradiol at 100%	4 rats/group; vehicle used only referred to as 'oil'. Synopsis of methods under figures, only. Unspecified whether wet or blotted uterine weights used. Control uteri appear to be ~25 - 28 mg in figure. All were partial agonists administered alone along with antagonists against oestradiol when co-administered.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ Time	Compounds (and other relevant data & endpoints)	Comments
Rosen *et al.* (1980)	Rat - CD	Immature, intact/ 21 day	Oral gavage/ 3 days	estrone, oestradiol, estriol, ethinyloestradiol, mestranol, and 415-oxaestrane derivatives	8-10 rats/group; sesame oil vehicle. Uterine weights after 'blotting on paper toweling.' In text, control means of 89 rats from 6 experiments: 29.3 mg. Results reported as dose effectively doubling uterine weights.
Routledge *et al.* (1998)	Rat - Alderly Park (Alpk:AP)	Immature, intact and adult OVX/ 21 -22 d, 38 - 55 g bw, ovx at 6 - 8 wks, used after 14 d.	Subcutan. and oral gavage/ 3 days	17β-oestradiol, dimethyl paraben, dibutylparaben (BP)/ Competitive binding assays (rat immature uterine cytosol), recombinant yeast	5-10 rats/group; arachis oil. Blotted weight, controls 27.1 ± 4.7, 30.3 ± 3.8, 33.8 ± 5.9, 28.2 ± 5.2 and 34.0 ± 4.6 mg in separate experiments for immature. ~4X increase 1.6 µg E sc or 16 µg E oral. BP at 1200 mg/kg s.c. leads to modest 40% increase in uterine weight. Vaginal cornification appearance coincident with increase in uterine weight in OVX (Figure 6). Dimethyl paraben negative.
Ruenitz *et al.* (1983a)	Rat - Sprague Dawley	Immature, intact/ 20-24 days	Subcutan./ 3 days	4-hydroxyclomiphene/ receptor-binding assays	7 rats per group; peanut oil vehicle. Blotted uterine weights used. Results reported as relative uterine weight gain to oestradiol benzoate dose as 100.
Ruenitz *et al.* (1983b)	Rat - Wistar	Immature, intact/ 21 days	Subcutan./ 3 days	3 nitromiphene derivatives and metabolites/ receptor-binding assays	min. 6 rats/group; peanut oil vehicle. Blotted uterine weights used. Results reported as relative uterine weight gain to oestradiol benzoate dose as 100.
Saeed *et al.* (1990)	Rat - no strain given	Immature, intact/ age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, LY-117018, and 10 synthesised derivatives of 2,3-diaryl-1-benzopyrans/ Receptor binding affinity up to 100 µM	6-9 rats/group; vehicle 1:1 propylene glycol: normal saline. Not specified whether wet or blotted uterine weights were used. Control uteri only 12.3 mg;. Agonist and antagonist screens; dose-response for certain compounds. Note: most compounds have aryl ring, but not hydroxyl. Metabolic role in activity may need review.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Safe and Gaido (1998)	Rat - Sprague-Dawley	Immature, intact/ 21 day	Oral gavage and i.p./ 3 days	17β-oestradiol, bisphenol A/ peroxidase and progesterone receptor (PR)	Corn oil vehicle. Not specified whether wet or blotted uterine weights were used. Control uteri ~40 mg, 4X induction using 0.02 µg E. Bisphenol not positive at 150 mg/ kg /day by oral route in uterotrophic (see Gould *et al.*), positive trend in peroxidase and PR. Combination of E and bisphenol A may be antagonistic.
Schmidt and Katzenellen-bogen (1979)	Rat - supply lab Holtzman, WI, strain not named	Immature, intact/ 20-23 days	Subcutan./ 3 days	oestradiol, testosterone, dihydroxy-testosterone, flutamide, CI-628, UII-100A/ receptor-binding assays, nuclear translocation of estrogen receptor	4-9 rats/group; sesame oil, DMSO, and saline-6% ethanol vehicles. Not specified whether wet or blotted uterine weights were used. Control uteri from several figures ~25-30 mg. Antiandrogens inhibited uterine wt increases of androgens; antiestrogens did not.
Segaloff and Gabbard (1984)	Rat - Fischer	Immature, intact/ age not given	Subcutan./ 3 days, 2X day	Eight esters of both estrone and oestradiol/ receptor-binding assay	Procedures only ref Gabbard and Segaloff 1983a. Not specified whether wet or blotted uterine weights were used. Results are reported relative to estrogen (100), so effectively the percentage increase relative to estrogen.
Sharma *et al.* (1990a)	Rat - no strain given	Immature, intact/age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, LY-117018, and 12 synthesised estrogenic derivatives of 2,3 diphenyl benzopyran / Relative binding affinities for ER up to 100 µM.	Min. 6 rats/group. Not specified whether wet or blotted uterine weights were used. Control uteri only 11.5 mg. Agonist and antagonist screens; dose-response for certain compounds. Note: most compounds have aryl ring, but not hydroxyl. Metabolic role in activity may need review.
Sharma *et al.* (1990b)	Rat - no strain given	Immature, intact/ age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, trioxifen, LY-117018, and 6 synthesised estrogenic derivatives of 2,3 diphenyl benzopyran/ Relative binding affinities for ER up to 100 µM.	Min. 6 rats/group. Not specified whether wet or blotted uterine weights were used. Control uteri only 11.5 mg. Agonist and antagonist screens; dose-response for certain compounds. Note: most compounds have aryl ring, but not hydroxyl. Metabolic role in activity may need review.
Snyder *et al.* (1989)	Rat - Sprague Dawley	Immature, intact/ weanling	Oral gavage/ 3 days	testosterone, flutamide, danazol, LY 156758// progesterone receptor levels and other assays	8-9 rats/group; vehicle not specified. Blotted weights used. Control ~30 mg uterine weight. See Fig. 3 testosterone induced increase in uterine weight was antagonised by flutamide.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Tinwell *et al.* (2000a)	Rat - Alderly Park (Alpk:AP)	Immature, intact and OVX (at 21-22 d used at 29-30 days) adult OVX (6-8 wks and used 14 days later/ intact: 21-22 days, 38-48 g bw	Oral gavage/ 3 days	oestradiol benzoate, coumestrol/ Wet and blotted uterus, also vaginal and cervical weights. Uterine cellular morphometry. BrdU labeling of uterine cells. Vaginal opening in all treatment groups, vaginal cytology (includes endometrial height, number of glands, and mitotic number in some groups).	6 rats/group; arachis oil vehicle. Blotted uterine weights used. Control immature uteri 28.3 ± 6.7 mg; immature OVX 19.1 ± 2.5 mg; adult OVX 98.7 ± 15.4 mg. Coumestrol 60mg/kg/day shows activity similar to oestradiol across the array of endpoints measured. See Fig. 2 and Table 4, intriguing suggestion that immature might be more sensitive across the array than the adult OVX.
Van de Velde *et al.* (1994)	Rat - Sprague Dawley	Immature, intact/ 18-19 days, 38-42 g bw	Subcutan. and oral gavage/ 3 days	tamoxifen, RU 58668, ICI 182780/ relative binding affinity to estrogen receptor, MCF-7 cell inhibition, antitumor activity in nude mice	5 rats/group; several vehicles (ethanol, methylcellulose, arachis oil). Not specified whether wet or blotted uterine weights were used. Results reported as relative decrease (antagonist assay) to controls.
Wade *et al.* (1997)	Rat - Sprague Dawley	Immature, intact/ 18 days	Subcutan./ 3 days	diethylstilbestrol, dieldrin, endosulfan/ receptor-binding assays, uterine peroxidase assay, MCF-7 growth assay	10 rats/group; corn oil vehicle. Reported uterus as % of body weight. Would result in 102 mg control uteri, which were reported to be blotted. Analyzed circulating estrogen levels in 18 day rats, 48 animals all <50 pg/mL, 36 were not detect at 20 pg/mL.
Wakeling and Bowler (1988)	Rat – not given in paper, method refers to previous work with reference	Immature and OVX/ no age given, used 14 days after ovx	Subcutan. and oral gavage/ 3 days	Oestradiol benzoate, tamoxifen, LY 117,018, ICI 160,325, ICI 163,964, ICI 164,275, ICI 164,384/ estrogen receptor binding, vaginal opening, LH hormone levels, MCF-7 and ZR-75-1 cell growth assays	Min. 5 rats/group; arachis oil for some and aqueous mix using Tween 80. Refer to manuscript where blotted weights were reported. See Figure 4, immature, adult OVX rats and immature, adult OVX mice all in same graphic. No substantive difference in response, variability, or sensitivity apparent. Agonist and antagonist assays.
Wakeling and Valcaccia (1983)	Rat - Alderly park	Immature, intact/ no age given, 35 -45 g bw	Oral gavage/ 3 days	Oestradiol benzoate, tamoxifen, trioxifene, LY 117018, LY 139481/ Mammary tumor response to administration	5 rats/group; arachis oil for some and aqueous mix using Tween 80. Uteri 'blotted dry after expulsion of uterine fluid and wet weight recorded' Expressed as mg uterine weight relative to 100 g body weight - estimate of ~25 mg for controls.

Table 1A-1. Uterotrophic bioassays in immature, intact rats. Assays involving 3 daily consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Wakeling *et al.* (1983)	Rat - Alderly Park	Immature, intact/ no age given, 35 - 45 g bw	Subcutan./ 3-6 days (see comment)	Tamoxifen, LY 117018/	5 rats/group; arachis oil for some and aqueous mix using Tween 80. In antagonist expts., oestradiol and oestradiol benzoate administered days 1-6; antiestrogen administered days 4-6. Uterine weight to body weight ratio used. Control uteri estimated at 25-30 mg. Blotted uterine weight used. Agonist and antagonist activities.
Wakeling *et al.* (1991)	Rat - Alderly Park	Immature, intact/ no age given	Subcutan./ 3 days	17β-oestradiol, ICI 164,384, and ICI 182,780	Min. 5 rats/group; arachis oil vehicle. Blotted uterine weight used. Expressed as uterine weight relative to body weight. Assuming 55 g weight, control uteri ~25 mg. Both compounds appear to be pure antagonists and to fully suppress oestradiol response.
Whitten *et al.* (1992)	Rat - Sprague Dawley	Immature, intact/ 20-21 days, 30-40 g bw	Subcutan. for 3 days and diet for 3, 4 and 8 days	coumestrol/ receptor binding, uterine progestrone receptor, uterine estrogen receptor	7 rats/group. Control uteri are ~50 mg as 'wet weight' and dry uteri are ~12 mg. Appear to be wet weights and not blotted. Semipurified diet used to avoid soy contamination. S.c. and dietary administration. Dietary administration appeared to be more effective over time (see p 102, column 1)
Williams *et al.* (1997)	Rat - Alderly Park (Alpk:AP)	Immature, / 21-22 days, 38-48 g bw	Subcutan. and oral gavage/ 3 days	oestradiol benzoate, polysorbate 80	6 rats/group; corn oil vehicle. Blotted uterine weights used. Both absolute and relative weights reported. No effect from the polysorbate.
Willson *et al.* (1997)	Rat - Sprague Dawley	Immature, intact/ 21 days, 30-35 g bw	Subcutan. and oral gavage/ 3 days	17β-oestradiol, tamoxifen, raloxifene, ICI 182780, GW 5638, GW 7604/ transfected cell response and bone mineral density studies	5 rats/group; sesame oil vehicle. Blotted uterine weights used. Uterine weight (mg) to body weight (g) used for results. Estimate uterine weights at 28-30 mg.
Yamasaki *et al.* (2000)	Rat - Sprague Dawley	Immature, intact/ 18 days, 36-37 g bw	Subcutan. and oral gavage/ 3 days	Bisphenol A only/ BPA plasma concentrations in some expts, wet and blotted weights, absolute and relative to body weight, time course after last administration (6, 12, 18, and 24 hours)	10 rats/group; sesame oil vehicle. Modest rend at high dose on body weights, appeared to impact statistics (absolute vs relative responiveness) in some cases. Multiple experiments show variability of the minimum effective dose.

Table 1A-2. Uterotrophic bioassays in adult OVX animals and 3 daily consecutive administrations of test compounds.

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
RAT					
Adult OVX animals and 3 days administration (another 'standard' uterotrophic bioassay)					
Ashby *et al.* (1997b)	Rat - Alderly Park (Alpk:AP); mouse Alpk:AP	Immature and adult OVX/ 21-22 days & 7 wk ovx used 2 wk later	Oral gavage/ 3 days, clofibrate 2X per day	17β-oestradiol, benzoic acid, clofibrate/ vaginal opening, vaginal cornification, and cell mitotic index included/ Dry as well as wet uterine weights. Timing of vaginal opening.	5 rats or mice/group; rat vehicle arachis oil. Blotted uterine weights used. Immature controls 25.5 to 37.5 mg. OVX 81.3 mg. Immature mice 9.7 - 10.1 mg. Neither test compound indicated response, i.e., failed to reproduce previously reported results in other labs.
Ashby *et al.* (2000a)	Rat - Alderly Park (Alpk:AP)	Adult OVX/ ovx 6-8 wks, 14 d recovery	Subcutan./ 3 days	17β-oestradiol, bisphenol A, 1-keto-1,2,3,4-tetrahydrophenanthrene, 4-keto-1,2,3,4-tetrahydrophenanthrene/ dry weight and vaginal cornification	3 rats for E. 4-7 rats/ group for test substance; sesame and arachis oil vehicles. Blotted uterine weights used. 4 control groups with uterine wts. Some one injection and sac on day 4, others one dose for 3 days, and other two doses per day for 3 days. Vaginal cornification matched uterine wt response.
Carthew *et al.* (1999a)	Rat - Wistar (Han)	Adult, OVX/ ovx at 6 wks, used after 3 wks, 225g bw	Subcutan./ 3 days	17β-oestradiol, tamoxifen, toremifene/ histopathology, vaginal weight, BrdU labeling of several uterine cell types, time course, α-estrogen receptor and progesterone immunolocalization	4 rats/time point group tricaprilin vehicle. Not specified whether wet or blotted weights were reported, both noted in methods. Control uteri ~140-150 mg. Comment in discussion notes water imbibition was only found with oestradiol, not other two substances. 72 hours required to see weight gain for tamoxifen and toremifene; not all oestradiol responses mimiced and responses typically weaker.
Diel *et al.* (2000)	Rat - DA/Han	Young adult OVX / ovx at 130 g bw – used 14 day after	Oral gavage/ 3 days	Ethinyl oestradiol, bisphenol A, *o,p'*-DDT, daidzein, octylphenol / PCR analysis for mRNA of androgen, estrogen and progesterone receptors; clustrin, complement 3, and GAPDH	6 rats/group; DMSO vehicle. Not specified whether wet or blotted weights used. Control uteri 451 ± 52; 'wet weight.' LOELs in Table 1 for uterine weights, although relative activity low. See gene expression fingerprint profile in Fig. 4.
Grese *et al.* (1997)	Rat – Sprague Dawley	Young adult, OVX/ 60 d ovx, 14 day wait	Oral gavage/ 4 days	17α-ethinyl oestradiol, tamoxifen, raloxifene, and 66 synthesised compounds/ receptor binding affinity, MCF-7 cell growth, cholestrol, bone density	5 rats/group; 20% cyclodextran vehicle. Blotted uterine weights used. Results reported as minimally effective dose to increase uterine weight relative to body weight and then as percentage increase over controls.

Table 1A-2. Uterotrophic bioassays in adult OVX animals and 3 daily consecutive administrations of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Gray *et al.* (1999)	Rat - Sprague Dawley	Adult, OVX/ no specifics	Subcutan. and oral gavage/ 2 days	oestradiol, octylphenol, bisphenol A, methoxychlor, dibutylphthalate/ lordosis behavior	At least 6 rats/group. Uteri weighed with luminal fluid (wet weight). Control ~90 mg. See Figure 4.
Hisaw *et al.* (1954)	Rat - albino	Adult OVX/ ovx at 100d or ~100 g bw	Subcutan./ 3 days	17β-oestradiol, estrone, estriol	Sesame oil vehicle. Used wet, blotted, and oven dry weights at different points. Control uteri ~120-130 mg. Competitive experiments with coadministration of estriol showing that estriol acts as antagonist, decreases uterine weights, when coadministered (Fig. 2). Also time course up to 15 days (Fig. 4)
Laws *et al.* (2000)	Rat - Long Evans	Young adult OVX/ ovx 60 d	Oral gavage/ 3 days	Octylphenol, nonylphenol, bisphenol A, methoxychlor, ethinyl oestradiol, and 17β-oestradiol/ Vaginal cornification	6 rats/group. Corn oil vehicle. Sacrifice 6 hours after last dose. 4 sets of control uterine wts (93-105 mg, see legend Fig. 4). Methods say both wet and blotted taken, data reports only the wet wts. Results expressed relative to controls. Vaginal cornification was less sensitive than uterotrophic wt (see Table 2 versus Fig. 4)
Odum *et al.* (1999a)	Rat - Noble	Young adult OVX/ ovx 4-5 weeks, used 2-3 wks after	Oral gavage/ 3 days	diethylstilbestrol and p-nonylphenol/ vaginal smears, BrdU staining and uterine histopathology, and mammary gland proliferation	5-7 rats/group; arachis oil vehicle used. Wet, blotted, and oven dry weights of the uterus. Control uteri ~50 mg. Vaginal cornification less sensitive (Fig. 1, 2) Also 3 vs 11 daily doses tested (Fig. 2)
Perel *et al.* (1970)	Rat - Wistar	Adult, OVX/ 3 mo., 207 g bw, used 14 d after ovx	Subcutan. 3.5 days, 2X daily, 7 doses	oestradiol, coumestrol, genistein/ effects on fertility (implantation)	10-12 rats/group; DMSO as vehicle. Not specified whether wet or blotted uterine weights used. Results reported as % increase over controls; no control wts.
Routledge *et al.* (1998)	Rat - Alderly Park (Alpk:AP)	Immature, intact and adult OVX/ immature 21 - 22 d, 38 - 55 g bw, ovx at 6 - 8 wks, used after 14 d, vaginal smear confirms.	Subcutan. and oral gavage/ 3 days	17β-oestradiol, dimethyl paraben, dibutylparaben (BP)/ Competitive binding assays (rat immature uterine cytosol), recombinant yeast	5-10 rats/group. Blotted weights used; controls 27.1 ± 4.7, 30.3 ± 3.8, 33.8 ± 5.9, 28.2 ± 5.2 and 34.0 ± 4.6 mg in separate experiments for immature. ~4X increase 1.6 µg E sc or 16 µg E oral. BP at 1200 mg/kg s.c. leads to modest 40% increase in uterine weight. Vaginal cornification appearance coincident with increase in uterine weight in OVX (Figure 6). Dimethyl paraben negative.

Table 1A-2. Uterotrophic bioassays in adult OVX animals and 3 daily consecutive administrations of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Velardo (1956)	Rat - Charles River	Adult, OVX/ 'adult' no age given, used 7 days after ovx	Subcutan./ 3 days	17β-oestradiol, Δ^1, 9α fluoro hydrocortisone/ Wet and dry uterine weights	9-20 rats/group; sesame oil and aqueous formula (CMS, polysorbate 80, sodium chloride and benzyl alcohol). If 2 materials, then injected at separate sites. Not specified whether wet or blotted weights used. Control uteri 129.4 ± 4.1 mg, oven-dry uteri 22.5 ± 0.8 mg (49 animals). Agonist and antagonist activities.
Velardo (1959)	Rat - Charles River	Adult, OVX/ 7 days after ovx at 90 days	Subcutan./ 3 days	17β-oestradiol, estrone, 16α-hydroxyestrone, 16β-hydroxyestrone, estriol, 16-*epi*-estriol, progesterone, 3α, 20β pergnanediol, 3β, 20β pergnanediol, pregnanedione, testosterone, desoxycorticosterone acetate, cortisone, hydrocortisone, 9α fluoro hydrocortisone, Δ^1 cortisone, Δ^1-hydrocortisone, Δ^1, 9α fluoro hydrocortisone/ Wet and dry uterine weights	11-20 rats/group; sesame oil. If 2 materials, then injected at separate sites. Not specified whether wet or blotted weights used. Control uteri 118 ± 7.0 mg, oven-dry uteri 22 ± 1.1 mg (all treated have both wet and dry weights). Typically ten points on dose-response curve of each chemical**.** Controls appear same in all figures, were controls run for each experiment? Antiestrogenic effects of substances also tested with wet and dry weights. Conclusion: Metabolic alterations of the uterus are due to all of hormones and their metabolites acting in concert.
Velardo and Sturgis (1955a)	Rat - Charles River	Adult OVX/ age not given, wait 7 days after ovx	Subcutan./ 3 days	17β-oestradiol, metacortandracin, metacortandralone/ Wet and dry uterine weights.	6-12 rats/group. Control uteri 115.5 ± 3.3 mg, oven-dry uteri 22.4 ± 0.7 mg (25 animals) - note: same as 1956 paper. Appear to have antagonist action.
Velardo and Sturgis (1955b)	Rat - strain not given	Adult OVX/ ovx 100 d, wait 7 days	Subcutan./ 3 days	17β-oestradiol, 16-*epi*-estriol Wet and dry uterine weights.	7-21 rats/group. Blotted weights. Control uteri 115.5 ± 3.3 mg, dry uteri 22.4 ± 0.7 mg (25 animals)note: same as other 1955 and 1956 papers. Dose responsive antagonism.
Velardo and Sturgis (1956)	Rat - strain not given	Adult OVX/ ovx 100 d, wait 7 days	Subcutan./ 3 days	9α-fluorohydrocortisone acetate, pregnane3α,20α-diol, 17-hydroxy-corticosterone acetate, ACTH/ Wet and dry uterine weights.	7-36 rats/group. Two vehicle controls - sesame oil and aqueous mix, control blotted uteri 115.5 ± 3.3 mg, dry uteri 22.4 ± 0.7 mg (25 animals) and vehicle control 116.4 ± 2.4 mg, dry uteri 22.8 ± 0.8 mg (15 animals). Corticosteroids functioned as antagonists. ACTH antagonist in intact OVX animals, ineffective in adrenalectomized animals.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats.

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Rats - Variations in the standard procedure of 3 days either s.c. or oral gavage					
AboulWafa *et al.* (1992)	Rat - strain not given	Adult OVX/ 100-150 g bw	Subcutan./ 4 days	17β-oestradiol and 18 synthesised compounds/ vaginal smears, antiimplantation activity	4-6 rats/group; olive oil vehicle. Uteri blotted. Results reported as mg uterine weight/100 g body weight.
Agrawal *et al.* (1977)	Rat - no strain given	Immature, no age given	I.p./ 3 days	diethylstilbestrol and nine 5-substituted thiosemicarbazido methoxy)-4-methyl coumarin derivatives	10 rats/group; propylene glycol vehicle. Oven-dry weights of uterus used.
Agrawal *et al.* (1978)	Rat - no strain given	Immature, no age given	I.p./ 3 days	oestradiol and seven 7-(4-substituted 2-oxazolidinethiones derivatives	10 rats/group; no vehicle given. Oven-dry weights of uterus used.
Arcaro *et al.* (1999)	Rat - Sprague Dawley	Immature, intact/ 21-22 days	I.p./ 2 days	17β-oestradiol, 2,2',6,6'-tetrachlorobiphenyl/ competitive estrogen receptor-binding assays, MCF-7 cell growth	10-30 rats per group; corn oil vehicle. Not specific whether blotted or wet weights used. Results also reported as mg uterine weight per 100 g bw. Olive oil control uteri 29.5 ± 1.7 mg (4 days)
Armstrong *et al.* (1976)	Rat - Sprague Dawley	Immature, intact and OVX/ ovx 22-25 days	Subcutan./ variable times	testosterone, dihydroxytestosterone/ uterine histology	4-5 rats/group; sesame oil vehicle. Uteri blotted 'on filter paper.' Control uterine weights 30-40 mg in graphs.
Ashby *et al.* (1997a)	Rat - Alderly Park (Alpk:AP)	Immature, intact and OVX / 21-22 days, 38-48 g bw; ovx at 6-8 wks, used 14 days later	Oral gavage/ 3 days	17β-oestradiol, raloxifene, ICI182,780/ vaginal opening, vaginal cornification, and cell mitotic index included/ Vaginal opening in all treatment groups, vaginal cytology (includes endometrial height, number of glands, and mitotic number in some groups).	5-7 rats/group. Immature slightly more sensitive than OVX. Vaginal opening in some 17β-oestradiol immature rats, vaginal cornification not observed with raloxifene individuals with increased uterine weights, ICI 182, 780 did inhibit uterine weight increase, cell mitotic figures were observed. 'Blotted to remove excess fluid' and oven dry uterine weights. Control immature uteri ~25 mg; sd estimate ± 5 mg. ovx ~80 mg.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats.

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Bachman n *et al.* (1998)	Rat - Wistar	Immature, intact/ 22 ± 1 days of age, 40-60 g b (heavier removed).	Oral gavage/ 2X daily for 4 days	diethylstilbestrol dipropionate and 23 samples of polystyrene extracts (primarily dimer and trimer) of standard, high impact, and expandable polystyrenes	10 rats/group. Vehicle control included. Wet, unblotted weights. Control uteri of 3 inhibits were 69, 59, and 79 mg; vehicle control 61, 68, and 76 mg; ; sd ± 15, 15 and 24 and 11, 33, and 28 mg., respectively Standard deviations were sometimes large relative to means for full wet weight. Weights randomised so groups variation did not exceed 10%.
Beri *et al.* (1998)	Rat - Sprague Dawley	Immature, OVX/ ovx ~21 days and used 5-7 days later	Subcutan./ 5 days	17β-oestradiol, testosterone, 7α-methyl-19-testosterone/ uterine peroxidase, progesterone receptor induction, vaginal cornification, preputial gland weights	5-6 rats per group; cotton seed oil vehicle. States that uteri were blotted dry. Control uteri appear to be ~35-40 mg in Fig. 1, but 21.4 ± 1.9 and 29.0 ± 2.3 in tables.
Bhargava (1986)	Rat - Wistar	Immature, OVX/ ovx at 15 days	Oral gavage/ 5 days	oestradiol and butin (phytoestrogen)	5-10 rats/group; water vehicle for butrin and olive oil for oestradiol. Results reported as relative: uterine wt in mg/bw (g). Not specified whether wet or blotted wts used.
Bhavnani *et al.* (1998)	Rat - Sprague Dawley	Immature, intact/ 18-19 days	I.p./ 3 days	equilin, 17β-dihydroequilin, 17β-oestradiol, estrone, Δ^8-17β-oestradiol, Δ^8-estrone, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin, 17α-dihydroeuilin, 17α-oestradiol/ human endometrial and rat uterine cytosol receptor assays,	20 rats per group; ethanol:saline (1:9) vehicle. Control uteri 33.8 ± 1.0 mg. Not specified whether wet or blotted weights used. Also expressed as mg uterine wt per 100 g bw. Potency of compounds with respect to metabolism was one objective of study.
Black *et al.* (1983)	Rat - no strain given	Immature OVX/ ovx at 21 d and used 7 d later	Subcutan./ 3 days	17β-oestradiol, tamoxifen, LY 139481/ Relative binding vs oestradiol over T range	6 rats/group; corn oil vehicle. If more than one compound, injections at separate sites. Blotted uterine weights used. Control uteri ~25 mg, no standard deviations given. Agonist and antagonist assays.
Black *et al.* (1994)	Rat - Sprague Dawley	Adult, OVX/ ovx 10-11 wk, 225 - 250 g bw	Oral gavage/ 5 weeks	Ethinyloestradiol, raloxifene/ bone density, serum parameters, four uterine histological endpoints (epithelial height, myometrial thickness, stromal expansion, stromal eosinophilia)	6-12 rats/group; 1.5% carboxymethyl cellulose as vehicle. Note: administration initiated immediately after OVX; 5-week administration period. OVX control 127 ± 5 mg. Not specified whether wet or blotted uterine weights were used. Raloxifene induced modest increases in uterine weights (~50% over OVX controls) with little or no modification of histological endpoints

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Branham *et al.* (1993)	Rat - Sprague Dawley (Crl:CD)	Immature, intact/ 20 days	Subcutan./ 5 days	17β-oestradiol, diethylstilbestrol, ethinyl oestradiol, clomiphene citrate, tamoxifen, monohydroxytamoxifen/ luminal epithelium height, glandular epithelium height, histology	6-8 rats/group (min. of 2 dams); sesame oil vehicle. Blotted uterine weight used; body weights 50 on d 21, 64 on d 24 - uterine controls ~25 mg. Up to 8X induction. Log dose response curves for several compounds.
Brownlee (1938)	Rat - no strain given	Immature, OVX / age not given, ovx ~40 g bw	Subcutan./ 3 and 4 days	estrone, oestradiol benzoate, unknown samples	5 rats/group. Uteri were blotted between filter paper after 24 hours in Bouin's fixative. Control uteri 18-20 mg. Note: was this somewhat lower mean wt. due to OVX?
Bülbring and Burn (1935)	Rat - no strain given	Two groups: a) adult OVX at bw of 120 g; animal used if bw <220 g and b) immature ovx at 40 g bw	Subcutan./ 3 or 4 days necropsy 48 hrs after last injection.	estrone	4-20 rats/group; olive oil vehicle. Study was performed blind on the quantity of compound (potency). Expressed as uteri per 100 g bw. The uteri were fixed in Bouin's solution, and were then blotted between two pieces of filter paper before weighing. Control uteri apparently averaged 19-25 mg by comments, back calculation.
Calhoun *et al.* (1971)	Rat - supply lab Holtzman, WI, strain not named	Immature, intact/ 19 days	I.p./ 1 days	pretreatment with various compounds twice daily for 3 days, followed by mestranol - one injection	10-27 rats/group; saline vehicle and corn oil vehicle for mestranol. Not specified whether wet or blotted weights used. Results reported as ration of uterine wt/body wt.
Chander *et al.* (1991)	Rat - Wistar	Immature, intact/ 19-20 days, 40-50 g bw	Subcutan./ 4 days	17β-oestradiol , tamoxifen, 4-iodotamoxifen, pyrrolidino-4-iodotamoxifen/ tumor response rates, estrogen receptor binding assays, vaginal cornification	5 rats/group; peanut oil vehicle. Uterine tissues were blotted. 'fluid content expelled.' Results reported as mg uterine weight per 100 g bw.
Clement and Okey (1972)	Rat - Wistar	Immature, intact/ 23 days	Dietary/ 7 days	diethylstilbestrol, *o,p'*-DDT/ wet and dry uterine weights, premature vaginal opening, uterine glycogen and protein	20 rats/group. Not specified whether wet or blotted weights were used; one horn oven dried. Control uteri ~58 mg; expressed as mg/g bw ~17. DDT increased all responses, including premature vaginal opening at doses ≥ 1000 ppm in diet (500 ppm did not elicit response or response not significant)

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Connor *et al.* (1997)	Rat - Sprague Dawley	Immature, intact/ 19 days	I.p./ 3 days	17β-oestradiol and 8 hydroxylated PCBs OH at 4 position (2,2',3',4',5'; 2,2',3',4',6'; 2,2',3',5',6'; 2,2',4',6'; 2',3,3',4',5'; 2',3,3',4',6'; 2',3,3',5',6'; 2',3,4',6'/ competitive binding rat and mouse uterine receptor, uterine peroxidase, MCF-7 proliferation, chloramphenicol acetyl transferase assay, stably transfected reporter in HeLa cells	4-5 rats/group; corn oil vehicle. Appears that uteri were weighed wet, then blotted before other analyses. Weak PCB binding, proliferation and other *in vitro* responses noted. None significantly increased uterine weights (doses 25 to 100 mg/kg/day). Increases in peroxidase and progesterone levels observed, but not always in dose responsive manner (see parallel experiments in mice; no increase in any parameter).
Cook *et al.* (1997)	Rat - Crl:CD	adult OVX/ not specified	Oral gavage and i.p./ 4 days	17β-oestradiol, estriol, tamoxifen, resperine, haloperidol, ICI-182,780, methoxychlor, bisphenol A	10-14 rats/group. Blotted weights, control uteri 75-85 mg, 200-225 g bw. Additional tissue, histopathologic, and hormonal endpoints.
Dahr *et al.* (1991)	Rat - Sprague Dawley	Immature, OVX/ 21 day OVX, 35-40 g bw, used 5 days later	Oral gavage/ implied 3 day dosing	CDRI-85/287/ several other tests of steroid activity	6 rats/group; test compound in gum acacia aqueous suspension and oestradiol in olive oil. Control uteri 19.66 ± 1.30 mg. Blotted weights: 'after expressing the uterine fluid between folds of a filter paper.'
Desaulniers *et al.* (1998)	Rat - not given	Immature, intact/ 20-21 days	I.p./ 2 days	N-oxydiethylene-2-benzothiazole sulfenamide, 17β-oestradiol/ cell proliferation expts (MCF-7-E3)	5 rats per group; corn oil vehicle. Control uteri 33.4 ± 3.0 mg. Blotted uterine weights used.
Dongfang and Bachmann (1998)	Rat - Sprague Dawley	Adult OVX/ ovx 10-11 wks 200-225 g bw	Oral gavage/ 7 days	17β-oestradiol, 2-hydroxyoestradiol, 4-hydroxyoestradiol, tamoxifen, 4-hydroxytamoifen, 20-methoxyestrone, 2-methoxyoestradiol/ cholesterol weights, uterine histology	4-5 rats/group; vehicle 1% methylcellulose. Blotted or wet uterine weights not specified. Control uteri 155 mg.
Dorfman *et al.* (1935)	Rat - albino	Immature, intact/ 25 days	Subcutan./ 5 days	theelin, theelol, human and equine male urine extracts	3-5 rats/group. Blotted uterine weights used. 5 day administration procedure, control uteri ~24 mg, bw less gut 41 g mean.
Duby *et al.* (1971)	Rat - Sprague Dawley	Immature, intact and OVX adult/ 21 day immature	Subcutan., dietary	estrone, *p,p'*-DDT, *o,p'*-DDT	Unknown group size; corn oil vehicle. 'all luminal fluids of the uterus were expressed.' ~27-32 mg control uteri. OVX rats were fed compounds for 175 days.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Dukes *et al.* (1994)	Rat - Alderly Park; mice	Immature, intact and adult OVX/ not specified, procedures in reference	Subcutan. and oral gavage/ Immature 3 day assays. OVX 7, 14, and 28 day treatment regimens.	17β-oestradiol benzoate, ZM189,154, tamoxifen	5 rats/group, 2 replicates. Control uteri ~25 mg in immature; 85 and 173 mg in two sets of adult animals of different ages. Comparative ZM 189,154 data: ED_{50} 0.09 mg/kg s.c. and 0.7 mg/kg oral in immature rat ED_{50} using oral administration was 0.7 mg/kg in immature rats, 1.3 mg/kg in OVX rats, and 6.2 mg/kg in OVX mice.
Duncan *et al.* (1963)	Rat - Sprague Dawley	Immature, OVX/ ovx 55 g bw or earlier	Oral gavage/ 10 days	oestradiol, U-10520A, U-11100A/	5 rats/group; 0.25% methylcellulose. Not specified whether wet or blotted uterine weights used. Control uteri 25-26 mg. Agonist and antagonist dose response.
Edgren and Calhoun (1960)	Rat - Sprague Dawley	Immature OVX / ovx at 30 days, used 10 days post op)	Subcutan./ 3 days	estrone, oestriol, 16-*epi*-oestriol	20 rats/ treatment group; corn oil vehicle. Uterus was 'scored and blotted to express contained fluid.' Mixtures of estrone and estriol: With six standard doses of estrone, three different levels of estriol were administered to each dose along with the dose response curve. Compounds were not additive, and appeared to depress estrone in the ascending portion of the dose curve.
Edgren and Calhoun (1961)	Rat - Sprague Dawley	Immature to adult, OVX/ (one set at 30 another at 100 days, used 10 days post op)	Subcutan./ 3 days	estrone, progesterone (P), desoxy-corticosterone (DC), cortisone acetate, testosterone propionate	12 rats/treatment group; corn oil vehicle. See Edgren and Calhoun (1960). Blotted uterine weights used. Additional matrices against estrone dose response curve (6 doses) with 3 levels of P and DC. Again, some materials not additive to estrone, others moderately additive, and others were modestly antagonistic during the ascending portion of the estrone curve (see Fig. 3).

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Edgren *et al.* (1966)	Rat - Charles River	Immature OVX / ovx at 30 days, used 10 days post op)	Subcutan./ (rats 3 days - mice for 7 days)	17β-oestradiol, 18-homoestriol (17β) (HE)/	Control uteri ~35 to 44 mg with vehicle in different experiments (some estrone doses 13 mg lower than vehicle control - Table 35); specific vehicle not given.. In addition to single compound administration, HE was coadministered with estrone. As single compound HE was estrogenic, but reduced estrone uterine weight gain (i.e., antiestrogenic) Blotted uterine weight used.
Edgren *et al.* (1967)	Rat - Charles River	Immature, OVX / ovx at 30 days, used 10 day post op	Subcutan./ Several time points	17β-oestradiol, 18-homoestradiol, propyl oestradiol/ Parallel studies in mouse, rat vaginal endpoints of cytology and day of opening (controls > 42 days), and chicken oviduct.	11-21 rats/group; specific vehicle not given. Modest comparison of 1, 2, 3, and 7 days of injection. Control rat uteri 31.9 and 32.8 mg (mean only given). See Fig. 9 where 95% confidence levels of 1 μg estrone are given. 0.3 μg 17β-oestradiol/rate/day resulted in 130 mg uteri after 3 days and 142 mg after 7 days. 0.01 μg doses were equivalent at 55 and 49 mg, respectively. Other compounds, gave similar trends. Blotted uterine weight used.
El-Tombay (1997)	Rat - strain not given	Immature, intact/ 21-23 days, 45-60 g bw	Subcutan./ 4 days	17β-oestradiol and a series of 30 synthesised compounds/ dry weights, antagonist activity, vaginal smears	4 rats/group; DMSO vehicle. Blotted weights. Results reported as mg uterine weight per 100 g bw.
Gabbard *et al.* (1981)	Rat - strain not given	Immature, intact/ age not given	Subcutan./ 5 days	11α-hydroxyestrone, 11α-hydroxy - βestrone, 11α-hydroxy-9β-estrone, 11β-hydroxy-9β-estrone	For procedures, etc., the paper refers to a previous publication.
Gabbard and Segaloff (1983b)	Rat - Fischer	Immature, intact/ age not given	Subcutan./ 4 days, 2X per day	nine estrogenic derivatives (9 beta-estrogens) / receptor binding assay	Group size not specified; cottonseed oil vehicle. Uteri were blotted. Results are reported relative to estrogen (100) so effectively percentage increase relative to estrogen.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Gellert *et al.* (1972)	Rat - Sprague-Dawley	Immature, intact and adult OVX/ 23 days	I.p./OVX 7 days, immature 27 days	*o,p'*-DDT, *p,p'*-DDA, *p,p'*-DDD, *p,p'*-DDE/ vaginal opening of immature rat	Group size 10 in immature and 12 in OVX. DMSO vehicle. 10 mg/day *o,p'*-DDT increased OVX uterine weights by ~75%. 500 micrograms per day for 27 days advanced vaginal opening and increased uterine weight in 27 day exposure. Possible wet weights, but unclear
Harnagea-Theophilus *et al.* (1999)	Rat - Wistar	Adult OVX/ 200-225 g bw	Subcutan. and oral gavage/ 4 days	17β-oestradiol (s.c.), acetaminophen (gavage)/ cell cycle stimulation and DNA incorporation, MCF-7 proliferation,	Rat data were not shown in paper. Mice were also administered the compounds and their results were reported.
Heller *et al.* (1938)	Rat - Sprague Dawley	Immature, intact/ 21 days 'exactly' 34-39 g bw	Subcutan./ 2X per day for 4.5 days	pituitary extract (gonadotropin) Note: includes photographs of uteri at different stages./ vaginal opening, weight and histology; ovarian weight	15 rats/group. Table 3 has frequency distribution of wts. Control uteri 19 mg (see Table 6 for summary). Uterine weight more sensitive on dose basis than vaginal opening. Uterine weight 'minus fluid.' Table 3 has uterine weight intervals at various doses, including number of animals in the intervals. Figure 6 is dose response plotted by individual animal points (not mean). Vaginal histology led to comment that cornification may be more sensitive than uterine weight.
Heller *et al.* (1942)	Rat - strain not given	Immature, OVX / ovx at 20 days	Subcutan.	Analysis of urine extractions and concentrates	Minimal data on procedures and no uterine weight data.
Heinrichs *et al.* (1971)	Rat - Sprague Dawley	Adult OVX/ ovx at 120 days	Subcutan./ 7 days	17β-oestradiol, DDT/ Number of assays in subchronic study on female estrus cyclicity	Rats administered high doses (>100mg/kg per day) DDT on neonatal days 2, 3 and 4; vehicle 1:1 dimethyl-sulfoxide:propylene glycol. Puberty, estrous cycle, ovarian histology, etc., then followed into adulthood. Response after OVX to oestradiol tested in control and test groups. Not specified whether wet or blotted weights used.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Huggins and Jensen (1954a)	Rat - albino, no strain given	Immature, HPX / (day not given), injections begun at 38 d	Subcutan./ 7 days	17β-oestradiol and 14 other 19-carbon steroids varying hydroxyl groups at carbons 3 and 17/ vaginal weight and opening	4 rats/group; sesame oil vehicle. Uteri 'blotted lightly.' Control uteri mean 19.3 and 21.6 mg in two experiments. Most results expressed as ratio to controls (>1 would indicate uterine weight increase). Maximum dosage 1 mg rat/day.
Huggins *et al.* (1954b)	Rat - Sprague Dawley	Immature, OVX, HPX, ADX / operation day 24, injections begun at 38 d	Subcutan./ 7 days	A total of 26 19-carbon substances./ organ weights and histopathology in some experiments with compounds	Min. 4 rats/group; sesame oil vehicle. 'During preliminary experiments, it was found that rations from 2 commercial sources induced estrus prematurely in adolescent rats, so that these foods could not be used.' Uteri 'blotted lightly.' Reported as ratio of uterine weight. Substances administered at levels of 1 mg/day.
Huggins and Jensen (1955a)	Rat - albino, no strain given	Immature, HPX / operation day 24, injections begun at 38 d	Subcutan./ 7 days	17β-oestradiol, estrone, equilin, 6-de-hydroestrone, d-equilenin, 4-hydroxy-17β-oestradiol, 7-ketoestrone, 17α-oestradiol, 17-desoxyoestradiol, estr-one-16, Δ-16,17-desoxyoestradiol, 16-keto-17β-oestradiol, 3-desoxy-17β-oestradiol, 3-desoxyestrone, 3-desoxy-17α-oestradiol, 16-ketoestrone, 6-keto-3,17β-oestradiol, 6-ketoestrone, 3,16α, 17β-oestradiol, 3,16α,17β-oestradiol, 3,16α,17β-oestradiol, 3,16β-oestradiol/ vaginal opening; histology of vagina; ovarian, preputial and vaginal wts.	Min. 6 rats/group; sesame oil vehicle. Dose response curves single compounds in Fig. 2 and 3. Std dev optimum doses (plateau) in Tab 2. Simultaneous administration with estrone of several compounds that were estrogenic alone reduced the estrone maximum 26-43% (i.e., antiestrogenic). 'This effect is remarkable since here one estrogenic substance is inhibiting the growth effect of another estrogen.' Comment that vaginal cornification and growth induced by estrone NOT reduced by coadministration. Increase in preputial weights not observed. Uteri 'blotted lightly.'
Huggins and Jensen (1955b)	Rat - albino, no strain given	Immature, HPX / operation at 24 days, injections begun at 38 d	Subcutan./ 7 days	Estrone, 18 steroid compounds, including series of flourinated compounds (usually C-9)/ Vaginal cytology.	6 rats/group. Uterus was 'blotted lightly' before weighing. Ten compounds acted as antagonists of estrone (0.5 μg rat/day) although quantities were 250 μg to 1 mg rat/day), another eight compounds were inactive as antagonists. Six compounds were tested in intact animals from days 23-50 with 18 to 51% reductions in uterine weights over the controls 341 (298 - 409) mg.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Huynh and Pollack (1993)	Rat - Sprague Dawley	Young adult, intact/ 50 days	Subcutan./ 2 days	tamoxifen, ICI 182780/ Insulin growth factor 1 expression	4 rats/group; peanut oil vehicle. Not specified whether wet or blotted weights used. Control uteri in these young adult animals ~320 mg., no apparent effort to control estrous cycle.
Ismail *et al.* (1996)	Rat - strain not reported	Adult OVX/ 100-180 g bw	Subcutan./ 4 days	oestradiol and seven synthesised estrogenic derivatives (steroidal 1,4-diketones and pyridazines) / antiimplantation activity	4-5 rats/group; DMSO vehicle. Blotted uterine weights. Results reported as mg uterine weight per 100 g bw.
Jansen at al. (1993)	Rat - Sprague-Dawley	Immature, intact/ 20 days	I.p./ 1 and 2 days	17β-oestradiol, Aroclor 1242, 3,4,3',4' tetachlorobiphenyl, 4OH-2,4,6, trichorobiphenyl, 2,5,2',5' tetra-chlorobiphenyl / pituitary cell line LH and FSH secretion	11-14 rats/group; corn oil vehicle. Control uteri ~21 mg. Uteri were 'blotted dry.' 10-70% uterine weight increases with various PCBs at 80-320 µg per day doses.
Johnson (1996)	Rat - Holtzman	Immature, OVX, 25-27 day ovx and used 4 days later	Subcutan./ 3 days	oestradiol benzoate, kepone (chlordecone)	5 rats per group. Vehicle benzyl benzoate:sesame oil (4:6). Expressed as mg uterine wt per 100 g bw. Not specified whether wet or blotted weights used.
Jones and Edgren (1973)	Rat - Charles River	Young OVX / ovx at 40 day, used 12 days later	Oral gavage/ 14 days	ethinyl oestradiol (EE), mestranol, lynesternol, norethynodrel, norethisterone acetate, ethynodial diacetate, norethisterone, methyl testosterone, hydrocortisone, progesterone, norgestrel/ Vaginal keratinization also measured. Claim in paper: that uterine growth is a 'nonspecific tests due to response with progesterone, testosterone, etc. Vaginal keratinization, then, is the most specific biologic test available for determining the estrogenic property of a compound. Figures include number of histologic sections.	5-20 rats/group; vehicle not specified other than 'oil.' Not specified whether wet or blotted weights used; controls 42 ± 1.8 mg at 215 g bw. 4.5 X at dosage of 10 µg daily EE. 'A significant response (uterine weight increase) was obtained with all compounds, although massive doses were required in some cases.' For more potent estrogens, uterine increase and vaginal response 'were within the same dose range.' Four compounds failed to induce vaginal keratinization; their uterine responses were at higher doses and very shallow. Some compounds active orally, but not i.p. Two tables include 95% confidence levels for responses and doses of different compounds.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Jones *et al.* (1979)	Rat - strain not given	OVX adult/	Subcutan. and oral gavage/ 7 days	estrone, [3,4-dihydro-2-(4-methoxyphenyl)-1-napthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phenyl]methanone, methanesulfonic acid salt / in vitro receptor binding, antifertility potency	5 rats per test group, 8 per control group. Reported as uterine weight increase over control. Corn oil vehicle. Not specified whether wet or blotted weights used. Compound shows anti-estrogenic activity with oestradiol co-administration.
Jordan (1976)	Rat - Sprague Dawley	OVX adult/ age not given	Subcutan./ 1, 2, 3 days	17β-oestradiol, tamoxifen	8 rats/group. Uterine weight at 1, 2, and 3 days. Control uteri 125 ± 5 mg. days
Jordan and Dix (1979)	Rat - Alderly Park	Immature, inact/ 30-50g bw, no age given	Subcutan. 1 dose 48h necropsy	oestradiol benzoate, tamoxifen, monohydroxytamoxifen/ progesterone receptor synthesis, cell division, endometrial cell morphometry	5 rats/group; peanut oil vehicle. Control uteri ~25 mg; very gentle removal of intraluminal fluid. Antiestrogen doses and ability to stimulate growth via cell division appears distinct.
Karkun and Mehrotra (1973b)	Rat - albino	Young adult OVX/ ovx at 150-170 g bw, used 16 d later	Subcutan./ 5 days	oestradiol-dipropionate (EDP), *cis*-clomiphene, *trans*-clomiphene / also wts of cervix and vagina as well as histopathology	Group size not given in this paper; olive oil vehicle. Not specified whether wet or blotted weights used. EDP stimulates weight increase in cervix and vagina in parallel to uterus (Table 1). Both clomiphene's increase, co-injected with EDP weights in all tissues only reach clomiphene target, not EDP.
Katsuda *et al.* (2000)	Rat - Crj:Donryu	Young adult OVX/ ovx 8 wks and used 3 wks later	Subcutan./ 2 and 14 day groups	oestradiol, *p-tert*-octylphenol/ uterine cell morphometry, serum levels of E and OP with injected dose, BrdU cell proliferation, vaginal cornification	5 rats/group; DMSO vehicle.2 day controls 143 mg uteri; 14 day controls 148 mg. Blotted uterine weights. Uterine weight of equivalent sensitivity; 14 day exposure decreases LOEL slightly.
Katsuki *et al.* (1997)	Rat - Wistar Imamichi	Immature OVX/ age not given, used 1 wk later	Subcutan. and oral gavage/ 3 days	17β-oestradiol, dienogest, danazol, medroxyprogesterone acetate/ progestationa activity, rabbit responses, estrogen receptor affinity, pregnancy and ovulation in rats, Hershberger in males, uterine DNA and protein	6 rats/group. Lauson procedure for uterine preparation cited. OVX control uteri 27.5 ± 2.7 mg (Table VII).
Kono *et al.* (1981)	Rat - Sprague Dawley	Adult OVX/ ovx 15 wks 200-220 g bw	I.v. (tail vein)/ 3 days	17β-oestradiol, 2-hydroxyestrone	6-10 rats group: propylene glycol vehicle. Uteri blotted. Dose response curves for agonist and antagonist activity. Control uteri ~80 mg.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Levin *et al.* (1951)	Rat - Sprague Dawley	Immature, intact; immature HPX, adult OVX/ 21 days for immature	Subcutan. and dietary/ variable on time	wheat germ oil, sesame oil, 0.6% α-tocopherol acetate/ pubertal timing by vaginal opening, histology of ovary and uterus	10-20 rats/group; test oils injected directly. Not specified whether wet or botted weights used. Wheat germ oil shows apparent estrogenic activity in premature puberty and uterine weight increase. However, in HPX animals, ovarian weight is also increased (nearly doubled) suggesting gonadotrophic activity.
Levin *et al.* (1968a)	Rat - Sprague Dawley	Immature, intact/ 19-20 day, 28 - 32 g bw	I.p. / 3 days pretreated, 3 days treat	17β-oestradiol, estrone. Pretreatments for 3-4 days to decrease in estrogenic response included phenobarbital, norchlorcyclizine, chloyclizine, pehylbutazone, orphenedrine, and chlordane.	8-9 rats/group; saline and corn oil vehicles. 3 day pretreatment with phenobarbital decreased oestradiol and estrone uterine response in a dose responsive manner. Not specified whether wet or blotted uterine weights were used. Control uteri 19-20 mg. All treatements reduced response.
Li and Hansen (1995)	Rat - Sprague Dawley	Immature, intact/ 20-22 days	I.p./ 2 days	17β-oestradiol, 2,2',5-trichlorobiphenyl/ liver microsomal metabolizing capacity and serum T4 levels	4-5 rats per group; corn oil vehicle. Analysis relative to body weight with high PCB doses (16-128 mg/kg total); question-able if body weights/rapid weight gain were changed. Not specified whether wet or blotted uterine weights were used. Expressed as uterine wt mg per g bw; corn oil vehicle.
Li and Hansen (1996)	Rat - Sprague Dawley	Immature, intact/ 21-22 days	I.p./ 3 days	Soil extracts containing PCBs, PCDDs, and PCDFs (and presumably other materials). However, results reported in regards to PCBs./ Liver P450 activities, serum T_4, and UDPGT activities.	5 rats/group; corn oil vehicle. Uterus to body weight ratio used. Presuming 50 gram bw then control uteri 25 mg. Not specified whether wet or blotted uterine weights were used. Response of soil extracts does not appear dose-dependent; greatest increase in uterine weight ~20%.
Li *et al.* (1994)	Rat - Sprague Dawley	Immature, intact/ 20 days, 40 g bw	I.p./ 2 days	17β-oestradiol, Aroclor 1242, 2,2',4,4',5,5'-hexachlorobiphenyl / Liver MFO activities, serum TT4 levels	4-7 rats per treatment group; corn oil vehicle. Not specified whether wet or blotted uterine weights were used. Results reported as mg uterine wt/100 g bw. Uterine preparation not specified.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Li *et al.* (1998)	Rat - Sprague Dawley	Immature, intact/ 21 days	I.p./ 2 days	2,3, 3',4',6-pentachlorobiphenyl/ liver MFO and UDP transferase activities	4-7 rats per group; corn oil vehicle. Not specified whether wet or blotted uterine weights were used. Reported as increase in uterine weight versus controls Purified fraction led to approximate doubling of uterine weight.
Loeber and Van Velsen (1984)	Rat - Wistar	Immature, intact/ purchased 21-23 days	Dietary/ 5 days	β-hexachlorohexane	10 rats/group; 10% ethanol in corn oil vehicle. Not specified whether wet or blotted uterine weights were used. Control uteri 18.4 mg. 15-50% increase near or at maximum tolerated dose (from 28 day study)
Lundeen *et al.* (1997)	Rat - Sprague Dawley	Immature, intact and OVX/ 20 days	Subcutan. and oral gavage/ 4 days	17α-ethinyl oestradiol, 17β-oestradiol, 17α-oestradiol, testosterone propionate, dexamethasone, progesterone, tamoxifen citrate, ICI 182,780/ concurrent analysis of serum cholesterol and lipids (circulating levels decreased by estrogen)	5-8 rats/group. Uteri were 'drained of fluid and stripped of fat and mesentery.' Immature uteri ~40 mg, adult OVX uteri ~80-100 mg (85 ± 7.2 mg in Table 2). Uterine weight increase occurs at lower dosage by s.c. than gavage, but route has no apparent effect on circulating cholesterol decrease (see Fig. 1 and 2)
Lyttle and DeSombre (1977)	Rat - Sprague-Dawley	Pubertal, intact/ 30 d, 54 g bw	Subcutan./ 2 days	17β-oestradiol/ peroxidase activity in both estrogen responsive and non-responsive tissues/ also mice, hamster, and guinea pig	4-10 per group; 25% ethanol-75% saline vehicle. Control uteri 25.2 mg; not stated whether wet or blotted weights used. 2 days of injections. 3.35 fold increase at 40μg of E/kg/day.
Markaverich *et al.* (1995)	Rat - Sprague Dawley	Immature – OVX/ ovx at 21 days, used 7-10 days later	Subcutan. and oral gavage/ time varied	17β-oestradiol, coumestrol/ Oven dry uterine weights; cytosolic and nuclear receptor competitive binding assays; time course of cytosolic to nuclear receptor levels, receptor affinity	5-6 rats/group; saline-2% Tween 80 vehicle. Not specified whether wet or blotted uterine weights were used; did include oven dry weights with conditions given. Control uterine weights ~25-30 mg. Most expts. 1 or 2 days. Authors propose that agonist or antagonist activity may be dose dependent and suggest interaction of competing estrogens with ER may be far more complex than previously thought.
Marlow (1936)	Rat - no strain given	Immature, intact/ 25 days	"Injected" / 5 days	theelin and dihydrotheelin (need to ref nomenclature)	Group size and vehicle not specified. Not specified whether wet or blotted uterine weights used. Ratio of mg uterine wt to g body wt 'less gut' Compounds had different slopes.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Martucci and Fishman (1977)	Rat - CD	Immature, intact/ 22 days	Paraffin pellet implants/2 4, 48, and 72 hrs	oestradiol, estriol, estrone, 2-methoxy-estrone, 2-hydroxyestrone, 15α-hydroxyestriol, 2-hydroxyoestradiol/ dry uterine weights	15 rats per dose, 5 rats per time point; paraffin implants. Uteri blotted before weighing. Control uteri 30-35 mg
Medlock *et al.* (1995)	Rat - Sprague Dawley	Immature/ postnatal days 1 through 10, sacrificed in sets up to day 25	Subcutan./ various times	diethylstilbestrol (DES), coumestrol, equol/ Wet and dry uterine weights, uterine gland development, and uterine estrogen receptor levels.	≥ 7 rats/group; sesame oil vehicle. Not specified whether wet or blotted uterine weights used; oven dry weights also recorded. Reported as ratio of uterine weight to body weight. DES> coumestrol > equol in several measures, including uterotrophic response and estrogen receptor levels. When plotted with the dose on a log basis, linear responses were observed (Figure 1).
Medlock *et al.* (1997)	Rat - Sprague Dawley	Immature/ pups from postnatal days 1 through 24 were sued	Subcutan./ pnd 1-5, develop-mental study	tamoxifen, toremifene	8-15 rats/group; sesame oil vehicle. ~25 mg control uteri at d 26, blotted weight after fixation. Objective was analysis of the development of specific uterine cell types (uterine glands) during postnatal days 10-14, and effects of antiestrogens.
Mirocha *et al.* (1978)	Rat - strain not specified	Immature, intact/ 20 days	Diet and dermal/ 3 days	*cis*-zearalenone, *cis*-zearalenol, *trans*-zearalenone, *trans*-zearalenol	3 rats/group. Vehicles not specified; not specified whether wet or blotted uterine weights used. Control uteri range stated as 27-40 mg in legend of Table 1. Compounds active by dermal route.
Nephew *et al.* (2000)	Rat - Sprague Dawley	Pubertal, intact/ 50 days	Dietary/ 28 days	17β-oestradiol, tamoxifen, toreminfene, DHEA, vorozole/ histo morphology of uterine cell types	10 rats/group. Tekald diet. Blotted uterine weights used. Both estrogenic and antiestrogenic effects evident compared to controls (uterine weights at 78 days, ~370 mg ± 20 mg, again, intact rats.)
Ng *et al.* (1994)	Rat - Sprague Dawley	Immature, OVX/ 18 days	Oral gavage/ 2X daily 3 days	17β-oestradiol , yuehchukene/ estrogen receptor binding affinity, MCF-7 cell proliferation, vaginal smears in mice, several uterine and liver enzyme assays	5 rats/group; vehicle not specified. Both wet ('with uterine fluid sealed inside') and blotted weights specified. Control uteri 17.2 ± 1.4 mg wet, 12.6 ± 0.8 mg blotted.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Nishino *et al.* (1991)	Rat - Wistar	Adult OVX/ ~200 g bw, used 14 days after ovx	Subcutan./ 5 days	17β-oestradiol, ZK 119010, ICI 164384/ Vaginal weights	6 rats/group. Vehicle not specified. Not specified whether wet or blotted uterine weights were used. Control uteri 38.0 ± 6.3mg per 100 g bw. Agonist and antagonist assays; agents mild agonists in rats for both uterus and vagina, and antagonists for oestradiol in both tissues. Mice also assayed in parallel.
Olson and Sheehan (1979)	Rat - Sprague Dawley	Adult, OVX/ ovx 175-200 g bw	Silastic implant/ 5 days	oestradiol and rotenone/ estrogen receptor binding	4 rats/group. Blotted uterine weights used (see table 1). Control uteri 96 mg. Data simultaneously given relative to body weight.
Omar *et al.* (1994)	Rat - strain not given ('albino rats')	Adult, OVX/ ovx at 100-180 g	Subcutan./ 4 days	oestradiol, 16 derivatives/ receptor binding affinity	4-5 rats/group; olive oil vehicle. Blotted uterine weights. Results also reported as weight per 100g bw and oven dried. Controls 26.6 and 14 mg blotted.
Omar *et al.* (1996)	Rat - strain not given	Adult, OVX/ ovx 160-240 g	Subcutan./ 4 days	oestradiol, estrone, 4',17-dioxo-5'H-estra-1(10),4-dieno[3,2-b]furan/ vaginal smears, implantation efficiency, receptor binding affinity	4 rats/group; DMSO vehicle. Blotted uterine weights were used. Results reported as weight per 100g bw. Oven dried weights also included. Controls 45 mg blotted.
Ostrovsky and Kitts (1963)	Rat - Wistar and Sprague Dawley	pre-pubertal, intact and post pubertal OVX/ no details of age, bw, or timing given	Dietary 4/ 6 days	Diethylstilbestrol and coumestrol acetate with whole forage and benzene extracted forage,	6 rats/group. Age of animals not given. Not specified whether wet or blotted uterine weights were used. Controls having mean body wts of 48 and 109 g had mean uteri wts of 101 and 109 mg, respectively. If OVX, bw 68, 82, and 117 g and uteri 29, 24, and 27 mg, respectively.
Rosenblum *et al.* (1993)	Rat - no strain given	Adult OVX/ no details given	Drinking water/ 30 days	β-sitosterol and bourbon/ receptor assays	11-34 rats/treated group (61 controls). Not specified whether wet or blotted uterine weights were used. Expressed as mg uterine wt/100 g bw.
Ruenitz *et al.* (1998)	Rat - Sprague Dawley	OVX/ 90 days, 250 g bw	Subcutan./ 5 days wk for 5 wks	17β-oestradiol, tamoxifen, clomiphene, 4-hydroxyclomiphene4-hydroxytamoxifen/ bone parameters	10 rats/group; vehicle 5% benzyl alcohol in corn oil. Not specified whether wet or blotted uterine weights were used. OVX control uteri 86 and 99 mg.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Ruh *et al.* (1995)	Rat - Sprague Dawley	Immature, intact/ 21 days	I.p./ 3 days	17β-oestradiol, naringenin/ progesterone receptor, uterine peroxidase, tritiated thymidine incorporation, cytosolic receptor depletion, ligand-receptor binding to DNA response element	4 rats/group; DMSO vehicle. Control uteri 41 ± 0.7 mg. Uterus blotted. Naringenin reduces (antagonises the action of oestradiol in preventing maximum increases in uterine weight and also in several complementary assays.
Saloniemi *et al.* (1995)	Rat - strain not given	Immature, intact/ age not given refers to another publication	Dietary/ 5 days	Extracts in diet equivalent to 3 grams of four different fodders.	8 rats/group. Control uteri 21.0 ± 2.0mg. Results as increase in uterine wt (mg), includes range for some treated groups. References are given procedures; no methods detailed in this paper.
Santell *et al.* (1997)	Rat - Sprague-Dawley	OVX / (groups 30, 60 and 70 d old)	Dietary/ 5 days	17β-oestradiol and genistein/ competitive binding analysis, plasma prolactin levels, wet and oven dry weights, uterine expression of *c-fos*, mammary gland growth	6-8 rats/group. Not specified whether wet or blotted weights used. Controls for 70 d animals 76.5 ± 3.2 mg. 3 X induction in OVX. Genistein generally active in other assays paralleling uterotrophic results, including mammary gland development.
Schlumpf *et al.* (2001)	Rat – Longs Evans	Immature, intact/ 21 days	Dietary/ 4 days	Ethinyl oestradiol and six sunscreen actives including 4-methyl-benzylidene camphor and octyl-methoxycinnamate/ MCF-7 cell proliferation assay	Group size adjusted with expected dose response (large group sizes, i.e., 19, in the lowest region of the dose response). Blotted control uteri ~25 mg. Several sunscreen actives had modest increase in uterine weights with very shallow dose-related slope as doses increased (highests doses > 1000 mg/kg/d).
Schneider *et al.* (1976)	Rat - possibly Sprague-Dawley	OVX / age unknown	Subcutan./ 7 days	17β-oestradiol, 3,9-dihydrobenz[*a*]anthracene (DHBA)/ relative binding affinity	5 rats/group; olive oil vehicle. Not specified whether wet or blotted weights used. Control weights not reported. Test compound (DHBA) ~3500 times less potent.
Segaloff and Gabbard (1984)	Rat - Fischer	Immature, intact/ age not given	Subcutan./ 4 days, 2X per day	17β-oestradiol, 11β-hydroxyoestradiol, estrone, and 17 11β oestradiol esters/ receptor binding assay	Indicates procedure detailed in Gabbard and Segaloff (1983b).
Shiverick and Muther (1982)	Rat - Holtzman	Young adult/ ovx 75-100 g bw at ovx, 7 days rest	Subcutan./ 4 days	estrone/ liver MFO induction and estrone serum concentrations	6 rats/group; corn oil vehicle. Control uteri ~30-35 g (Fig. 2). Uteri immersed in Bouin's solution 24hrs before weighing. Not specified if wet or blotted weights.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Skidmore *et al.* (1972)	Rat - Alderly park	Not given / (OVX animals used for receptor assays)	Not given	17β-oestradiol, ICI 46,474, ICI 47,699/ rat uterine receptor binding (rat, rabbit, mouse)	Compares association constant with half maximal dose of compound in uterotrophic bioassays and mice (Tables 4 and 5). Not specified whether wet or blotted weights used. Discussion of differences in vaginal cornification p. 297.
Sreenlvasulu *et al.* (1992)	Rat - Sprague Dawley	Immature OVX/ 25-30 g at ovx, rested 7 days	3 days	CD 85/287/ vaginal opening and vaginal smears, antiimplantation activity.	Group size and vehicle not specified. Not specified if blotted or wet weights. Control weights in figures ~20 mg.
Tullner (1961)	Rat - Sprague Dawley	Immature OVX + ADX and HPX/ final bw 55 - 73 g	Oral gavage/ 3 days	Methoxychlor, estrone, anisole/ vaginal cytology	10 rats/group; sesame oil vehicle. Control uteri 17 ± 2 and 24 ± 5. Not specified whether wet or blotted uterine weights used. ~400 mg/kg/day methoxychlor elicited 2-fold uterine increase. Vaginal cornification also observed. Experiments performed due to observed uterine increase when lab animals were dusted with pesticide containing methoxychlor.
Turnbull *et al.* (1999)	Rat - Wistar	Immature/ 17 days	Dietary/ 4 days	diethylstilbestrol, wood stanol, vegetable oil stanol (stanol composition not analyzed)/ MCF-7 proliferation	10 rats/group. Not specified whether wet or blotted uterine weights used; control uteri 50 ± 1 mg. DES LOEL 1.2 μg/kg/day.
Umberger *et al.* (1958)	Rat - Osborne-Mendel	Immature, intact/ 20-22 days	Dietary/ 7 days	stilbestrol, 17β-oestradiol, estrone	10 rats/group. Blotted uterine weights used. Control uteri 43.7 ± 1.1 mg. Diets were chicken muscle to which estrogenic chemicals were added as authors sought assay for estrogens in diet.
Wade *et al.* (1993)	Rat - CD	Adult OVX/ age not given	Subcutan./ 3 and 4 weeks	17β-oestradiol benzoate, tamoxifen, ICI 182780/ tissue uptake of labeled oestradiol, receptor binding affinity, estrous, estrous behavior	5 rats/group; sesame oil vehicle. From Fig. 5, uterine weights ~90 mg for controls and high anti-estrogen dose groups. Not specified if wet or blotted weights.
Wakeling *et al.* (1991)	Rat	Immature, intact and OVX adult/	Subcutan./ 3 days	17β-oestradiol, ICI 164,384, and ICI 182,780 / receptor binding affinity	Min. 5 rats/group. Previous publication referenced for methods, including uterine preparation and treatment. Control immature uterine wt ~40 mg; control OVX uteri ~75 mg.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Washburn *et al.* (1993)	Rat - Fisher 344	OVX adult/ 11 wk OVX	Dietary/ 120 days	premarin, estrone sulfate, 17α-oestradiol, 17α-dihydroequilenin sulfate/ plasma cholesterol and lipoprotein fractions	8-10 rats/group. Control uteri 124.6 ± 12.0 mg. While uterine weights are increased in dose responsive manner, there is no time course or comparison with s.c. to address issue of extended dosing. Not specified whether wet or blotted uterine weights used.
Welch *et al.* (1969)	Rat - Sprague Dawley	Immature, intact/ 19- 20 days, 30 32 g bw	I.p./ 3 days	DDT technical grade, *o,p'*-DDT, *p,p'*-DDT, *m,p'*-DDD, *o,p'*-DDD, *p,p'*-DDE, *p,p'*-DDE/ Competitive effect on tritiated oestradiol uptake.	6 rats/group; DMSO vehicle. Not specified whether wet or blotted uterine weights used. Control uteri 18.2 ± 0.6, 20.4 ±0.4 and 20.8 ± 0.7 mg in three expts. Increases 151% (*o,p'*-DDT) and 75% technical DDT at 50 mg/kg/day. Note: Age at start ≤ 20, tight bw range, and very low s.d.
Welch *et al.* (1969)	Rat - Sprague Dawley	OVX adult/ 2 wks after OVX	I.p./ 3 days	DDT technical grade and *o,p'*-DDT/	7 rats/group; DMSO vehicle. Not specified whether wet or blotted uterine weights used. Control uteri 89 ± 4.9 mg at 200-210 body wt, used 2 week after OVX
Welch *et al.* (1971)	Rat - Sprague Dawley	Immature, intact/ 19-20 d, 28 - 32 g bw	I.p./ 7 day pre-treat and 3 day admin	γ-chlordane, dieldrin, heptachlor, lindane, *p,p'*-DDD, *p,p'*-DDE, toxaphene pretreatments. ± estrone/ Uptake of tritiated oestradiol into uterus, metabolism of oestradiol *in vivo*, metabolism of tritiated 17β-oestradiol in liver microsomes.	6-8 rats/group; corn oil vehicle. Not specified whether wet or blotted uterine weights used. 7 d pretreatment with insecticides to induce liver monooxygenases. No increase in uterine wt over controls (doses 3-25 mg/kg) in 7 d of treatment (Table 3). All reduced estrone weight increase when estrone injected 24 hrs after last insecticide.
Wenzel and Rosenberg (1956)	Rat - Sprague Dawley	Immature, intact/ 21-23 day	Subcutan./ (2X per day for 4 days) and oral (4 days)	estrone, osajin, pomiferin, 4',6dihydroxyflavone (DHF), liqueritigenin, naringenin, hesperitin	8 rats/group; cottonseed oil vehicle. Only estrone and DHF (sc) were active. Not specified whether wet or blotted uterine weights used. Control uteri 36 ± 2.5mg. Total dosage for 4 days, 20 mg flavinoids sc, 40 mg oral.

Table 1A-3. Uterotrophic bioassays with variations in the standard protocol with intact, immature; OVX immature, and adult OVX rats. (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Whitten *et al.* (1992)	Rat - Sprague Dawley	Intact, immature/ 20-21 days, 30-40 g bw	Subcutan. for 3 days and diet for 3, 4 and 8 days	coumestrol/ receptor binding, uterine progesterone receptor, uterine estrogen receptor	7 rats/group. Control uteri are ~50 mg as 'wet weight' and dry uteri are ~12 mg. Would appear that wet weights are not blotted. Semi-purified diet used to avoid soy contamination. S.C. and dietary administration. Dietary administration appeared to be more effective over time (see p 102, column 1)
Zacharewski *et al.* (1998)	Rat - Sprague Dawley	OVX, immature/ ovx day 19, received 24-25 and acclimated	Oral gavage/ 4 days	ethinyl oestradiol, eight phthalates: di(n-butyl), dihexyl, diethylhexyl, diisoheptyl, di(n-octyl), diiosnonyl, diisodecyl, and dibenzyl/ rat uterine estrogen receptor binding, transient transfection in MCF-7 and HeLa cells using reporter genes, estrogen uracil necessary growth in uracil deficient yeast	10 rats/group; sesame oil vehicle. Control uteri 13 to 51 mg when expressed mg/ 100g bw. Uterine stubs (incomplete OVX) suspected as cause of variation. Uteri 'nicked, blotted, and weighed.' Three phthalates very weak ER competitors, same three slightly active in MCF7, only one active in HeLa and uracil dependent yeast. No significant dose dependent responses observe red *in vivo* using uterotrophic bioassays with doses up to 2000 mg/kg in two replicate experiments.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds.

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
MOUSE					
Ashby *et al.* (1997b)	Mouse - Alderly Park Alpk:AP_fCD-1	Immature, intact/ 21-22 days	Subcutan./ 3 days	ethinyl oestradiol, benzoic acid	5-10 mice group; corn oil vehicle. Blotted weights used. Ontrol uteri 9.7 ± 2.3 mg. Benzoic acid did not respond at 1 mg/kg.
Bartlett *et al.* (1948)	Mouse - no strain given	Immature, intact/ age not given	Subcutan./ 3 days (2X daily)	α-oestradiol and 6 extracts of herbage	Necropsy 18 hrs after last injection. Not specified whether wet or blotted uterine weights used. Control uteri 5.3 ± 0.4 mg and 7.8 ± 0.8 mg in two experiments. and arachis oil vehicle 6.4 mg. Extracts up to 3X uterine weight and oestradiol nearly 4X.
Berger *et al.* (1986)	Mouse - Swiss	Immature, intact/ 18 days	I.p./ 3 days	estrogen and six synthesised compounds/ mammary tumor proliferation and receptor binding assay	8 mice/group; DMSO vehicle. Uteri removed fixed in Bouin's solution and washed in alcoholic LiCl, then weighed. Results reported as mg dried uterine wt/bw g * 100.
Bickoff *et al.* (1959)	Mouse - Dalswiss	Immature, intact and adult, OVX/ 19-21 days, 8 - 10 g bw and 6 wks, respectively	Dietary or oral gavage/ 6-8 days	coumestrol, stilbestrol, diethylstilbestrol, ladino clover extract	5-10 mice/ group. Wet uterine weights were used without blotting. Statistical view of assay over one year in Figure 1 for both vehicle control (9.41 mg uteri) and DES control at 0.1 μg (31.96 mg uteri). Comparison of immature and OVX mouse in Table 7, indicates immature is more sensitive than OVX using oral administration route for DES.
Bickoff *et al.* (1960a)	Mouse - no strain given	Immature, intact/ 19-21 days	Dietary / 4-6 days	coumestrol, stilbestrol (? diethylstilbestrol - noted only in statistical experiments), 35 different types and samples of alfalfa meal from various locations and cuts, 7 clover samples, 9 grass samples, plus others including stability study from cutting, through drying and aging of clover	5-10 mice/ group. Statistical review of assay in Table 4. Not specified in this paper whether wet or blotted uterine weights used. Control uteri ~ 9 mg. Feeding was 4-6 days, varied until researchers indicated a constant equivalence of coumestrol in the forage diet was consumed. Wide range in forage potency observed.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Bickoff *et al.* (1960b)	Mouse - no strain given	Immature, intact/ 19-21 days	Dietary/ 4-6 days - until 10 g diet was consumed	Coumestrol	Uterus weighted 'without preliminary fixing or blotting.' Expressed only as activity relative to coumestrol (1000), a number of compounds reported as having 0 activity.
Bickoff *et al.* (1962)	Mouse - no strain given	Immature, intact/ 19-21 days, 8 - 10 g bw	Dietary/ 4-6 days - until 10 g consumed	Diethylstilbestrol, estrone, coumestrol, coumestrol diacetate, genistein, daidzein, biochanin A, formaonenetrin	5 mice/group. Control uteri 9.6 ± 0.3 mg. Reported as not blotted. Substances reported as relative affinity to genistein.
Black and Goode (1980)	Mouse - Standard Cox	Immature, intact/ 11-13 gm bw, age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, trioxifene and LY-117018	10 mice/group. Control uteri ~12 mg. 'uteri were dissected free of extraneous tissue and fluid contents expelled.' Tamoxifen antagonist activity observed.
Black and Goode (1980)	Mouse - CD-1	OVX/ used 7 days after OVX, age and bw not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, trioxifene and LY-117018	10 mice/group. Control uteri ~25 mg. 'uteri were dissected free of extraneous tissue and fluid contests expelled.' Tamoxifen antagonist activity NOT observed (consistent with other investigators).
Bohl *et al.* (1987)	Mouse - ABD2-F_1	Immature, intact/ no age specified	Oral gavage/3 days	Thirty estrogenic derivatives (steroids substituted at C14, C15)/ receptor binding activity and implantation efficiency	Group size not specified; sesame or peanut oil vehicles. Not specified wheter wet or blotted weights used. Uterotrophic results as concentration required to double uterine weight (plotted on log scale against implantation in Fig. 3)
Booth *et al.* (1960)	Mouse - no strain given	Immature, intact/ no age given	Dietary and subcutan./ 4 days	17 vegetable oil samples (various sources, refined, etc.)	5 mice/group. Not specified whether wet or blotted uterine weights used. Control uteri 9.5 mg. Almost all vegetable oils when included at 10% in the diet increased the uterine weight. Also corn oils injected subcutaneously at 0.4 ml, increased uterine weights (control 9.9, saline 10.3, 5 oil samples 13.2 - 18.2 mg). Suggest vehicle control and attention to total fat in diet (other references).

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Breinholt *et al.* (2000)	Mouse - C57B6 female crossed to DBA2J male (B6D2F1)	Immature, intact/ 17-18 days	Oral gavage/ 4 days	17β-oestradiol, genistein, equol, apigenin, kaempferol/ increase in total cellular estrogen receptor and translocation from cytoplasm to nucleus.	5 mice/group, DMSO. Uterus excised, trimmed free of fat and pierced to remove excess fluid. Cut above junction with cervix and junction of horns with ovaries. Reported only as relative to body weight (Figure 2)
Carter *et al.* (1953)	Mouse - no strain given	Immature, intact/ 3 week old	Subcutan. 2X daily and dietary/ 3 days	diethylstilbestrol, genistein, soybean meal, extracted soybean meal	6 mice/group; peanut oil vehicle. Uterus ' pressed lightly on filter paper to eliminate uterine fluid.' Control uteri 8.2 mg. Commercial soybean meal via diet led to nearly 200% increase.
Carthew *et al.* (1999b)	Mouse - CD1	Adult OVX ovx at ~30 g bw	Subcutan./ 3 days	oestradiol benzoate, tamoxifen, toremifene, raloxifene/ vaginal weight, vaginal cornification, luminal epithelium morphometry, BrdU labeling	4 mice/group; DMSO and tricaprilin as vehicles. Uteri weighted with and without fluid.
Chae *et al.* (1991)	Mouse - CD1	Adult OVX, sacrifice at 8-10 wks age	Subcutan./ 3 days	17β-oestradiol, diethylstilbestrol, indenestrol A, 4'-deoxyindenestrol/ receptor binding affinity, uterine DNA synthesis	3 mice/group; corn oil vehicle. Not specified whether wet or blotted weights used. Results reported as uterine mg/body weight g ratio.
Cheng *et al.* (1953a)	Mouse - no strain given	Immature, intact/ 3 week old	Subcutan. and dietary 3 day (2X day for sc)	diethylstilbestrol, genistein, unextracted and extracted soybean meal	6-20 mice/group; olive oil vehicle. Statistical review in Table 2. Dietary time not given. Control uteri ~8.2 mg. Uteri were fixed in Bouin's solution for 24-hours and blotted with filter paper.
Cheng *et al.* (1953b)	Mouse - no strain given	Immature, intact/ 3 week old	Subcutan. and dietary/ 4 days	genistein (both oral and s.c.), diethylstilbestrol	5-6 mice/group. s.c. versus dietary comparison. Sacrifice 24 hrs after administration. Control uteri ~9.7 mg. Uteri were fixed in Bouin's solution, but blotting was not specified.
Cheng *et al.* (1954)	Mouse – no strain given	Unknown - method in previous paper not yet reviewed	Oral gavage/	biochainin A, diadzein, formonoetin, genistein, stilbestrol	6 mice/group; olive oil vehicle. Control uteri mean 6.4 mg. Fixation and uterine preparation such as blotting were not specified in this paper. Isoflavone administration in feed estimated at 2.5 mg/day.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Claussner *et al.* (1992)	Mouse - Swiss	Immature, intact/ 18-19 days	Subcutan. and oral gavage/ 3 days	RU 50667, RU 51625, RU 53637, RU 50667, RU 51625, RU 53637, RU 54485/ receptor binding assay, MCF-7 and Ly2 cell assays, tumor inhibition assay in mouse	Group size not specified; oral vehicle 5% methylcellulose and s.c. vehicle sesame oil with 5% benzylic alcohol. Not specified whether wet or blotted uterine weights used. Results expressed as percentage of uterine weight of controls. Three doses of each test compound.
Coldham *et al.* (1997)	Mouse - CFLP	Immature, intact/ 18 days	Subcutan./ 3 days	17β-oestradiol (E), diethylstilbestrol, 4-nonylphenol (NP - tech. Sigma), 4-octylphenol, coumestrol, benzylbutyl-phthalate, dibutylphthalate, α-zearalanol, bisphenol A (BPA)/ yeast reporter gene assays and receptor binding data from literature	7 mice/group; corn oil vehicle. Control uteri expressed as uterine weight/bw - 0.097 ± 0.030. Not specified whether wet or blotted weights used. E max induction 5.5 fold. In general, potency of compounds using *in vivo* uterotrophic were lower by one or more orders of magnitude versus *in vitro* potency. Two compounds, NP and BPA, elicited acute toxic effects at higher doses (~400 mg/kg/day).
Connor *et al.* (1997)	Mouse - B6C3F1	Immature, intact/ 19 days	I.p./3 days	17β-oestradiol and 8 hydroxylated PCBs OH at 4 position (2,2',3',4',5'; 2,2',3',4',6'; 2,2',3',5',6'; 2,2',4',6'; 2',3,3',4',5'; 2',3,3',4',6'; 2',3,3',5',6'; 2',3,4',6'/ competitive binding to rat and mouse uterine receptor, uterine peroxidase and progesterone receptor levels, MCF-7 proliferation, chloramphenicol acetyl transferase assay, stably transfected reporter in HeLa cells	6-9 mice/group; corn oil vehicle. Uteri nicked and blotted. At doses of 100 mg/kg/day, none of the PCBs increased weight, peroxidase activity, or level of progesterone receptor. Reported as percent increase over controls, so direct weights not reported.
Day *et al.* (1991)	Mouse - Swiss Webster	Immature, intact/ 17-19 days	Subcutan./ 3 days	17β-oestradiol, tamoxifen, and 13 synthesised compounds/ estrogen receptor binding assays	6 mice/group; sesame oil vehicle. Uteri blotted 'to remove tissue fluid.' Results reported as percentage decrease versus controls (antagonist assay).
Dorfman and Kincl (1966)	Mouse - Swiss albino	Immature, intact/ 21-23 days	Subcutan. and oral gavage/	In excess of 50 structurally related compounds are compared against estrone and 17β-oestradiol as standards (nine tables).	18-107 mice/group; sesame oil vehicle. Not specified whether wet or blotted uterine weights used. Comparisons expressed relative to estrone and oestradiol (uterine weights are NOT given), some oral and s.c. comparisons.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Drane *et al.* (1980)	Mouse - MF1	Immature, Intact/ 18 days, 7-9 g bw	Dietary/ 3.5 days	16 samples of soya meal and ethyl acetate extracts of 11 lots.	6 mice/group. Blotted uterine weights. Controls not given, non-statistically significant test animal weights 7.7-11.7 mg.
Edery *et al.* (1985)	Mouse - C3Hf(VVII)	Immature, intact/ 20 days, 11-13 g bw	Subcutan./ 3 days	17β-oestradiol, tamoxifen, cis-broparoestrol, trans-broparoestrol/ nucleic acid and protein assays of uterus	10-14 mice/group; peanut oil vehicle. Not specified whether wet or blotted weights used, but reference Rubin (used blotted weights). Control mouse uteri in two experiments. were 10.7 ± 2 and 9.2 ± 1 mg.
Edgren and Calhoun (1957)	Mouse - no strain given	Immature, intact/ age not given	Subcutan. and oral gavage/ time not given	estrone, testosterone propionate, progesterone, 17-ethyl-19-testosterone/	8-10 mice/group; corn oil vehicle. Control uteri 9.8 mg (test). Results reported as relative to increase observed with given dose of estrone. Not specified whether wet or blotted weights used.
Edgren (1956)	Mouse - no strain given	Immature, intact/ 23-25 days	Subcutan./ 3 days	estrone, estriol, 17β-oestradiol, diethylstilbestrol, vallestril, 16-oxoestrone, SC-6370, SC-3402	8-10 mice/group; corn oil vehicle. Uteri scored and blotted to express contained fluid. Expressed as regression coefficient.
Edgren (1958)	Mouse - no strain given	Immature, intact/ age not given	Subcutan. and oral gavage/time not given	estrone, 17α-ethynyl-17-hydroxy-5(10)-estren-3-one (norethynodrel), 17α-ethynyl-19-nortestosterone/ antagonist activity against estrone	8-10 mice/group; vehicle not specified. Not specified whether wet or blotted uterine weights used. Control uteri stated as 12 mg. Fig. 1 compares s.c. and gavage (slopes parallel; s.c. has lower effective doses but degree varies). Some repetition has shown - two to six groups for up to five dosages of each compound in Figure and in Table 1. Route of administration did not affect antagonist assays against s.c. administered estrone (Tables 2 and 3).
Edgren *et al.* (1966)	Mouse - Charles River	Immature, intact/ 23-25 days	Subcutan./ 7 days	17β-oestradiol, estrone, estriol, 18-homoestrone, 18-homoestradiol, 18-homoestriol	Uterine growth is normally expressed as a ratio to body weight and the results of all stated experiments are not reported. Not specified whether wet or blotted uterine weights used. Vehicle controls are uteri appear very high in weight; specific vehicle not given.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Edgren *et al.* (1967)	Mouse - Charles River	Immature, intact/ 23-25 days	Subcutan./ 3 days	17β-oestradiol, 18-homoestradiol, propyl oestradiol/ mouse vaginal studies OVX as adults, 23 - 27 g bw, injections for 4 days, relative potency vs 17β-oestradiol using approximately 10 doses	9-25 mice/group; specific vehicle not given. Control uteri 27 mg; mean only; 'unusually high average for the oil vehicle control value.' Showed only about 2X increase in weight over controls. 4 doses oestradiol used; 7 doses of other compounds used. Other comparative work on vaginal assay, rat assays, chick oviduct also done
Evans *et al.* (1941)	Mouse - albino	Immature, intact/ no age given, 6-8 g bw	Subcutan./ 3 days (2X daily)	17β-oestradiol, estrone, estriol, 17α-oestradiol, stilbestrol, 17α-oestradiol diacetate, 17α-oestradiol benzoate, 17α-oestradiol dipropionate, 17β-oestradiol (from estrone), 17β-oestradiol (mare's urine), 17β-oestradiol benzoate, 17β-oestradiol diacetate, equilin, Δ6-equilin, α-dihydroequilin, β-dihydroequilenin, progesterone, testosterone, testosterone propionate, androsterone, dehydroisoandrosterone	5 mice/group; sesame oil vehicle. Plots are mg increase over controls. Not specified whether wet or blotted uterine weights used. Control uteri in one experiment 4.6 mg. Non-estrogens do increase uterine weight at high doses - see Figure 5 and Table 2. Coadministration of weak estrogen agonist can reduce estrone (i.e., appears as antiestrogen or antagonist). One experiment shows wide variation within lab over time (see Table 1).
Farmakalidis and Murphy (1984a)	Mouse - three different strains: ICR, B6D2F, $B6C3F_1$	Immature, intact/ 20-21 days	Oral gavage/ 4 days	diethylstilbestrol	12 mice/group; 5% Tween 80 vehicle. Control uteri varied among strains (ICR 27.30 mg, B6D2F 12.66 mg, $B6C3F_1$ 13.70 mg). Uteri 'dissected out and weighted immediately without blotting'. Apparent strain differences in weight gain when dosed with DES, but dose in μg/kg bw differed due to differences in body weights among strain groups.
Farmakalidis and Murphy (1984b)	Mouse - CD1 strain	Immature, intact/ 20-21 days	Oral gavage/ 4 days	Genistein, diethylstilbestrol, genistin, daidzein	8-16 mice/group. Uteri 'dissected out and weighted immediately without blotting'. Control uteri 25.1 mg. Gavage with 5% Tween 80 vehicle. Indicate that CD-1 strain of mouse not as responsive.
Farmakalidis *et al.* (1985)	Mouse - B6D2F1	Immature, intact/ 20-21 days	Oral gavage/ 4 days	Genistein, diethylstilbestrol, genistin, daidzein	7-24 mice/group. Uteri 'dissected out and weighted immediately without blotting'. Control uteri 14.88 mg. Daidzein activity not statistically significant at 3 mg/mouse/day.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Feldman *et al.* (1984)	Mouse - Swiss Webster	OVX/ ovx at 6 wks, used 3 wks later	Subcutan./ 3 days	oestradiol, extract of baking yeast/ receptor binding assays	Group size unclear; DMSO vehicle. Uteri 'blotted dry.' Control uteri ~16-18 mg.
Fielden *et al.* (1997)	Mouse - CD1	OVX received at 12 wks and mean bw 41 g, acclimated for 6 days	Oral gavage/ 4 days	Ethinyl oestradiol, 2,4,6,2',6'-penta-chlorobiphenyl (PCB 104)/ receptor binding assay, MCF-7 cell proliferation assay, vaginal cornification	5 mice/group; sesame oil vehicle. Control uteri expressed as mg/g bw: 1.1 ± 0.1. 'Uteri … blotted to remove water and weighed.' PCB 104 at 202 mg/kg/day increased uterine weights ~70% (less than 1-fold) and also increased vaginal cornification.
Folman and Pope (1966)	Mouse - BSVS	Immature, intact/ 21 to 28 d (3-4 weeks of age)	Subcutan./ 2X daily for 3 days	17β-oestradiol, diethylstilbestrol, estrone, norethisterone acetate, megestrol acetate, coumestrol, genistein and dimethylstilboestroel/ vaginal weights also included	9 mice/group; "aqueous propan-1,2-diol" vehicle. ~7-12 mg control uteri. Blotted uterine weights were used. For 11 experiments, mean and range of bw given (mean range was 9.6 to 11.1 g), some administration of two substances in mixed solutions and some coadministered at different sites; dose reported as total administered over 3 days. Typically, the 'weaker' compound was not additive with 'potent' compounds, but appeared to reduce the response of potent compounds (i.e., antiestrogenic)
Galey *et al.* (1993)	Mouse - Swiss Webster	Immature, intact/ 10-12 g bw, age not given	Dietary/ 5 days	17β-oestradiol, diethylstilbestrol, coumestrol, forages/ histopathology of uterus	3 mice/group. Graphically, control uteri appear to be large and to have high variability (estimated mean 20 mg). Not specified whether wet or blotted weights used. Some hay forages showed positive increase; others did not
Greenman *et al.* (1977)	Mouse - BALB/c, C57BL/6, $B6CF_1$, and monohybrid cross	Immature, intact/ 18 - 20 days	Dietary/ 6 days	diethylstilbestrol/ vaginal cytology	11-12 mice/group. Changes in vaginal cytology and uterine weight compared to DES dietary administration. C57BL/6 strain was slightly more sensitive in both the vaginal and uterine assays. Uteri were weighed 'after expressing luminal fluid onto absorbent paper.'

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Greenman *et al.* (1979)	Mouse - 4 strains (BALB/cStCrl C3Hf/Nctr, C57BL/6JfC3Hf /Nctr, F_1 hybrid of the two, and F_2 crossing F_1	Immature, intact/ 18 - 20 days	Dietary/ 6 days	diethylstilbestrol/ thymic weights and histopathology	11-12 mice/group. Figure 2 shows comparisons - about 400 animals per strain. BALB/c dose response slope slightly less. Some differences in thymic weight and, in subchronic studies, body weight were observed. Differences in pituitary and testicular lesions were observed at necropsy after subchronic administration (up to 50 wks - see Table 4). Blotted uterine weights were used.
Harnagea-Theophilus *et al.* (1999)	Mouse - Swiss Webster	Adult OVX/ 20-25 g bw	Subcutan. and oral gavage/ 4 days	17β-oestradiol (s.c.), acetaminophen (gavage)/ cell cycle stimulation and DNA incorporation, MCF-7 proliferation,	Group size not specified. Uterine weights were blotted. However, control uteri (Fig. 8) appear to be ~28mg.
Hartmann *et al.* (1980)	Mouse - NMRI	Immature, intact/ 20 days	Subcutan./ 3 days	derivatives of 1,1,2,2'-tetraalkyl-1,2-diphenylethanes/ receptor binding assays and inhibition of mammary tumor growth	10 mice/group; olive oil vehicle. Uteri fixed in Bouin's solution, dried at 100° C for 24 hrs, then weighed. Results expressed as ratio of uterine wt to body wt.
Hartmann (1986)	Mouse - NMRI	Immature, intact/ 20 days, 15 g bw	Subcutan./ 3 days	derivatives of 1,2-dialkylated 1,2-bis(4- or 3-hydroxyphenyl)ethane estrogens/ receptor binding assays	10 mice/group; olive oil vehicle. Uteri fixed in Bouin's solution, dried, and then weighed. Results expressed as ratio of uterine wt to body wt. Cites Hartmann *et al.* 1980.
Hartmann *et al.* (1983)	Mouse - NMRI	Immature, intact/ 20 days, 14.5 ± 1.2 g bw	Subcutan./ 3 days	derivatives of 1,2-dialkylated 1,2-bis(hydroxyphenyl)ethane estrogens/ receptor binding assays	10 mice/group; olive oil vehicle. Uteri fixed in Bouin's solution, dried, and then weighed. Results expressed as ratio of uterine wt to body wt..
Hartmann *et al.* (1985)	Mouse - NMRI	Immature, intact/ 21 days	Subcutan./ 3 days	Eleven estrogenic compounds were synthesised by two routes and tested/ receptor binding assay and mammary tumor growth inhibition	10 mice/group; olive oil vehicle. Results reported as uterine wt (mg)/body wt (g) * 100. Uteri fixed in Bouin's solution, dried, and then weighed. Cites Hartmann *et al.* 1980.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Hilgar and Palmore	Mouse - PHS/NCI studies	Immature, intact 21 days, 8-11 g bw	Subcutan. and oral gavage/ 3 days	Estrone as standard reference. Generated data on 745 steroids and 360 non-steroid compounds. However, the maximum s.c. dose was 0.5 mg/kg/day and about 20 fold higher for oral. This oral max was rarely used,	10-12 mice/group; sesame oil vehicle. Uteri lightly compressed and blotted. Fig. 1 and 2 show quality control, but control uterine means are given as 11.5 and 19.0 mg. Control data given on pages 5-9 shows variation in vehicle control and estrone curve.
Ho and Levin (1986)	Mouse - CD-1	Immature, intact/ obtained at 21 days	Subcutan/ 2 days, uteri removed on day 4	17β-oestradiol, 3β-hydroxy oestradiol, Δ5-hydroxy oestradiol, 3α-hydroxy oestradiol/ receptor binding activity	6 mice/group; mineral oil vehicle. Only one uterine horn was weighed, not specified if blotted (but uterus apparently opened to obtain a single horn).
Hossaini *et al.* (2000)	Mouse – F_1 of C57B6 x DBA2J	Immature, intact/ 18-20 days, 10 ± 2 g bw	Subcutan. and oral gavage/ 3 days	Oestradiol benzoate, *p*-hydroxybenzoic acid, methyl parabens, ethyl parabens, propyl parabens, butyl parabens/	5-10 mice /group; peanut oil vehicle. Results reported as both absolute and relative uterine weights. Control uteri were 5.0 to 6.5 mg average. Uteri were 'exicsed, trimmed free of fat and pierced to remove excess fluid, and subsequently weighed.' No parabensactivity at 100 mg/kg/day s.c. or oral. Ethyl paraben negative at 1000 mg/kg/day by gavage.
Jones and Pope (1960)	Mouse - no strain given	Immature, intact/ exact age not known, 8 - 10 g bw	Oral gavage, i.p. and subcutan./ 3 days (2X daily)	17β-oestradiol, estriol, stilboestrol, miroestrol	10 mice/group; 1:1 aqueous propylene glycol. Comparison of routes of exposure and time (also single injection). Preparation of uterus e - 24 hrs in Bouin fluid then 48 hours in 70% alcohol, then uterus weighed after 'blotted to constant dryness' (see Astwood 2ubchapter for that method)
Jones *et al.* (1979)	Mouse - Cox	Immature, intact/ age not given, 11-13 g bw	Subcutan. and oral gavage/ 3 days	estrone, [3,4-dihydro-2-(4-methoxyphenyl)-1-napthalenyl][4-[2-(1-pyrrolidinyl)ethoxy]-phenyl]methanone, methanesulfonic acid salt / in vitro receptor binding, antifertility potency	10 mice/ group; corn oil vehicle. Reported as increase over control (no absolute weights reported). Not specified whether wet or blotted weights used.
Kallio *et al.* (1986)	Mouse - NMRI	Immature, intact / 18-20 days 8-10 g bw	Refers to Terenius, indicates subcutan. for 3 days	17β-oestradiol, Fc-1157a / receptor affinity binding, nuclear receptor translocation	Route and time not clearly specified – manuscript only mentions 'injection.' Group size not specified; sesame oil vehicle. Results are reported as mg uterine weight per 100 g bw. Not specified whether wet or blotted weight were used.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Katzenellen-bogen *et al.* (1975)	Mouse - Cox	Immature, 11-13 g bw	Subcutan./ 3 days	estrone, hexestrol and five iodinated hexestrol derivatives/ receptor binding assays, series of molecular and biochemical responses in the uterus to estrogens	5-10 mice group; corn oil vehicle. Uteri preparation desicribed as 'carefully blotted.' Control uteri 8.5 ± 0.8 mg. Two to three doses used.
Kitts (1987)	Mouse - Swiss UBC strain	Immature, intact/ no age given, 12-18 g bw	Oral gavage/ 3 days	oestradiol and coffee extracts/ receptor binding displacement of tritiated oestradiol	5 mice/ group in at least one experiment.; 2% ethanol in saline. Uteri were 'blotted dry.' Most results expressed as uterine to body weight ratio. In Table 2, control uteri 27.8 mg!
Korach *et al.* (1978)	Mouse - CD1	immature - intact 23 days	Subcutan./ 3 days	17β-oestradiol, diethylstilbestrol, indenyl-DES, α-dienestrol, DES-epoxide, α,α'-dihydroxy DES, β-dienestrol, DES-phenanthrene/ mouse uterine estrogen receptor competitive binding and sucrose gradient assays	5-19 mice/group; vehicle not specified. Not specified whether wet or blotted weights used. Dosage in μg/kg necessary to double uterine weight reported; control weights not reported. No specifics for uterine preparation and weighing given.
Korach *et al.* (1979)	Mouse - CD1	Immature, intact 21 days	Subcutan./ 2 or 3 days	diethylstilbestrol, pseudo-DES, indenestrol A, indenestrol B, inanestrol/ competitive estrogen receptor binding curves, sedimentation binding curves, cytosolic and nuclear receptor level time course after administration, uterine histology	Expressed as quantity of compound required to double uterine weight. Vehicle not specified. Although relatively high binding affinity was observed, differences in uterotrophic activities were also observed (no direct correlation between receptor affinity and biological activity).
Korach *et al.* (1987)	Mouse - CD-1 [ICR]BR	OVX, not specific on adult or immature	Not specified	17β-oestradiol, indanestrol, indanestrol A, indanetrol B, diethylstilbestrol, E-pseudo DES, Z-pseudo DES/ receptor binding activity and various molecular and biochemical responses in uterus.	No specific description of uterotrophic procedures. Results in Fig. 1 reported as dose of compound required to double uterine weight.
Korenman (1969)	Mouse – not specified	Immature, intact 21 days, 8-11 g bw	Subcutan./ 3 days	utilises data of Hilgar and Palmore and compares to rabbit uterine cytosol binding affinity for compounds	All data expressed in relative terms.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Kranzfelder *et al.* (1982)	Mouse - NMRE	Immature, intact/ 20 days	Subcutan./ 3 days	Derivatives of 3,4-bis(3'-hydroxyphenyl)hexane. Estrogen receptor binding assays, tissue culture growth stimulation or inhibition, breast tumor growth inhibition	10 mice/group; olive oil vehicle. Uteri were first fixed in Bouin's solution for 12 hours and washed in a saturated alcoholic solution of LiCl, then were weighed wet or oven dried at 100° C. Results are expressed as uterine dry weight (mg)/ bw (g)*100.
Kumar and Pakrasi (1995)	Mouse - Parkes	Immature, intact and adult, OVX/ 15 and 30 g bw	I.p./ 3 days	17β-oestradiol, clomiphene citrate/ luminal cell height from morphometry	At least 5 mice/group; saline for test substance and olive oil for oestradiol. Oestradiol subcutaneously. Not specified whether wet or blotted weights used. Immature controls 6.83 ± 0.06 mg per 15 g bw. OVX controls 15.12 ± 0.57 per 30 g bw. Weight and morphometry parallel on doses in both test systems.
Legg *et al.* (1951)	Mouse - albino, no strain given	Immature/ ovx ~ 30 days, used 48 hours later	Unclear, ref to Robinson, where 2 routes used	Extracts of four plant materials including red clover, leaf and stem, seasonal cuts from Feb. - June (Australia)	Estrogenic activity expressed as µg equivalents of estrogen per 100 g plant material. Vehicle and whether wet or blotted weights used was not specified.
Lemini *et al.* (1995)	Mouse - CD1	Immature intact (20 days, 10 - 12 g bw) and adult OVX (34-40 g bw at OVX used 3 wks later)	Subcutan./ 3 days	17β-oestradiol, oestradiol benzoate, benzoic acid.	6-10 mice/group; corn oil vehicle. Results expressed uterine mg/100 g bw, so no control values available directly. If one assumes a 10 g body weight, then control uteri are ~30 mg (Fig. 2 and 3) which is unrealistically high, especially as these are not wet weights. Same true for OVX adults at 40 g bw, ~181 mg uteri. Uteri 'blotted to release intraluminal fluid'
Lemini *et al.* (1997)	Mouse - CD1	Immature, intact (21 days, 10 - 12 g bw) and adult OVX (vaginal smears 3 wks after OVX before use)	Subcutan./ 3 days	17β-oestradiol, *p*-hydroxybenzoic acid/ Vaginal cornification.	9-18 mice/group; corn oil vehicle. Not specified whether wet or blotted weights used. Immature ~38-40 mg; adult OVX ~58 mg. 1.5X weight increase 10 µg daily E is very unusually small. Weights appear high and increase only modest. *p*-hydroxybenzoic acid increase equivalent to oestradiol (unusual for weakly estrogenic compounds).

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Lerner *et al.* (1958)	Mouse - strain not given	Immature, intact and OVX/ 6-8 g bw	Subcutan/ 2x per day for 3 days	oestradiol, MER-25, (estrone, estriol, chlorotrianisene, diethylstilbestrol - one dose)/ vaginal cornification in rats and a battery of endocrine and repro assays Also data on monkeys, chickens, and rabbits	10-37 mice/group; olive oil vehicle. Control uteri ~7mg for both intact and OVX. Uterine fluid expressed before weighing.
Levin and Tyndale (1937)	Mouse - Bagg albinos	Immature, intact/ 21 - 23 d, 7 - 10 g bw	Subcutan./ 3 days	gonadotrophic substance/ Vaginal opening	15 mice/group. Control uteri 5.9 ± 0.3 mg. Uterus 'freed of intro-uterine fluid by pressure against a piece of dry filter paper.' Ovarian growth and uterine weight increase measured, injection in OVX mice did not induce uterine weight increase (therefore, considered estrogen free)
Loeber and Van Velsen (1984)	Mouse - Swiss	Immature, intact/ 'animals purchased 14-17 days old'	Dietary/ 5 days	β-hexachlorohexane	Not specified whether wet or blotted uterine weight. Not specified whether wet or blotted uterine weights used. Control uteri 8.4 mg. 10-25% increase near or at maximum tolerated dose (from 28 day study)
Lyman and Jordan (1985)	Mouse - ICR	Adult OVX/ OVX at 10 wks	Subcutan./ 3 days	Tamoxifen and four derivatives or metabolites	5 or more mice/group; peanut oil vehicle. Uterine intraluminal fluid was pressed out. Control uteri ~20 mg. Dose response curves in Fig. 2.
Lyttle and DeSombre (1977)	Mouse - Swiss Albino	Pubertal, intact/ 28 d, 19 g bw	Subcutan./ 2 days	17β-oestradiol/ peroxidase activity in both estrogen responsive and non-responsive tissues/ also rat, hamster, and guinea pig	4-10 per group; 25% ethanol-75% saline vehicle. Control uteri 7.8 mg; not specified wether wet or blotted weights used. 2 days of injections. 4.49 fold increase at 40μg of E/kg/day.
Marin *et al.* (1996)	Mouse - NIH	Adult, OVX/ ~10 g bw at ovx	Subcutan./ 2 days with necropsy 4 d after last	oestradiol, etolame, pentolame / in vitro contractions of excised uterus	4 mice/group; corn oil vehicle. Not specified whether wet or blotted uterine weights used. Very high control uterine wts for mouse: 24.0 ± 2.0 and 27.9 ± 2.5 mg. Also waiting 4 days after injection unusual.
Mariotti *et al.* (1998)	Mouse - Swiss Webster	Adult, OVX/	Subcutan./ 3X per week for 3 wk	bisGMA (2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl]-propane	5 mice/group; peanut oil vehicle. Uteri were blotted. Results reported as mg uterine wt/g body weight.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Markey *et al.* (2001)	Mouse – CD1	Immature, intact – 23 days (too old)	Subcutan./ 3 days	17β-oestradiol, Bisphenol A/ uterine histology including luminal cell height, lactoferrin, vaginal opening, proliferating cell nuclear antigen	Used osmotic pumps. Group size varied from 4 to 22; DMSO vehicle. Uterine blotted weight 19.01 ± 1.16 (dissected out, each uterus was bloted, and the wet weight recorded). General concordance of makers with uterine weight.
Martel *et al.* (1998)	Mouse - BALB/c	Adult OVX/ ovx ~60 days	Subcutan. and oral/ 9 days	Estrone, EM-800, ICI 182,780/ estrogen binding, uterine uptake of tritiated reference estrogen, vaginal weight	10 mice/group; s.c. vehicle 1:1 PEG 600:ethanol. Graphically, control immature uteri ~12-23 mg, OVX ~18 mg. EM-800 is potent and apparently pure antiestrogen.
Mehmood *et al.* (2000)	Mouse - CD-1	Immature, intact/ 21 days	Subcutan./ 3 days	diethylstilboestrol, α-zearalanol, methoxychlor, bisphenol A, coumestrol, genistein, naringenin, chlordecone, *o,p'*-DDT/ includes lactoferrin (LF) expression, peroxidase activity, and bromodeoxy-uridine (BrdU) labeling	Minimum 4 mice/group; corn oil vehicle. Clear dose response experiments 4 chemicals, single dose others. LF and BrdU useful to confirm, may be 2-3X more sensitive. Uteri blotted, but increase expressed relative to body weight.
Meyers *et al.* (1988)	Mouse - C3H/MTV (im) and C3H/He (OVX)	Intact, age not given / and adult OVX (11 weeks)	Subcutan./ 5-7 days	oestradiol 3-one and 44 doisynolic acid derivatives/ relative estrogen receptor binding, vaginal cornification	4-16 mice/group; corn oil vehicle. Not specified whether wet or blotted weights used. Control uteri absolute weight average was 16.5 mg.
Micheli *et al.* (1962)	Mouse	unknown	Dietary/	diethylstilbestrol, coumestrol, and 42 other isoflavone derivatives and structural analogs	Group size not given. Control uteri ~10 mg. Dose levels in various tables are quantities necessary to achieve a 25 mg uterine weight (2.5 fold increase).
Mittal *et al.* (1985)	Mouse - no strain given	Immature, intact/ age not given (see Fig. 1 legend)	Subcutan./ e days	oestradiol and 24 synthesised oestradiol compounds/ receptor binding assay	6-10 mice/group; propylene glycol:saline vehicle 1:1. Table II as relative weights. Table III control 17.6 ± 4.6 mg; not specified whether wet or blotted weights used.
Nesaretnam *et al.* (1996)	Mouse - CD1	Immature - intact/ 21 and 25 days	Dermal/ 3 days	17β-oestradiol, 3,4,3',4'tetrachlorobiphenyl (PCB77),	6 mice/group; 0.5 M ethanol. Results not fully reproducible between experiments. Age varied between experiments (21 vs 25 d). Sd's in 25 d controls considerably higher (see Fig. 10). May be example where age is critical to reducing variability in older animals. Control uteri appear to vary between 15 and 70 mg (see Fig. 10)! Ethanol used in dermal application.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Newbold *et al.* (2001)	Mouse -	Immature, intact/ 17-19 days	Subcutan./ 3 days	17β-oestradiol , diethylstilbestrol, tamoxifen, 4-hydroxytamoxifen, nonylphenol, bisphenol A, methoxychlor, HPTE, *o*,p'-DDT, kepone, chordecone/ uterine histology markers (including epithelial gland number), lactoferrin, complement C3, proliferating cell nuclear antigen (PCNA)	Minimum 5/mice per group; corn oil vehicle. Imbibed fluid uterine wet weight used. Weights are reported relative to body weight. Most markers concordant when dosages yielding maxiumum uterine weight are used.
Newman and Moon (1969)	Mouse - Swiss Webster	Immature, intact/ 19 days	Subcutan./ 3 days	estrone, 3-methylcholanthrene	Group size not given; sesame oil vehicle. Not specified whether wet or blotted uterine weights used. Results reported as uterine wt mg/ body wt grams.
Nique *et al.* (1994)	Mouse - Swiss	Immature, intact/ 18-19 days, 9-11 g bw	Subcutan./ 3 days	7 synthesised compounds, tamoxifen, and 4-hydroxytamoxifen/ receptor binding assays	Group size not specified; sesame oil vehicle. Results expressed relative to controls. Not specified if uteri were blotted.
Nishino *et al.* (1976)	Mouse - NMRI	Adult OVX/ ~30 g bw, used 10 days after ovx	Subcutan. and oral/ 3 days	17β-oestradiol and 19 other derivatives of estrogen including 8α-hydroxy derivatives/ included vaginal response in parallel.	6 mice/group; vehicle not specified. Not specified whether wet or blotted weights used. Results reported as relative potency to oestradiol (1.0).
Nishino *et al.* (1991)	Mouse - NMRI	Immature, intact/ ~15 g bw	Subcutan./ 3 days	17β-oestradiol, ZK 119010, ICI 164384	6 mice/group; castor oil vehicle. Not specified whether wet or blotted uterine weights used. Control uteri 5.9 ± 3.4 and 7.6 ± 1.7 mg per 19 g bw in two experiments. Agonist and antagonist assays. Failed to duplicate antagonist activities of ICI 164384 reported by Wakeling and Bowler (1987)
Ostrovsky and Kitts (1962)	Mouse - Swiss albino	Immature, intact/ no age given, 8-11 g bw.	Dietary / 3 days	Diethylstilbestrol and extracted red clover forage,	10-12 mice/group. Controls had mean uteri wts of 13.59, 13.63, and 15.52 mg in 3 experiments. Not specified whether wet or blotted weights used. Extraction and fractionation procedures for red clover tested in mouse bioassay.
Papaconstant-inou *et al.* (2000)	Mouse - B6C3F1	Juvenile OVX/ 35-60 d and 20 g bw	Subcutan./ 3 days	oestradiol, bisphenol A/ cell heights in various uterine tissues	Avg. 10 mice/group; corn oil vehicle. Uteri blotted. Note very low control uteri weights of 4.54 mg in study 6. BPA from 1 to 400 mg/kg/day; LOEL 40 mg/kg/d.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Paria *et al.* (1994)	Mouse - CD1	Adult OVX/ 7-8 wks, used 7 days later	Subcutan./ 7 days	17β-oestradiol, Δ-9-tetahydro-cannabinol/ tritiated thymidine incorporation	4-5 mice/group; sesame oil vehicle. Not specified whether wet or blotted weight used. Control uteri 24 mg.
Pavlik *et al.* (1986)	Mouse - CF-1	Immature, intact/ 23-35 days	Subcutan./ 3 days (after cytotoxic agents on day 1)	17β-oestradiol and 34 cytotoxic agents/ estrogen and progesterone receptor binding assays, induction of progesterone receptor, DNA content of uterus	5 mice/group; sesame oil vehicle for oestradiol, varies for other agents. Uteri 'pierced and water content was expelled and uteri blotted.' Control in Fig. 2, column B, ~20 mg.
Pento *et al.* (1981)	Mouse - Swiss Webster	Immature, intact/ 21 days, 10-14 g bw	Subcutan./ 3 days	oestradiol and ten cyclopropyl derivatives/ histology of uterus and effects on fertility	5-6 mice/group; sesame oil vehicle. Uteri blotted 'lightly.' Results reported as relative to oestradiol in tables. No apparent control values in figures.
Poirier *et al.* (1991)	Mouse - CD-1	Adult, OVX/ 19-20 g bw	Subcutan./ 4.5 days, 2X daily	EM-139, ICI 164384, and five synthesised compounds/ relative receptor binding activity, proliferative activity in breast cancer cell lines, DNA synthesis in uterine tissues	9-10 mice group; oestradiol in ethanol, other compounds in saline vehicle with 1% gelatin. Not specified whether wet or blotted weightsused; however, controls are ~40 mg (see Fig. 7)
Preston *et al.* (1956)	Mouse – Rockland CFW	Immature, intact/ 20 d, 8-10 g bw	Dietary/ 10 days	Beef carcass additions to diet	9-10 mice/group. Uteri were dissected, fixed in Bouin's, then blotted before weighing. Control uteri had wide range 4.5-16.0 mg. No effect from DES treatment of cattle.
Ramamo orthy *et al.* (1997)	Mouse - B6C3F1	Immature, intact/ 21 days of age	I.p./ 3 days	17β-oestradiol, toxaphene, dieldrin (endosulfan and chlordane in some systems, but not uterotrophic)/ Progesterone and estrogen receptor binding assays (uterine source), estrogen receptor (MCF-7 source), MCF-7 cell proliferation assay, MCF-7 transient transfection, uterine peroxidase, yeast reporter gene with both murine and human receptor	6-9 mice/group; corn oil vehicle. Control uteri 13 ± 1.6 mg wet weight, 3X induction to 42.6 ± 8.4 mg at 0.0053 μM Oestradiol/kg dose Uterine tissue was blotted before weighing. Table 1 indicates neither dieldrin nor toxaphene is uterotrophic individually or together; consistent with no peroxidase or progesterone receptor induction, and no competitive binding to estrogen receptor or cell responses.
Robinson (1949)	Mouse - albino, no strain given	Immature, OVX / ovx ~ 30 days, used 48 hours later, < 15 g bw	Dietary and s.c./ 4 days, necropsy on day 6	Oestradiol and extracts of various plant materials/ Vaginal smears.	6 mice/group. Control uteri 5.6 - 7.1 mg. Uteri fixed in Bouin's and then dried between filter papers. Oestradiol dose response curve (nine doses, some multiple groups). Earlier work with guinea pigs noted, but noted that response not specific for estrogens - so work with species discontinued.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Roper *et al.* (1999)	Mouse - three strains	Adult OVX/ ovx at 5-6 wks, rested for 1 wk	Subcutan./ 2 days	17β-oestradiol	C57BL/6J, C3H/HeJ, and B6C3F$_1$ strains used. Control uteri 17.5, 19.5, and 17.6 means. Procedure reported to include blotting. C3H/HeJ strain appears to be less responsive.
Rubin *et al.* (1951)	Mouse - Swiss	Immature intact/ 23-35 days	Subcutan./ 3 days	17β-oestradiol, estrone, estriol	10-54 mice/group; corn oil vehicle. Mean bw 9.6 g, uterine / bw ratio used (at ratio of 111, therefore, presumably uterine weight ~10 mg). >2X increase at 0.06 μg 17β-oestradiol /animal/day. Uteri blotted. Statistical analysis of assays performed on precision, etc. 'using 40 mice the limits of error were ± 13%' See Table 9 for summary of rodent methods analyzed by these authors.
Schneider *et al.* (1985)	Mouse - NMRI	Immature, intact/ 20 days, 10-12 g bw	Subcutan./ 3 days	Acetoxy substituted 1,1,2-triphenylbut-1-enes/ receptor binding assays and mammary tumor inhibition	10 mice/group; olive oil vehicle. Uteri fixed in Bouin's solution, dried, and then weighed. Results expressed as ration of uterine wt to body wt. Cites Hartmann *et al.* 1980.
Schneider (1986a)	Mouse - NMRI	Immature, intact/ 20 days, 10-12 g bw	Subcutan./ 3 days	Acetoxy substituted 1,1,2-triphenylbut-1-enes/ receptor binding assays and mammary tumor inhibition	10 mice/group; olive oil vehicle. Uteri fixed in Bouin's solution, dried, and then weighed. Results expressed as ration of uterine wt to body wt. Cites Hartmann *et al.* 1980.
Schneider (1986b)	Mouse - NMRI	Immature, intact/ 20 days, 10-12 g bw	Subcutan./ 3 days	derivatives of 2-Alkyl-substituted 1,1-bis(4-acetoxyphenyl)-2-phenylethenes / receptor binding assays	10 mice/group; olive oil vehicle. Uteri fixed in Bouin's solution, dried, and then weighed. Results expressed as ration of uterine wt to body wt. Cites Hartmann *et al.* 1980.
Seinen *et al.* (1999)	Mouse - BALB/c	Immature, intact/ begun at 21 days	Dietary/ 2 weeks (E by sc)	oestradiol, AHTN: 6-acetyl-1,1,2,4,4,7-hexamethyltetraline, HHCB: 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benopyran	6 mice/group; dietary incorporation. Fed 2 weeks (into puberty). 2 and 6.5 mg/kg/day for AHTN; 6 and 40 mg/kg day for HHCB. Uterus expressed relative to bw (but final bw available). No direct evidence for activity.
Sharma *et al.* (1990a)	Mouse - no strain given	Immature, intact/ age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, LY-117018, and 3 synthesised derivatives of 2,3 biphenyl benzopyran / Relative binding affinities for ER up to 100 μM (rat ER.)	6-9 mice/group; propylene glycol:normal saline 1:1 vehicle. Control uteri only ~4 mg. Agonist and antagonist screens; dose-response for certain compounds. Note: most compounds have aryl ring, but not hydroxyl. Metabolic role in activity may need review.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Sharma *et al.* (1990b)	Mouse - no strain given	Immature, intact/ age not given	Subcutan./ 3 days	17β-oestradiol, tamoxifen, trioxifen, LY-117018, and 4 synthesised derivatives of 2,3 diphenyl benzopyran/ Relative binding affinities for ER up to 100 μM (rat ER.)	6-9 mice/group; propylene glycol:normal saline 1:1 vehicle. Control uteri only 3 mg. Agonist and antagonist screens; dose-response for certain compounds. Note: most compounds have aryl ring, but not hydroxyl. Metabolic role in activity may need review.
Shelby *et al.* (1996)	Mouse - CD-1	Immature, intact/ day 17 start	Subcutan./ 3 days	17β-oestradiol, diethylstilbestrol, tamoxifen, 4-hydroxytamoxifen, methoxychlor, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane*, endosulfan, *p*-nonylphenol, *o,p'*-DDT, kepone * methoxychlor metabolite/ competitive binding assays, HeLa cell transfection assay	Min. 5 mice/group; corn oil vehicle. Plotted as uterine ratio to body weight. Up to 1000 fold of estrogen, methoxychlor, endosulfan, and kepone did not bind ER. Kepone weakly uterotrophic (~40-50% increase in weight at high doses). General consistency among assays.
Standeven *et al.* (1994)	Mouse – B6C3F1	Adult OVX / ovx at 8 wks age	I.g../ 3 days	17β-oestradiol , tamoxifen, unleaded gasoline/ competitive estrogen receptor binding, peroxidase activity, liver metabolism	9 mice/group; corn oil vehicle. Uteri were weighed wet, with luminal fluid. Uteri were reported as % of body weight (control ~0.05%).
Terenius (1970)	Mouse - no strain given	Immature, intact/ age not given, 8 - 10 g bw	Subcutan./ 3 days	17ß-oestradiol, *meso*-butoestrol, U-11100A, ICI-46,474/ Tissue uptake competition against tritiated oestradiol in uterus, vagina, and mammary tumors	5-8 mice/group. Control weights not given. Only disclosed as maximum weight gain of group uteri (in mg) in a dose range (Table I)
Terenius (1971)	Mouse - NMRI strain	Immature, intact/ age not given, 8 - 10 g bw	Subcutan./ 3 days	17ß-oestradiol and 14 other steroidal and non-steroidal compounds/ estrogen receptor binding competition, tissue uptake competition,	5-6 mice/group; olive oil vehicle. Uterine dry weights only; control uteri ~1 mg. Pooled dose response curves in Figure 2.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Thigpen *et al.* (1987a)	Mouse - CD1	Immature, intact/ different ages tested for applicability in assay	Dietary/ Weaned d 15, fed diets 3, 5, and 7 d	Diet and diethylstilbestrol	Most detailed report of investigation of dissection, uterine preparation, and uterine weighing in the literature. 15-20 mice/group. Clear directions on removal of excess tissue (Fig. 1) and blotting of uterus (pp. 597, col. 1). Effect of uterine dehydration prior to weighing investigated (observed to decrease weights - Table 1). Variability due to apparent entry into early phases of puberty observed in somewhat older animals (after 22 days of age when injections were given at 24-26 days; individual data plotted in Figure 3, p. 599). Recommends studies in mice conclude before reaching 23 days. Control uteri at 15 days 8.2 ± 2.7 mg; at 17 days 12.2 ± 3.4 mg.
Thigpen *et al.* (1987b)	Mouse - CD1	Immature, intact/ 15 days	Dietary/ Weaned d 15, fed diets 3, 5, and 7 d	diethylstilbestrol, certified rodent chow #5002, rodent laboratory chow #5001, mouse chow #5015, AIN-76A, NIH-07, NIH-31	25 mice/group. Blotted weights used. All diets except #5001 appeared to increase the uterine weights some statistically significant. #5002 uteri 13.8 mg and #5001 uteri 10 mg (statistically different)
Thigpen *et al.* (1987c)	Mouse - CD1	Immature, intact/ 15 days	Dietary/ Fed diets 7 days.	NIH-31 diet with diethylstilbestrol, sucrose, dextrose, corn starch, corn oil, and soybean oil	14-45 mice/group. #5002 uteri 15.2 mg. Blotted weights used. Soybean oil mean was higher but not statistically significant, all other supplements and 6 µg statistically significantly higher. Correlated with higher body weights.
Tinwell *et al.* (2000b)	Mouse - Alderly Park Alpk:AP_fCD-1	Immature, intact/ 19-20d; max bw 18 g	Subcutan. and oral gavage/	diethylstilbestrol, bisphenol A/ vaginal cytology, uterine morphometric analyses, BrdU labeling and histopathology	4-20 mice/group. Arachis oil vehicle. Nine control groups; minimum uterine wt 7.7 ± 2.1 mg and maximum 14.6 ± 5.1 mg. Full DES dose response 0.02 to 40 µg/kg, included (Fig. 1a). BPA range from 0.02 µg to 300 mg/kg for s.c. route and 500 µg to 300 mg/kg for oral gavage. Parallel analysis of BrdU labeling indexes and uterine cell morphometric measurements in several experiments. indicated these were equivalent and complementary to uterine wt.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Tullner (1961)	Mouse - NIH general purpose strain	OVX / final bw 9 - 10 g	Oral gavage/ 3 days	Methoxychlor, estrone, anisole/ vaginal cytology	9 - 10 mice/group; sesame oil vehicle. Control uteri 5.2 ± 0.6 mg. Uterine procedure specifics not given. ~500 mg/kg/day methoxychlor elicited 6-fold uterine wt increase. Some vaginal cytology changes observed. Experiments performed due to observed uterine increase when lab animals were dusted with pesticide containing methoxychlor.
Turner (1956)	Mouse – no strain given	Immature, intact/ Age not given	Dietary/ 10 days	Various tissues and organs from beef cattle fed DES were incorporated at 10% levels in the diet	5-12 mice/group. Control uteri 12.0-16.4 mg. Cervix was included in weight.
Turner (1956)	Mouse – no strain given	Immature, OVX/ 3-5 weeks for involution	Dietary/ 10 days	Various tissues and organs from beef cattle fed DES were incorporated at 10% levels in the diet	5-12 mice/group. Control uteri 12.0-16.4 mg. Cervix was included in weight. Where cattle were allowed to feed 10 days after DES treatment, no effects on uterine wt were seen.
Van de Velde *et al.* (1994)	Mouse - Swiss	Immature, intact/ 18-19 days, 9-11 g bw	Subcutan. and oral/ 3 days	tamoxifen, RU 58668, ICI 182780/ relative binding affinity to estrogen receptor, MCF-7 cell inhibition, antitumor activity in nude mice	5 mice/group; several vehicles (ethanol, methylcellulose, arachis oil). Not specified whether wet or blotted weights used; however, 21.5 ± 2.8 mg control uteri reported in Table 3. Results reported as relative decrease (antagonist assay) to controls.
von Angerer *et al.* (1982)	Refers to Hartmann *et al.* 1980			Seven N,N'-diethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)-ethylenediamine derivatives/ receptor binding assays and mammary tumor growth inhibition	8-10 mice/group. Uterine ove-dry weight with uterine weight change results reported relative to body weight. ref. Hartmann *et al.* 1980 and Dorfman
von Angerer *et al.* (1980)	Refers to Hartmann *et al.* 1980			Four N,N'-Dialkylbis(dichloro-phenyl)ethylenediamine derivatives/ receptor binding assays and mammary tumor growth inhibition	ref. Hartmann *et al.* 1980 and Dorfman
Wakeling and Bowler (1988)	Mouse – not given in paper, method refers to previous work with reference	Immature and OVX	Subcutan. and oral gavage/ 3 days	Oestradiol benzoate, tamoxifen, LY 117,018, ICI 160,325, ICI 163,964, ICI 164,275, ICI 164,384/ estrogen receptor binding, vaginal opening, LH hormone levels, MCF-7 and ZR-75-1 cell growth assays	See Figure 4, immature, adult OVX rats and immature, adult OVX mice all in same graphic. No substantive difference in response, variability, or sensitivity apparent.

Table 1B. Uterotrophic bioassays in mice. Assays involving consecutive, multiple administration of test compounds (continued).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Wani *et al.* (1975)	Mouse - CF-W	Immature, intact/ 21 day	Oral gavage/ 3 days	diethylstilbestrol, mestranol, ethinyloestradiol and 14 flavinoid derivatives/ antifertility effects in rats	5 mice/group; sesame oil vehicle. Uteri blotted and lightly compressed. Control weights for 75 mice in Figure 1, indicating control wts just over 10 mg.
Welch *et al.* (1971)	Mouse - Swiss Webster	Adult OVX/	I.p./ 7 d pre-treat - 3 d admin	γ-chlordane, *p,p'*-DDD pretreatments. ± estrone and 17β-oestradiol.	6-8 mice/group; corn oil vehicle. Pretreatment to induce liver monooxygenases. Neither increased uterine weight over controls. Chlordane reduced estrone weight increase when estrone and 17β-oestradiol injected 24 hours after last insecticide. *p,p'*-DDD did not. Metabolism of tritiated 17β-oestradiol in liver microsomes.
Zarrow *et al.* (1953)	Mouse - no strain given	OVX/ 30 g bw	Subcutan/ 7 days	animal laboratory rodent diet	Group size and vehicle not stated. Control uteri were 11.2 ± 1.4 mg and 13.3 ± 1.3 mg. The petroleum ether extracts of the diet increased uterine weights to 53.6 mg after 7 consecutive injections.

Table 2A. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
RAT					
Anderson *et al.* (1972)	Rat - Purdue-Wistar	Immature, intact/ 21-23 days, 50-60 g bw	Subcutan.	17ß-oestradiol, estriol/ Nuclear receptor complex formation in uterus with dose over 1-24 hour period	Group size unclear. Both wet (<24hrs) and oven dry (@24hrs) uterine weights. Controls ~30 mg. wet and 6 mg dry. Oestradiol 0.1 µg dose response ~50% increase at 24 hrs.
Astwood (1938)	Rat - 'albino - no specific strain noted	Immature, intact/ 21-23 days, 25-49 g bw	Subcutan./ 0-54 hour time course	17β-oestradiol/	Blotted weight, then oven dried. One injection. 22 mg control uterine wts, 36 gram bw. Uterine water, blotted weights, and dry weights recorded.
Bitman and Cecil (1970)	Rat - Wistar	Immature, intact/ 21-23 days, 36-48 g bw	Subcutan./ 18 hours	53 DDT analogues and other compounds screened/ uterine glycogen content	Figure 1 compares glycogen and wet uterine weight response with set of o,p'-DDT dose responses. All other data are glycogen response, but presumably parallel to uterine increase.
Bitman *et al.* (1968)	Rat - strain not identified	Immature, intact/ 22-25 day	Subcutan.	17β-oestradiol, *o,p'*-DDT, *p,p'*-DDT/ glycogenic response of uterus and chicken oviduct weight response	4-14 rats/group. 4 mg DDT and 0.4 µg oestradiol injections. Data not shown. "The *o,p'*-DDT stimulated characteristic estrogenic responses in the uterus, increases in wet weight, water content …" *p,p'*-DDT exhibited only slight activity.
Bo *et al.* (1971)	Rat - Holtzman	Adult OVX/ at 150 g bw, used 10 d later	Subcutan. 24 hours	17β-oestradiol, progesterone/ Glycogen content, dry weight	Vehicle uteri 92.5 ± 7.0 mg. Cottonseed oil vehicle. Blotted uterine weights. Progesterone coadministration can significantly lower water imbibition.
Bowman *et al.* (1981a)	Rat - Sprague Dawley	Adult OVX/ at 250 g bw	I.p./ 1 injection	chlomiphene	5-8 rats/group; arachis oil vehicle. Control uteri 99 ± 10 mg after blotting; luminal fluid 10 ± 1 mg in controls. Also recorded for other doses. Actual sacrifice 72 hours after chlomiphene injection followed by oestradiol benzoate at 24 hrs.

Table 2A. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Bowman *et al.* (1981b)	Rat - Sprague Dawley	OVX/ at 250 g bw	I.p./ one injection	chlomiphene/ receptor binding assay	2-3 rats/group; arachis oil vehicle. Time course after injection from 0-24 days. Both uterus after excluding luminal fluid and the actual luminal fluid weights are recorded.
Branham *et al.* (1988)	Rat - Sprague Dawley	Immature, 14 days and immature OVX 26 d	Subcutan. and i.p.	17β-oestradiol, tamoxifen, monohydroxytamoxifen/ orthinine decarboxylase activity(ODC)	Group size unclear; sesame oil vehicle. Time course of weight increase and ODC up regulation. Reported as relative to body weight.
Branham and Sheehan (1995)	Rat - Sprague Dawley (Crl:CD)	Immature - intact, OVX, ADX/ 2-29 days	Subcutan.	17β-oestradiol, desoxycorticosterone acetate/ luminal epithelium height,	Blotted uterine weights, control uteri ~25 mg at 20 days. Typically as ratio of uterine weight to body weight. 2X with one injection. Difference in intact and OVX grows after 20 days. OVX at early days were later E responsive.
Bulger *et al.* (1978)	Rat - Sprague Dawley	Immature/ ovx at 28 d	I.p./ Astwood	Methoxychlor, 17β-oestradiol, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)/ ornithine decarboxylase activity, receptor binding inhibition	4-10 rats/group; corn oil vehicle. Sacrificed 7 hours after injection. Uterine mg per 100 g bw. Microsomal demethylation of methoxychlor investigated.
Cecil *et al.* (1971)	Rat - albino, no strain given	Immature, intact and adult OVX/ 22 days 35-50 g bw and 230-270 g bw adult	Subcutan.	17β-oestradiol, *o,p'*-DDT, *p,p'*-DDT/ glycogen content and other uterine endpoints	Olive oil vehicle. Control uteri 24.7 ± 0.3 mg. Time course with single injection. *o,p'*-DDT peaked at 24-36 hours. Mature OVX uterine increase approx same at 6 and 18 hours.
Dickerson *et al.* (1992)	Rat - Sprague Dawley	Immature, intact/ 25 days	i.p.	17β-oestradiol, various dibenzo furan derivatives, chorolbiphenyl derivative, and TCDD/ other receptor assays	Group size not clear. Uteri reported as % of body weight (corn oil control was 0.102% - compare to Wade *et al.* 1997)
Ecobichon and MacKinzie (1974)	Rat - Wistar	Immature, intact/ 21-26 days	i.p./ 18 hr rather than 24 hr	*o,p'*-DDT; Aroclors 1016, 1221, 1232, 1242, 1248, and 1260; biphenyl; 2, 3, and 4 chlorobiphenyl; 2,2', 2,4', 3,3', and 4,4' dichlorobiphenyl/ glycogen content of uterus, water content	8-33 rats/group (average about 9); DMSO vehicle. Average uterine weight ~20 mg (note that only one day, 18 hrs after 1st injection). Uteri were bisected before weighting, presumably fluid lost. Increases appear to be marginal.

Table 2A. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Edgren *et al.* (1967)	Rat - Charles River	Adult OVX/ 180 g bw, used after at least 14 days	Subcutan.	17β-oestradiol, 18-homoestradiol, propyl oestradiol/ time course up to six days on vaginal response with three compounds with single injection, two injections and examination on day 3 also analyzed Approximately 7 doses in each protocol for each compound.	Other comparative work on uterine growth assay, vaginal opening, mouse assays, chick oviduct also done
Galand *et al.* (1984)	Rat - Wistar	Immature, intact/ 21 22 days, ~45g bw	I.v.	17β-oestradiol, nafoxidine/ DNA, RAN, protein, glycogen content of uterus, eosinophils	5 rats/group; saline vehicle. Unclear if wet or blotted. Plotted as % increase in uterine weight over controls (control levels not given). Time course 0-72 hours (Fig. 2A)
Galand *et al.* (1987)	Rat - Wistar	Immature, intact/ 21 22 days, ~45g bw	I.p.	17β-oestradiol, *o,p'*-DDT, *p,p'*-DDT/ protein content, DNA content, glycogen, cytosolic protein induction	5 rats/group; vehicle 1:1 DMSO:propylene glycol. Plotted as % increase in uterine weight over controls (control levels not given).
Gellert (1978a)	Rat - Sprague-Dawley	Immature, intact/ 22 d 50-60 g bw	Subcutan.	17β-oestradiol, Aroclors 1221, 1242, 1254, and 1260/ also administered on days 3 and 4 to neonate and sexual development (vaginal opening) and estrous cycle followed	5 rats/group. Sesame oil vehicle. Control uteri 26.1 ± 1.1 mg. 1 microgram oestradiol/kg induced increase in uterine weight after one injection. Aroclor 1221 at 1000 mg/kg was positive, other PCBs were not.
Gellert (1978b)	Rat - Sprague-Dawley	Immature, intact/ 22 d 50-60 g bw	Subcutan.	Kepone, mirex, aldrin, dieldrin, 17β-oestradiol / Parallel experiments on neonatal development after s.c. injection on pnd 2 and 3 including vaginal opening, estrous cycle, etc.	5 rats/group. Sesame oil vehicle. Control uteri 28.1 ± 1.0 and 23.1 ± 0.6 mg. Blotted on filter paper. Kepone positive and accelerated vaginal opening. Other pesticides were negative.
Grunert *et al.* (1986)	Rat - Sprague Dawley	Immature, intact/ 40-50 g bw/ adult OVX 280-320 g bw	I.v. (jugular)	17β-oestradiol, diethylstilbestrol/ Series of endpoints including eosinophil recruitment,, uterine cell morphometry, mitotic number in uterine cell types, cell density	5-24 rats per group; ethanol vehicle. Time points of 6 and 24 hours. Unclear if wet or blotted weights, expressed relative to body weight and as % of controls. Dose response studies six orders of magnitude.

Table 2A. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Hisaw (1959)	Rat - 'Harvard' strain	Immature, intact/ 22 day, 40-50 g bw	Subcutan.	17β-oestradiol, diethylstilbestrol, estrone, estriol, equilin, equilenin/ Wet and dry weights	Single injection, wet (some blotted) and oven dry; sesame oil vehicle. ~20 ± 0.3 mg control uteri (51 animals. All compounds effective in µg (1-10) range. Up to 16 doses used for response curve. Time course of single injection up to 72 hours. Technique comments: 'Where expressed uterine weights were desired, … then nicked with scissors, the luminal contents gently pressed out on mist paper toweling' if uterine were distended with luminal fluid … it was impossible to prevent the loss of small portion of the fluid when the uterine cervix is cut.'
Katzenellen-bogen and Ferguson (1980)	Rat - supply lab Holtzman, WI, strain not named	Immature, intact/ 21-25 days old	Subcutan./ time course	17ß-oestradiol, three antiestrogens: CI-628 Parke-Davis, U-11,100A or nafoxidine Upjohn, and MER-25 Wm. Merrell	5-6 rats/group; 0.15 M saline with 1% ethanol vehicle. Uteri excised for metabolic experiments. Control uteri ~ 27 mg in 24 hr experiments.
Kaye *et al.* (1971)	Rat - Wistar	Immature, intact/ 20 days, mean bw ~ 33 g	I.p./ time course	17β-oestradiol, 17α-oestradiol, diethylstilbestrol, testosterone, genistein, coumestrol/ Ornithine decarboxylase induction in uterus (versus liver) and *S*-adnosylmethionine decarboxylase induction.	4 rats/group; ethanol and DMSO vehicles. Sacrificed hours after administration, control uteri 22 and 25 mg. Enzyme induction was up to 30 fold when uterine weight increase was less than 1 fold (this is early in uterine growth response and water imbibition is the primary weight increase).
Kitts *et al.* (1983)	Rat - Wistar	Immature, intact/ 21-24 days	Dietary, subcutan. and i.v.	17β-oestradiol, coumestrol, zearalanol/ cytosolic and nuclear receptor level time course, uterine receptor levels, and total cytosolic protein.	Uterine weights recorded as mg/gm body weight. If 50 g bw, uterine controls would be ~22-30 mg among experiments. Coadministration of coumestrol could reduce oestradiol response (i.e., antiandrogen)
Lan and Katzenellen-bogen (1976)	Rat - supply lab Holtzman, WI, strain not named	Immature, intact/ 21 days for uterotrophic	Subcutan./ 3 days	17ß-oestradiol, estriol, 17α-ethinyloestradiol, 17α-ethinylestriol-3-cyclopentyl ether/ Receptor binding assays, cytosolic and nuclear receptor distribution, 2-deoxy-*D*-glucose phosphorylation and tritium labeled thymidine incorporation into DNA	Control uteri ~ 28 mg; aqueous 2% ethanol in saline (0.9%). Note: the cyclopentyl ether did not bind receptor, appears to be metabolically activated.

Table 2A. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Lee and Lee (1996)	Rat - Sprague Dawley	Immature, intact/ 20-21 d	I.p./ 12 and 24-hour time points	17β-oestradiol, nonylphenol/ Uterine peroxidase, uterine DNA content, uterine protein	3 rats/group; vehicle not specified. Birth time known within 6 hrs due to cage inspection. Wet weight, control uteri ~67 - 74 mg/100 gm bw (apparently high, no details on uterine preparation whether full wet weight with luminal fluid). ICI 182,780 inhibited weight increase in both compounds. NP at 1 mg/rat/day began to increase uterine wt and peroxidase activity. NP coadministered with E reduced E alone weight increase.
Levin *et al.* (1968b)	Rat - Sprague Dawley	Immature, intact/ 19-20 days, 30-32 g bw	Sacrified 4 hrs after s.c.	phenobarbital pretreatment followed by dose range of ethinyl oestradiol, ethinyl oestradiol-3-methyl ester, diethylstilbestrol, norethynodrel, norethindrone	i.p. for phenobarbital and oral for estrogens; 4 day 2X daily pretreatment, 1 estrogen, sacrifice 4 hrs later. 6-7 rats/group; saline vehicle for phenobarbital and 10% ethanol in saline for estrogens. Control uteri groups from 18.5 to 19.8 mg. Not specified if wet or blotted.
MacLusky *et al.* (1989)	Rat - Sprague Dawley	Immature, intact/ 22 days and adult OVX/ 5-60 days	I.v./ 1-48 hr time course	17β-oestradiol, oestradiol stearate, oestradiol sulfate, oestradiol cyprionate, oestradiol glucuronide	Time course to 48 hours. 4-8 rats/group; normal saline with 3% bovine serum albumin vehicle. Blotted or wet weights not specified. Immature control uteri: 42.7, 42.1, 41.8, and 37.4 mg. Adult OVX control uteri 104.4 mg.
Noteboom and Gorski (1963)	Rat - Holtzman	Adult OVX/ ovx at ~175 g bw, used 3 wks later	I.p/ 6-hr after injection	Oestradiol, coumestrol, genistein/ Tritiated glycine incorporation into protein, ^{32}P incorporation into phospholipid, RNA, and DNA	4 rats/group; propylene glycol vehicle. Control uteri 63 ± 7 mg. Injections 6 hrs before sacrifice.
Perel *et al.* (1970)	Rat - Wistar	Adult, OVX/ 3 mo., 207 g bw, used 14 d after ovx	Subcutan./ 6 hours	oestradiol, coumestrol, genistein/ effects on fertility (implantation	10-12 rats/group; DMSO as vehicle. Not specified if wet or blotted uterine weights. Results reported as % increase over controls; no control wts.
Smith and Quinn (1992)	Rat - Sprague Dawley	Immature, intact/ age and wt not given	I.p./ not all times given 1-72 hr time course in one expt.	diethylstilbestrol, amsonic acid, and several amsonic acid derivatives/	3-6 rats per group; saline and corn oil vehicles. See Table 2where at 2 of 8 doses 4-nitrotoluene gives low response (~30% increase). Results reported as relative to controls (effectively a % increase).

Table 2A. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Tang and Adams (1980)	Rat - Wistar	Immature, intact/ 21-22 days	Subcutan./ 1-72-hr time course	Estrone/ DNA synthesis, protein synthesis, receptor binding,	12-16 rats/group; ethanol:saline (1:9) vehicle. Control uteri at 24 hrs ~20 mg; after 3 days 21.1 ± 1.0 mg. Not specified how uterus was handled and prepared.
Welch *et al.* (1969)	Rat - Sprague Dawley	Immature, intact/ 19- 20 days, 30 32 g bw	I.p. / uteri removed 6 hrs after injection	DDT technical grade, *o,p'*-DDT, *p,p'*-DDT, *m,p'*-DDD, *o,p'*-DDD, *p,p'*-DDE, *p,p'*-DDE, methoxychlor	6 rats/group.
Zhu *et al.* (1997)	Rat - Sprague Dawley	Immature, intact/ 23 days, 50-60 g bw	Subcutan./s ac. 24 hrs	17β-oestradiol, 17β-oestradiol-3-sulfate	8 rats/group; aqueous ethanol and saline vehicles. Reported as mg uterine wt per g bw. Not specified if blotted. Based on starting wts, uteri about 55-60 mg.

Table 2B. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
MOUSE					
Jones and Pope (1960)	Mouse	Immature. Intact/ exact age not known	Oral gavage, i.p. and subcutan./ (2X daily)	17β-oestradiol, estriol, stilboestrol, miroestrol	8-14 rats/group. 3 days, comparison of routes of exposure and time (also single injection). Preparation - uterus and vagina removed, uterus with fluid weighed quickly, 'each uterine horn was then nicked with scissors, the uterine fluid expressed by gently pressing between filter paper, and the tissue reweighed. Note: different routes of administration summarized in Table 4.
Korach *et al.* (1979)	Mouse - CD1	Immature, intact/ 21 days	Subcutan./	diethylstilbestrol, pseudo-DES, indenestrol A/ competitive estrogen receptor binding curves, sedimentation binding curves, cytosolic and nuclear receptor level time course after administration, uterine histology	Expressed as ratio of uterine weight to body weight. Control ratio ~1, presuming 10 g bw, the uterus ~10 mg.
MacLusky *et al.* (1991)	Mouse - CD1	Adult, OVX/ 7 weeks, used 7 days later	Subcutan./ 1 injection with 20 day follow up	17β-oestradiol , estriol, 16α-estriol=stearate, 17β-estriol- stearate, 17β-oestradiol-stearate/ estrogen receptor binding, alkaline phosphatase induction in Ishikawa cells	Time course; esters show max induction at later times(~5 days) and return to control baseline by 20 days (Fig. 3). Control uterine weights ~25 mg.
Milligan *et al.* (1998)	Mouse - Swiss albino	OVX/ ovx at 3 months, used after 2 weeks	Subcutan./ 4 hours	17β-oestradiol, estriol, bisphenol A, octylphenol, nonylphenol, coumestrol, genistein, daidzein, dioctyl phthalate, benzylbutyl phthalate, dibutyl-phthalate, 3,4,3',4'-tertachlorobi-phenyl, formononetin/	Unique procedure using changes in uterine vascular permeability measured by extracellular ^{125}I-labelled albumin accumulation. Control uteri 13.00 ± 1.25 mg. ICI 182,780
Mizejewski *et al.* (1983)	Mouse - Nya:NYLAR	Immature, intact/ 15-18 days	I.p./	17β-oestradiol ± purified α-fetoprotein	4-44 mice/group; ethanol:phosphate buffered saline (1:20) vehicle. Results expressed as uterine wt to body wt ratio. Fetoprotein appears to inhibit response, but dose curve erratic.

Table 2B. Uterotrophic bioassays in rodents. Assays involving single administrations of test compounds (Astwood type assay).

Citation	Species/ Strain	Condition/ Age	Route/ time	Compounds (and other relevant data & endpoints)	Comments
Pollard and Martin (1968)	Mouse - Q.S.	Adult, OVX/ age not given	Subcutan./ 24 hours later	oestradiol and ten synthetic compounds / vaginal assays	5-10 mice/group; vehicle only described as 'oil.' Not indicated if uteri were wet or blotted. Control uteri appear high 27.7 mg mean.
Zhu *et al.* (1997)	Mouse - CD1	Immature, intact/ 23 days, 10-13 bw	Subcutan./sac. 24 hrs	17β-oestradiol, 17β-oestradiol-3-sulfate	10-12 mice/group; aqueous ethanol and saline vehicles. Reported as mg uterine wt per g bw. Based on starting wts, uteri about 12-14 mg.

ATTACHMENT TO THE ANNEX:

REFERENCES FOR EXTRACTED LITERATURE FOR UTEROTROPHIC BIOASSAYS (LABORATORY RODENTS - ONE OR MORE ADMINISTERED DOSES)

[Omits vaginal keritization and cornification literature (Allen-Doisy assay)]

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