NICEATM and ICCVAM COMMENTS on the REVISED DRAFT OECD GUIDELINE for the TESTING of CHEMICALS
The Uterotrophic Bioassay in Rodents

Submitted as Discussion Items for the OECD Validation Management Group (VMG) meeting scheduled for 17-18 January 2007 in Slovenia

Note: ICCVAM would like the VMG to consider the general and technical comments previously submitted to OECD regarding the Uterotrophic Bioassay Test Guideline (attached as ICCVAMcomments19Jun06.doc and ICCVAMcomments20Sep06.doc). It is especially important that the VMG be cognizant of the following ICCVAM position and recommendation regarding adequacy of the validation of the Uterotrophic Bioassay (additional comments are also provided below):

"The data generated in the OECD validation program demonstrated the ability of the rat uterotrophic bioassay to reproducibly detect a small number of estrogenic substances when laboratories were instructed to use specific doses for each non-coded test article in one of four assay protocols. Because OECD test guidelines should be based on adequately validated test methods (OECD GD 34, 2005), the validation database would appear to be insufficient for this purpose.

"Therefore, it is recommended that the Uterotrophic Bioassay be issued as an OECD Guidance Document that could be used as the basis for further studies that could lead to an adequate demonstration of validation; such tests could be conducted as part of a U.S. EPA or OECD testing program and could use the ICCVAM recommended list of reference substances for estrogen and androgen receptor activity as a basis for comparative performance. It is also recommended that a comprehensive retrospective evaluation be conducted that integrates the OECD validation study data with historical data to better define the performance characteristics and the limitations of the test method, and to identify (any) data gaps that would need to be filled.

"In any test guideline developed for the uterotrophic bioassay, it is strongly recommended that the context for utilization of the data from this test method within a regulatory framework be clearly stated. For example "the uterotrophic bioassay is intended to be used only as a screen to identify substances with potential estrogenic activity (i.e., estrogen agonists) as part of a battery of in vitro and in vivo tests to identify substances with the potential to interact with the endocrine system."

Comments based on the technical issues previously identified as concerns by member countries on the original draft OECD Test Guideline are as follows:

Measurement and influence of the phytoestrogen level in the diet and bedding.

- The US continues to recommend that measurements of phytoestrogen content in food be mandatory.
- Analysis of phytoestrogen content in feed can readily be performed by new, *in vitro* methods.
- A statement that investigators should select diets based on the suspicion that sensitivity to dietary estrogens (e.g. soy) is highest in immature mice>mature ovariectomized mice>immature rats>mature ovariectomized rats is not sufficient. Sensitivity to phytoestrogens varies significantly from species to species, within species, and across animal strains. Phytoestrogen content in feed can also vary significantly from lot to lot. (Note: we have attached an article [FeedFactor.pdf] from the November Environmental Health Perspectives reporting on the workshop that was held at NIEHS in August, "DIET II – The Effect of Variability in Estrogenic Activity of Commercial Animal Feeds: Interaction with Manufacturers, NIH Officials, and Scientific Societies to Develop a Solution," that reviews and comments on a number of these concerns and request that this article be provided to the VMG as a reference for their discussions of phytoestrogens in rodent diets).

Controls

- The proposal to run one dose level of the strong positive control as an ongoing positive control is not acceptable. Either a weak positive control or a low dose of a strong positive control should be run concurrently with experiments. A weak positive control allows an ongoing evaluation of the sensitivity of the assay and the ability of the assay to correctly identify substances that have weak estrogenic activity.
- The running of concurrent controls to support the validity of data obtained using an experimental method cannot be considered to be an egregious or excessive use of the additional animals required and should not cause animal welfare concerns. The primary consideration should be the scientific integrity of the protocols.

Criteria for significance of positive results.

- There is a need to demonstrate the ability of the test method to detect weakly positive substances. It is not sufficient to extrapolate from the ability to detect strong substances.

Criteria used to evaluate the pubertal status of immature rats after dosing.

- Agree that the VMG should consider and discuss better methods for determining pubertal status.

Ovariectomy procedure.

- Agree with the U.S. national position that these results should be considered, as if the uterus does not fully regress, the dynamic range of the assay will be diminished resulting in a loss of assay sensitivity.
- In regards to the use of an analgesic on days following the ovariectomy, it is important to eliminate pain and suffering, but it needs to be shown that providing analgesics during recovery does not have an effect on uterotrophic response.

Summary Table.

- We concur with the request for the provision of a guidance document.

Comments regarding the draft report, "Additional Data on the Specificity of the Uterotrophic Bioassay" are as follows:

The comparison of the data obtained from the uterotrophic test method versus that obtained from ER binding and transcriptional activation methods does not address a critical difference between the data sets. Of the 65 chemicals tested in binding, TA, and uterotrophic assays, only 8 (24%) of the 34 chemicals indicated as negative in the uterotrophic bioassay were also negative in both *in vitro* assays. This lack of concordance for chemicals testing negative in the uterotrophic assay but not in the *in vitro* assays should be addressed. It is possible that one of the reasons for this lack of concordance is that substances can be tested to much higher concentrations *in vitro* than *in vivo* (i.e., uterotrophic bioassay). Therefore, it might be of use to perform a comparison of ranked EC- or IC_{50} values for all substances tested in the uterotrophic assay. This would allow a comparison of relative sensitivity of the three assays.

Comments regarding the draft report, "Validation of the Uterotrophic Biossay in Mice by Bridging Data to Rats" are as follows:

An evaluation of data from the mouse and rat uterotrophic test methods comparing the relative sensitivity of the rat and mouse models, as well as a comparison of the two *in vivo* systems to the two *in vitro* systems would better support the conclusion that the two methods are equivalent.

No negative substances were used in the bridging studies, therefore a comparison of specificity is considerably compromised.

The conclusion that "interlaboratory concordance is good" is also compromised, as this conclusion is based on evaluation of vehicle control and the ethinyl estradiol reference standard only.

Also of concern is the authors' conclusion that weak estrogens and substances without information on their potency require administration for seven days in the mouse uterotrophic bioassay, but strong estrogens only require administration for three days. On what basis does one determine, *a priori*, whether a test substance is a strong or weak estrogen and what quantitative criteria does one use to classify a strong versus a weak estrogen in a particular test method? It is recognized that there may be substances that are strongly positive in certain *in vitro* assays that will be negative or weakly positive in the uterotrophic bioassay for a number of reasons (e.g., issues related to ADME). It would seem more practical to simply administer all test substances for seven days. This would prevent the retesting of substances in a seven-day test that were indicated as strongly positive *in vitro* but were negative after three days of administration in the mouse uterotrophic bioassay.

Attachments:

1) ICCVAM Comments on draft OECD Uterotrophic Bioassay Test Guideline,19 June 2006 (attached as ICCVAMcomments19Jun06.doc).
2) ICCVAM Comments on revised draft OECD Uterotrophic Bioassay Test Guideline, 20 September 2006 (attached as ICCVAMcomments 20Sep06.doc).
3) November 2006 Environmental Health Perspectives report on the workshop that was held at NIEHS in August 2006, "DIET II – The Effect of Variability in Estrogenic Activity of Commercial Animal Feeds: Interaction with Manufacturers, NIH Officials, and Scientific Societies to Develop a Solution" (attached as FeedFactor.pdf).