The purpose of this workshop was to bring together the collective experience of many disciplines to discuss and evaluate techniques for the detection of functional estrogenicity. Estrogenicity was first defined as a physiological response to a compound that induced estrus in vivo. The bioassay for induction of estrus developed into a uterotrophic assay. The classical tissue response evaluated in this assay was an estrogen-induced increase in wet weight and tissue mass. Tissue response follows a time course of stimulation, including an early phase at 2 to 4 hr, followed by a second later phase. A higher dose of compound generates a stronger response. Weak estrogens exhibit the early response phase, but then the response falls off; however, multiple low doses of weak estrogens mimic the full activity of strong estrogens. A host of chemical and molecular biological factors are involved in the uterotropic response.

**Gene Activation**

Hormone receptor mechanisms exist—both membrane and nuclear receptor pathways—that affect gene regulation and interact with one another. Certain genes are specifically regulated by estrogen acting through the estrogen receptor (ER). These effects appear to be mediated by the interaction of the estrogen/ER complex with estrogen response elements (EREs) in regulatory regions of those genes. For instance, the vitellogenin A2 estrogen-responsive sequence has been cloned and can be used to detect and measure estrogen response. The DNA sequence is closely analogous to the sequence of a glucocorticoid responsive element with four base changes.

Korach’s group (1) cloned an estrogen-responsive sequence and inserted it into a reporter gene vector. The construct became an estrogen “inducible promoter” linked to bacterial chloramphenicol acetyltransferase (CAT), which can be detected in a transfected cell to measure estrogen-related gene activity. When an ER-positive target cell such as MCF-7 is transfected with this receptor-specific reporter-gene construct, CAT expression can be measured following estrogen treatment. In a test against a non-responsive (ER negative) cell, transfection with the receptor-specific reporter alone does not lead to estrogen-induced CAT expression. It must be transfected with ER to get the hormonally induced CAT production, demonstrating that the system is specific for ER-mediated gene expression and regulation. This system will respond to treatment with stilbestrol estrogens with CAT gene expression as well. Phospho-imaging of the CAT gene product can be used for easy quantitation.

Phytoestrogen compounds were tested with this ERE-CAT construct–transfection system. CAT stimulation by genistein, coumestrol, and zearalenone was compared with the stimulation produced by estradiol. CAT induction by genistein was about 20% that of estradiol; coumestrol activity was about the same; zearalenone produced about 1,7-fold stimulation compared to estradiol. The addition of a commercially available estrogen antagonist (ICI 164,384) resulted in almost complete inhibition of CAT induction by these compounds. It is important to keep in mind that the active compound may be a metabolite that the cell might not see in an in vitro assay but which would show activity in vivo. Future studies using different mutants of the receptor can evaluate more precise regulatory mechanisms.

The gold standard at this time is still the mouse uterine bioassay, precisely because it replicates a living situation and incorporates the effects of metabolism, serum binding, and pharmacokinetics. However, a complete dose–response assay can be expensive and time consuming; it is also a difficult process for testing a large number of chemicals. In vitro assays may be cheaper, quicker, and more reproducible; however, the difficulties already mentioned must be considered and dealt with to allow their use in exposure studies and possible risk assessments. A question was posed by a number of participants: How do we come up with a way to design the best assays for screening water or other contaminated sources to determine estrogenic activity?

**Proliferation Controls**

The question that has guided the efforts of Soto’s laboratory in the area of proliferation has been how to screen for a variety of chemicals quickly (2). It is important to remember that environmental contaminants of widely diverse chemical structure mimic estrogen actions. From a public safety concern, estrogenicity should be tested before chemicals are released into the environment.

Screening should be based on the end point of estrogenic action that has the greatest physiological relevance. A crucial end point can be based on the definition of an estrogen promulgated by Roy Hertz in 1985 (3): an estrogen is a substance that can elicit the mitotic stimulation of the tissues of the female genital tract; therefore, measuring cell proliferation is of key importance in assessing estrogenicity. To determine whether chemical “X” is an estrogen, one must test its ability to induce proliferation of estrogen-responsive target cells, even though not all estrogen responses or target tissues respond with proliferation.

The question of whether fish in lake “Y” or birds in region “Z” are exposed to estrogenic xenobiotics can be answered at an initial level of screening by examining...
endogenous vitellogenin (VTG) production. When VTG levels are increased, the next question is which chemicals caused the observed estrogenic effects? To answer this question, isolation and chemical analysis are required. When dealing with compounds that are not classically described as estrogens, a bioassay is necessary to evaluate their estrogenicity. Estrogenicity cannot be deduced solely from chemical structure.

The classic method for measuring estrogen induction of cell proliferation is to determine the increase of mitotic indices of epithelia in rodents. As mentioned by Korach, this is an accurate but labor-intensive, time-consuming approach, totally unsuitable for the screening of large numbers of chemicals.

Soto et al. (2) have introduced a cell proliferation assay termed the E-SCREEN test. For this bioassay, MCF-7 breast cancer cells were chosen. These cells are genuine human estrogen-sensitive cells; they remain quiescent when inoculated into ovariectomized hosts. They require the presence of estrogen to grow as tumors in hosts. When MCF-7 cells are grown in culture in medium supplemented with nonestrogenic charcoal-stripped human serum, proliferation is prevented. When estrogen is added, the cells proliferate. The E-SCREEN assay compares the cell yield achieved after 4 to 6 days of culture in medium supplemented with 5 to 10% charcoal–dextran stripped human serum in the presence (positive control) or absence (negative control) of estradiol and with diverse concentrations of xenobiotics suspected of being estrogenic. When cell yield is examined over a wide range of test–compound concentrations, it is possible to distinguish agonists, partial agonists, and inactive compounds from one another. There is general agreement about cell yield being a more sensitive marker than gene expression. A 6-fold increase can be measured in cell yields after 5 days of exposure; no false positives and no false negatives have been found among the test compounds of known estrogenicity. Methoxychlor, which requires metabolic activation, is positive in this assay; obviously, MCF-7 cells can provide the appropriate metabolic transformation. The E-SCREEN cell–yield assay is easy to perform, requiring only standard eukaryotic cell culture equipment plus an ELISA reader or an electronic particle counter. For regulatory purposes, an assay that is biologically valid is needed; that is, the end point examined should have wide but unambiguous biological meaning.

McLachlan was concerned with the binding proteins and whether they were still present in the charcoal-stripped serum. It appears that the binding proteins are present (sex hormone-binding globulin [SHBG] and serum albumin); however, a significant amount of SHBG is denatured during heat inactivation of serum (final concentration in 10% charcoal–dextran human serum is less than 3 nM (4)). Approximately 85% of estradiol is bound to plasma proteins in this assay (5).

Screening for estrogens using males as a model should not be done because estrogens are primarily defined by their ability to increase the mitotic activity of female secondary sex organs. There are reports of effects on male fertility due to exposure to estrogens. In males, estrogens are believed to act via a negative feedback to inhibit gonadotropins; this results in the lowering of androgen production by Leydig cells and inhibition of spermatogenesis.

There is no endogenous estrogen in this assay; we are measuring direct effects. An enzyme inhibitor could be an effector of an estrogen response in a system where there is a source of estrogens, such as in any animal model. Moreover, this assay can be used as a first screen for estrogenicity; any positive response would require further study to determine an underlying mechanism of action.

It was pointed out by McLachlan that in the 1930s CCl4 was described as an estrogen because of the enhanced estrogen-like sequelae of CCl4 administration. It was later learned that these estrogen-like effects occurred because of CCl4 destruction of the liver, which resulted in decreased turnover and an elevation of the level of estradiol in the circulation.

An MCF-7 variant has been modified to grow in serum-free and nonestrogenic medium by Briand et al. (6). This variant behaves similarly in the E-SCREEN assay when exposed to 10% charcoal–dextran stripped human serum (estradiol [E2] concentration in the medium is below 0.001 pg/ml; this is below 4 fM or about 1/1000 of the dose needed for the smallest significant proliferative response). MCF-7 cells are exposed to practically no estrogen in this "estrogenless" condition.

**Vitellogenin as a Biomarker**

Could a universal assay for vertebrate vitellogenins be used as a new test for environmental estrogens? Sullivan described a screen that defines an estrogen as something that induces vitellogenesis [Heppell et al., this volume; (7)]. Vitellogenin is a classic steroid-inducible protein; if you expose an oviparous vertebrate to estrogen, it will synthesize VTG. VTG is synthesized in the liver under the control of estrogen. This usually occurs in females but can also be induced in males. VTG is taken up into the oocyte by receptor-mediated endocytosis. It is present in the plasma of females several months before ovulation and is related to cholesterol and mineral transporters. In serum collected from brown bullhead, Sullivan observed a massive induction of a protein in tumor-bearing males and females, which is not present in control females; this induction appeared to be the result of an environmental insult. The induced protein was shown to be VTG by N-terminal amino acid sequencing.

For the purposes of a universal assay, VTG itself is hard to work with since it varies in structure between species. So, the paradigm includes creation of monoclonal antibodies to vertebrate VTG. Sullivan’s group screened first for antibodies that recognize both rainbow trout and striped bass VTGs. The second screen was against VTG from fish, amphibians, and mammals. A positive screening result from one clone included cross-reactivity to VTG from bass, perch, trout, tilapia, sturgeon, chicken, tuatara, and platypus; there was a questionable result in this assay for ratlenske VTG due to a high background.

Antibodies to specific portions of the molecule can be generated. It is possible to select conserved sequences, do structural analysis, select a portion of the peptide that seems likely to be on the outside in native conformation, synthesize it and conjugate it to carriers, generate antibodies, and repeat first and second screens. The N-terminal sequence of striped bass VTG shows homology with various fish, amphibian, and avian species. Demonstrated homology was between 40 to 100%. Using VTG and related proteins like von Willebrand factor, you can generate an assay that would be applicable across species from fish to mammal.

The physiological significance of vitellogenesis in males is not known. VTG receptors in the testes and in muscles in males have been found, and VTG is found in spermatocytes. VTG is a generic, ancient lipoprotein; it is not surprising that it is found in an abundance of tissues. The VTG receptor might also be involved in endocytosis of other lipoproteins.

**Lactoferrin as a Biomarker**

What would be a good biomarker for estrogenicity in mammals—similar to what
Sullivan described in fish or reptiles (see Heppell et al., this volume). Ideally, one would choose a natural product of the cells that is sensitive to estrogenic compounds, one that is present in mammals—both humans and wildlife, and one that is convenient to study, i.e., a well-characterized estrogen-responsive protein or gene. Work and studies by Teng showed that, of the 30 to 40 total uterine proteins, one band makes up approximately 15% of the total; this band is lactoferin (LF). As seen by immunolocalization, LF is made in response to diethylstilbestrol (DES) treatment. It is an iron-binding glycoprotein with a molecular weight of 68 to 70 kDa. It is a basic protein with a pI of 9 to 10 belonging to the transferrin gene family. Lactoferin has 2.6 kb mRNA; the gene is 33 kb in the human and 22 kb in the mouse. LF’s biological function is antibacterial and antiviral; it also has a growth-stimulating factor activity. Low levels exist in biological fluids and wet surface mucosa, whereas high levels exist in neutrophils, lactating mammary gland, and uterus. LF is regulated under various control mechanisms in different tissues. In the uterus, it is inducible by estrogen. Looking at putative regulatory elements of the mouse lactoferin gene promoter region, Teng’s group identified and characterized an estrogen response element (8). More recently, they found a response element that was responsive to cAMP and epidermal growth factor (EGF) (C Teng, this volume). LF induction seems to be a complex interaction, not a simple one-way cause and effect. The human LF gene has an ERE in the same location, but it also has some different response elements.

As pointed out by Adlercreutz, SHBG is another potential marker. SHBG increases when estrogens are given to women orally and not if they are given parenterally; 200 nM estradiol is needed to get this response, but with a weak estrogen, much more may be needed. Several tests have been made with enterolactone. Are there any studies of effects of thyroxine or insulin? Could other hormones have an effect? SHBG is a good marker in females. Another protein marker is ceruloplasmin (CP), a copper-containing protein. Treatment with a nonestrogenic oral contraceptive stimulates CP, which is also increased in pregnancy.

Regarding estrogenicity, biology is a hierarchical science whereby questions should be answered at the same hierarchical level at which they are posed. Estrogens were defined before the development of molecular biology and the discovery of the estrogen receptor. It is important to understand mechanisms, but we are far from agreeing on them. In the meantime, estrogens are reliably defined by their effect on the female genital tract. Moreover, estrogens exclusively induce cell proliferation in the E-SCREEN assay. These same estrogens also induce progesterone receptor and displace estradiol from the estrogen receptor.

**Toxicological Approaches**

Toxicological approaches rely on the chemical attributes of estrogens and use the study of the carcinogenic effects of DES as a background. Investigators have asked whether DES, acting as a carcinogen, causes genetic damage. This approach has been tested by Metzler’s laboratory (9). One sort of genetic change that has been reported in many systems is aneuploidy or altered chromosome numbers following exposure to DES and other estrogens. Furthermore, it has been shown that DES has colchicine-like effects and disrupts mitotic spindles. This suggests tubulin as a possible additional target for estrogen action and aneuploidy as a measure of such action. Metzler showed there are at least two binding sites on the tubulin molecule: one is the colchicine-binding site, which can also be bound by DES; another binding site can be occupied by estradiol or by DES but not by colchicine and has no effect on microtubule assembly (MA) (9). The assay for turbidity measures MA under cell-free conditions. All stilbene estrogens can bind and inhibit MA directly, but the steroid estrogens and the phytoestrogen the Metzler group has tested so far cannot bind or inhibit MA. Some steroid estrogens are inhibitory after metabolic activation. Therefore, tubulin binding and MA disruptions are not estrogenic markers per se. However, all the estrogens tested were positive in inducing micronuclei, an indication of chromosomal damage. Coumestrol, the only phytoestrogen tested so far, was also active in micronuclei induction. The mechanism may be different from effects of compounds that are capable of binding directly to tubulin and disrupting microtubule assembly. Binding to tubulin itself is not a biomarker, but many estrogens can cause chromosomal damage by different mechanisms.

This introduces the concept of bipolar characteristics of estrogens. Some of the same compounds, which may be more or less weak estrogens, may have different activities in spindle disruption or may have other activities that have yet to be discovered. Consider, in addition, another factor—time of life stage of exposure. 17α-Estradiol does not cause uterine growth in an adult, but exposure during a critical period of growth leads to manifold effects including aneuploidy. 17α-Estradiol is a strong effector in neonatal or fetal tissue.

**Conclusion**

At the end of the presentation and workshop, one of the participants posed two overlying problems. First, if we are concerned about exposure to the male, the critical period is *in utero*. Second, how does the male fetus protect itself against estrogens, either maternal or from other sources? We have no answer right now. We need an overall picture of how we are exposed to estrogens, and we need a method to determine our exact levels of exposure.

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