



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES,
AND TOXIC SUBSTANCES

May 7, 2004

MEMORANDUM

SUBJECT: Transmittal of the Report and Meeting Minutes of the Endocrine Disruptor Methods Validation Subcommittee under the National Advisory Council for Environmental Policy and Technology (NACEPT) held December 10-12, 2003.

TO: Dorothy Bowers, Chair
National Advisory Council for Environmental Policy and Technology
Office of Cooperation and Environmental Management
And
Mark Joyce & Sonia Altieri
Designated Federal Officials
National Advisory Council for Environmental Policy and Technology
Office of Cooperation and Environmental Management

FROM: Jane Scott Smith, Designated Federal Official
Endocrine Disruptor Methods Validation Subcommittee
Office of Science Coordination and Policy, OPPTS

THRU: Joseph Merenda, Chair
Endocrine Disruptor Methods Validation Subcommittee
Director, Office of Science Coordination and Policy, EPA

Please find attached the minutes of the NACEPT Endocrine Disruptor Methods Validation Subcommittee Ninth open meeting held in Washington, DC from December 10-12, 2003. This meeting summary covers the pubertal assay and aromatase assay prevalidation results and recommend next steps. Specifically: received an introductory presentation on the adult intact male assay, updates on androgen receptor binding assay, efforts to finalize reference chemicals, update of OECD Fish Drafting Group, and review of activities regarding in vitro fish assays.

Information about this NACEPT EDMVS meetings and activities can be obtained from the website at <http://www.epa.gov/scipoly/oscpendo> or the OPPT Docket, Docket Number OPPT-2003-0064 online (www.epa.gov/edocket) or at (202) 566-0280. Interested persons are invited to contact Jane Smith, EDMVS Designated Federal Official (DFO), via e-mail at smith.jane-scott@epa.gov.

cc: Charles Auer, OPPT
Daiva Balkus, OCEM
Sandra Evalenko, OPPTS
Elaine Frances, ORD
Susan Hazen, OPPTS
James Jones, OPP
Bill Jordan, OPP
Joseph Merenda, OSCP
Margaret Schneider, OPPT
Adam Sharp, OPPTS
OPPT Docket # OPPT-2003-0064

**REPORT
OF
ENDOCRINE DISRUPTOR METHODS VALIDATION SUBCOMMITTEE MEETING
A Subcommittee of the National Advisory Council for
Environmental Policy and Technology
December 10 – 12, 2003
AT
Resolve
1255 23rd Street, N.W., Suite 275
Washington, DC 20037**

This meeting was a review and discussion of the pubertal assay and aromatase assay prevalidation results and recommend next steps. Specifically: received an introductory presentation on the adult intact male assay, updates on androgen receptor binding assay, efforts to finalize reference chemicals, update of OECD Fish Drafting Group, review of activities regarding in vitro fish assays; and a discussion on the next step as well as the agenda for the next meeting of the EDMVS.

_____/s/_____
**Jane Scott Smith, DFO
Endocrine Disruptor Methods
Validation Subcommittee under
The National Advisory Council for
Environmental Policy and Technology
Date: ___May 10, 2004_____**

_____/s/_____
**Joseph Merenda, Chair
Endocrine Disruptor Methods
Validation Subcommittee under
The National Advisory Council for
Environmental and Technology
Date: ___May 13, 2004_____**

TABLE OF CONTENTS

	Page
Official Signatures.....	3
Attendees.....	5
Presenters	6
Public Commenters	7
Notice.....	8
Meeting Agenda.....	9
Meeting Introduction and Summary.....	12
Attachment A	33

EDMVS Members - December 2003 Meeting

Joseph Merenda, Chair
U.S. EPA

Rodger Curren, Ph.D.
Institute for In Vitro Sciences, Inc.

William Benson, Ph.D., Vice Chair
U.S. EPA

Gerald A. LeBlanc, Ph.D
North Carolina State University

Theodora Colborn, Ph.D
World Wildlife Fund

Ron Miller, Ph.D
The Dow Chemical Company

Robert D. Combes, Ph.D. (via Telecon)
Scientific Director, FAME

Susan C. Nagel, Ph. D.
U. Missouri - Columbia

Peter L. deFur, Ph.D
Commonwealth University

James W. "Willie" Owens, Ph.D.
The Procter & Gamble Company

James T. Stevens, Ph.D.
Wake Forest U. School of Medicine

Thomas L. Potter, Ph.D.
USDA-Agriculture Research Service

David Hattan, Ph.D.
Food and Drug Administration

William Stokes, D.V.M.
NIEHS

Robert J. Kavlock, Ph.D.
U.S. EPA

Glen Van Der Kraak, Ph.D.
University of Guelph

Timothy Kubiak, M.P.A.
U..S. Fish and Wildlife Service

Mildred Christian, Ph. D.
Argus International

Shane Snyder, Ph. D.
Southern Nevada Water Authority

William Kelce, Ph. D.
Pharmacia

Charles Eldridge, Ph. D.
Wake Forest U. School of Medicine

Penelope A. Fenner-Crisp, Ph. D.
International Life Sciences Inst.

Dorothy Bowers, (Temporary Stand-In)
Chair - NACEPT Council

**Valerie Wilson, Ph. D.
NACEPT

**Nancy Kim, Ph. D.
NY ENV. Health

**Ted Schettler, Ph. D.
WWF, OEHN, NRDC

**James Yager, Ph. D.
NRDC

**** Members not Present at Meeting**

Facilitator
Paul De Morgan
RESOLVE

Designated Federal Official
Jane Scott Smith
Office of Science Policy and Coordination

Presenters

In Order of Presentation

December 10, 2003

Jane Smith, DFO
EPA, OSCP

Dr. Vickie Wilson
EPA, Office of Research & Development (ORD)

Gary Timm
EPA, OSCP

December 11, 2003

Dr. L. Earl Gray
EPA, Office of Research & Development (ORD)

Dr. Tammy Stocker
EPA, Office of Research & Development (ORD)

Jim Kariya
EPA, OSCP

Dr. Susan Laws
EPA, Office of Research & Development (ORD)

December 12, 2003

Gary Timm
EPA, OSCP

Dr. John C. O'Connor
DuPont Haskell Laboratory

Dr. Les Touart
EPA, OSCP

Oral Public Comment

In Order of Presentation:

December 10, 2003

Rick Becker, Ph.D.
American Chemistry Council (ACC)

Chris Borgert, Ph.D.
Applied Pharmacology And Toxicology, Inc

December 11, 2003

Rick Becker, Ph.D.
American Chemistry Council (ACC)

NOTICE

This meeting summary has been written as part of the activities of the National Advisory Council on Environmental Policy and Technology (NACEPT), Endocrine Disruptor Methods Validation Subcommittee (EDMVS). This meeting summary has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of the meeting summary do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The NACEPT EDMVS was established in partial fulfillment of a Congressional statute. When Congress amended the Federal Food Drug and Cosmetics Act (FFDCA) in the Food Quality Protection Act (FQPA) of 1996, it directed the U.S. Environmental Protection Agency (EPA) to develop a screening program to determine whether certain substances may have hormonal effects in humans. To ensure that EPA has the best and most up-to-date advice available regarding the validation of the screens and tests in the EDSP, EPA established the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) under the NACEPT. The EDMVS provides independent advice and counsel to the Agency through NACEPT on scientific and technical issues related to validation of the EDSP Tier I and Tier II assays, including advice on methods for reducing animal use, refining procedures involving animals to make them less stressful, and replacing animals where scientifically appropriate. The EDMVS held their first meeting in October of 2001. This was the ninth meeting of the EDMVS.

The December 10 - 12, 2003 open meeting of the EDMVS was announced in the Federal Register on November 21, 2003 (Volume 68, Number 225). Further information about NACEPT EDMVS meetings and activities can be obtained from its website at <http://www.epa.gov/scipoly/oscpendo> or the OPPT Docket number OPPT-2003-0064 online at www.epa.gov/edocket or at (202) 566-0280. Interested persons are invited to contact Jane Smith, EDMVS Designated Federal Official (DFO), via e-mail at jane-scott@epa.gov.

**National Advisory Council for Environmental Policy and Technology (NACEPT)
Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Plenary Meeting
December 10 – 12, 2003
DRAFT Agenda**

RESOLVE
1255 23rd Street, N.W., Suite 275
Washington, DC 20037
(202) 944-2300

Meeting Objectives:

1. Discuss the pubertal assay and aromatase assay prevalidation results and recommend next steps.
2. Receive introductory presentation on adult intact male assay.
3. Receive updates on:
 - androgen receptor binding assay,
 - efforts to finalize reference chemicals,
 - OECD Fish Drafting Group, and
 - activities regarding in vitro fish assays.

Wednesday, December 10, 2003

- 1:00 – 1:10** **Welcome and Opening Comments**
Joe Merenda, EDMVS Chair and Director, Office of Science Coordination and Policy (OSCP), EPA
- 1:10 – 1:30** **Introduction, Agenda Review, and Review of Previous Meeting Summary**
Paul De Morgan, Facilitator, RESOLVE
- 1:30 – 2:00** **Review of EDMVS Work Plan**
Jane Smith, EDMVS Designated Federal Official, OSCP, EPA
- 2:00 – 3:00** **Update on Androgen Receptor Binding Assay**
Vickie Wilson, Ph.D., Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory (NHEERL), Office of Research and Development (ORD), EPA
- 3:00 – 3:15** **Break**
- 3:15 – 4:15** **Update on Reference Chemicals**
Gary Timm, OSCP, EPA
- 4:15 – 4:45** **Public Comment**
Members of the public will be given an opportunity to comment on any aspect of the EDMVS work. The amount of time given to each individual will depend on the number of people wishing to provide comment.

4:45 Adjourn

Thursday, December 11, 2003

8:30 – 8:45 Settling In

8:45 – 11:45 Presentation and Discussion of Reproductive Endpoints of the Pubertal Assay
(with break)
L. Earl Gray, Jr., Ph.D., Reproductive Toxicology Division, NHEERL, ORD, EPA

11:45 – 1:00 Lunch

1:00 – 1:45 Presentation of the Pubertal Assay Thyroid Endpoints
Tammy Stoker, Ph.D., Reproductive Toxicology Division, NHEERL, ORD, EPA

1:45 – 3:45 Discussion of Issues Associated with the Pubertal Assays and Interlaboratory Variability
Jim Kariya, OSCP, EPA

3:45 – 4:30 Presentation of Aromatase Assays
Susan Laws, Ph.D., Reproductive Toxicology Division, NHEERL, ORD, EPA

4:30 – 5:00 Public Comment
Members of the public will be given an opportunity to comment on any aspect of the EDMVS work. The amount of time given to each individual will depend on the number of people wishing to provide comment

5:00 Adjourn

Friday, December 12, 2003

8:30 – 8:45 Settling In

8:45 – 9:45 Discussion of Aromatase Assays
Gary Timm, OSCP, EPA

9:45 – 10:45 Preliminary Presentation on the 14-Day Adult Intact Male Assay
John O'Connor, Ph.D, DuPont Haskell Laboratory for Health and Environmental Sciences And the American Chemistry Council
Gary Timm, OSCP, EPA

10:45 – 11:00 Break

11:00 – 11:45 Update on OECD Ecotoxicity Activities
Les Touart, OSCP, EPA

- Update from OECD Fish Drafting Group
- Update on activities regarding *in vitro* fish assays

11:45 – 12:00 Next Steps and Agenda for Next Meeting

12:00 Adjourn

Introduction

The Office of Science Policy and Coordination's Endocrine Disruptor Screening Program established the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) under The National Advisory Council for Environmental Policy and Technology (NACEPT). The first EDMVS meeting was held in October 2001. That initial meeting brought the members together to review the mission statement and discuss subcommittee roles and responsibilities. The second meeting, held in December 2001, was the first time the subcommittee members were presented with specific questions regarding assay protocols. This third meeting, held March 2002, continued discussions on protocols as well as some discussions on the validation process, Core Chemicals, 'low dose' and means of assessing human health effects. The fourth meeting, held as a teleconference, was wholly concerned with the Steroidogenesis assay. The fifth meeting held July 23-24, 2002, was concerned with screening criteria, core chemicals, In Vitro ER/AR assays, and dose setting as well as test results of two special studies, a pubertal study involving restricted feeding, and a mammalian 2-generation study involving PTU. Detailed review papers were presented on amphibian metamorphosis and invertebrate assays. The sixth meeting, held as a teleconference, was to receive comments and advice on the Fish Lifecycle DRP (Tier II). The seventh meeting was held June 5 – 6, 2003 to review and discuss prevalidation results for the steroidogenesis assay, aromatase assay and the mammalian two generation assay as well as the validation plans for each. The eighth meeting held August 18 – 20, 2003 reviewed and discussed the status/results of the prevalidation work on:

- the fish screening assay, specifically: the survey of vitellogenin methods in Fathead Minnow, Zebrafish and Medaka; the comparative evaluation of the Fathead Minnow assays; and the Fish Screen (non-spawning) assay; and
- the steroidogenesis assay optimized protocol.
Provided input and advice on the:
 - EDSP's validation plans for the fish screening assay and steroidogenesis assay;
 - strain/species white paper;
 - chemicals used in EDSP's prevalidation and validation.
 - avian detailed review paper; and
 - issues related to the pubertal assays.
- Receive an update on the amphibian workshop conducted recently.

This ninth meeting held December 10 - 12, 2003 reviewed and discussed the status/results of the prevalidation work on:

- Discuss the pubertal assay and aromatase assay prevalidation results and recommend next steps.
- Receive introductory presentation on the adult intact male assay.
- Receive updates on:
 - androgen receptor binding assay,
 - efforts to finalize reference chemicals,
 - OECD Fish Drafting Group, and
 - activities regarding in vitro fish assays.

**Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Ninth Plenary Meeting
December 10-12, 2003**

Meeting Summary

On December 10-12, 2003, the U.S. Environmental Protection Agency (EPA) convened the ninth meeting of the EDMVS. The meeting objectives included:

1. Discuss the pubertal assay and aromatase assay prevalidation results and recommend next steps.
2. Receive introductory presentation on the adult intact male assay.
3. Receive updates on:
 - androgen receptor binding assay,
 - efforts to finalize reference chemicals,
 - OECD Fish Drafting Group, and
 - activities regarding in vitro fish assays.

Copies of presentation slides and other materials distributed at the meeting may be obtained by contacting Jane Smith at smith.jane-scott@epa.gov or 202/564-8476. Many of the materials also are available on the EPA website at <http://www.epa.gov/scipoly/oscpendo>. EPA has established an administrative record for this meeting under docket control number OPPT– 2003 – 0064. The official public docket is the collection of materials that is available for public viewing at the EPA Docket Center, Rm. B102-Reading Room, EPA West, 1301 Constitution Ave., NW., Washington, DC. The EPA Docket Center is open from 8:30 a.m. to 4:30 p.m., Monday through Friday, excluding legal holidays. The EPA Docket Center Reading Room telephone number is (202) 566-1744 and the telephone number for the OPPT Docket, which is located in EPA Docket Center, is (202) 566-0280.

I. Welcome and Opening Comments

Joe Merenda, director of the EPA Office of Science Coordination and Policy (OSCP) and chair of the EDMVS, welcomed the EDMVS and members of the public. He thanked members for their service on the subcommittee.

II. Introductions, Agenda Review, and Review of Previous Meeting Summary

Paul De Morgan, senior mediator with RESOLVE and facilitator of the meeting, reviewed the meeting agenda and groundrules. He encouraged members to submit written comments to EPA on EDMVS issues in addition to comments offered during the meeting discussion. He noted that a final draft of the August meeting summary will be distributed by the end of the meeting. He also welcomed Dorothy Bowers, the Chair of NACEPT.

III. Review of EDMVS Work Plan

Jane Smith, EDMVS Designated Federal Official (DFO) described the progress of the Endocrine Disruptor Screening Program (EDSP) and provided the status of each assay in tier 1 and tier 2. (As indicated above, copies of slides from Ms. Smith's presentation may be obtained from the

docket or EPA website.) Potential topics for spring and summer of 2004 include amphibian DRP, amphibian screen protocol, fish-screen multi-chemical results, Organization for Economic Cooperation and Development (OECD) phase 1B fish screen assay, the mysid transferable protocol, in utero through lactation, and avian species comparison results on the Bobwhite quail and Japanese quail study.

With regard to the subcommittee's status, Ms. Smith explained that the Endocrine Disruptor Screening Program staff was given permission to pursue committee status versus subcommittee status under NACEPT. She indicated they are currently working on the Federal Advisory Committee Act (FACA) package to elevate the subcommittee to an Agency full FACA Committee (referred to as Tier I) and expects to submit it for approval by early January. As proposed, the new committee would have full committee status with its own bylaws, and would send recommendations directly to the Agency rather than a parent committee. Should full committee status be confirmed, then a nomination notice will be distributed. This would be one of the main ways the public would be informed EPA is seeking nominations for the Tier I committee. If committee status is not confirmed, then EPA would come out with a nomination request for the subcommittee, and the subcommittee would continue. The appropriate nomination notice will appear on the EDMVS website. The timing of the next committee meeting will depend on the committee member selection process, whether it is a reconstitution of the EDMVS or a Tier I committee, and the status of material available to be discussed at the meeting. EDMVS members urged EPA not to slow progress on activities related to the EDMVS while working on formation of a new committee. Ms Bower assured the group that the EDMVS is still under the umbrella of NACEPT. She urged the group to continue as a subcommittee under NACEPT if this will eliminate holding up the work activities during the transition stage.

IV. Update on Androgen Receptor Binding Assay

Vickie Wilson, EPA, presented an update on the androgen receptor (AR) binding assay. (As indicated above, copies of slides from Dr. Wilson's presentation may be obtained from the docket or EPA website.) She began with a general introduction to binding assays and then reminded participants of some of the key points of the ICCVAM Expert Panel Report. Among other findings, the panel acknowledged the lack of a standardized in vitro AR binding assay protocol, recommended as high priority the development of an assay using purified, recombinant full-length AR, and suggested that an AR sequence from a species closely related to human may be necessary given patent issues with human AR.

Dr. Wilson summarized the work completed thus far on the AR binding assay using rat ventral prostate cytosol. She said that the protocol has been re-optimized to make it clearer and to incorporate some of the recommendations of the expert panel. She reported that a comparison of the rat ventral prostate cytosol (RPC) and the Pan Vera (PV) binding assays using 19 chemicals that were picked over a range of potencies, showed that the PV assay is two-fold more variable and, therefore, would require more replicates to achieve the same level of sensitivity (the PV assay uses recombinant receptor). Due to this and other considerations, EPA went forward with work on the RPC assay.

Dr. Wilson reported that a reference chemical comparison was conducted in which two technicians ran the binding assay sixteen times in three "batches" using R1881. The comparison was generally considered a success as each run provided an excellent fit and in the

worst case, the IC₅₀ values varied by two fold. In an AR binding protocol comparison run at the same time, it was concluded that there were only slight differences between the older protocol run in work assignment 2-19 and the modified protocol run in WA 2-22; however, further analysis showed that several of the differences were statistically significant. Dr. Wilson said that EPA concluded that the comparison was separated considerably in time, so the results should be qualified until the hypothesis is tested in a true side-by-side experiment.

Dr. Wilson also shared the results of the classification of sixteen chemicals. The original report from Battelle classified fourteen chemicals as binders and two as non-binders. EPA's review reclassified ten chemicals as binders, four as equivocal, and two as non-binders. Dr. Wilson commented that additional experiments are needed to define the equilibrium dissociation constant K_i (equilibrium dissociation constant for the chemical and receptor) for the equivocal binders.

Dr. Wilson explained that work is ongoing at EPA to develop a recombinant AR assay. There are no standard data sets for comparison purposes. Also comparative performance criteria are needed so researchers know what to look for in any newly developed assays. She reported that other future work includes supplementing the binding data of the sixteen chemicals with additional runs and conducting statistical analysis (interlaboratory), and moving forward with the RPC assay.

Discussion/Clarifications

Several members commented on the statistically significant results of the study and expressed support for its general strategic direction. One member noted that in designing the protocol, researchers may have set the performance criteria too tightly. A member added that a next useful step is to use the results of the study to define categories to describe the results as positive, equivocal, or negative and develop a prediction model. Another member suggested that, for the use of these studies to develop performance criteria, the protocols are actually quite close statistically and questioned that any biological differences would be seen.

A member asked why a rat receptor was not used in the assay. He suggested that since the rat ligand binding domain is 100% homologous to human LBD, a rat recombinant LBD modified in a couple of places known not to interfere with function could be used. This would avoid the patent issues that now exist for using human rAR. A member inquired about the legal implications of patent issues in the use of receptors if cloned. EPA staff clarified that while discussions on this legal issue have taken place, researchers did not wait for resolution before proceeding with the assays. EPA will work to find a path to avoid legal issues.

A member commented that the disadvantage to the Pan Vera is the higher variability. The advantage is that rat tissue is not required to conduct the assay. To reduce variability in the protocol, the suggestion was made to combine the Pan Vera chimeric receptor with the ventral prostate protocol. Dr. Wilson explained that there are probably adjustments that could be made, for example, using a naked receptor. In the cytosol assay, group shock proteins help stabilize the receptor and the Pan Vera receptor could be run using the same buffers used for the rat cytosol prep; however, the tissue would be missing. The protocols actually are very similar in some terms but there are slight differences in the buffers used.

Dr. Wilson provided the following additional clarifications to member questions about the AR binding assay:

- The same solvent (ethanol) was used in both assays.
- The time lapse between assays is days or weeks.
- Cytosol is prepared in batches and can be frozen for 6 months before activity drops.
- The four chemicals that showed equivocal results were tested to the maximum limit for solubility.
- Lyophilization works well for ER.

Prevalidation Next Steps

Gary Timm, EPA, outlined the next steps in prevalidation and validation of the rat prostrate cytosol AR binding assay:

1. Complete analysis of the intralaboratory variability by performing replicates studies on some of the sixteen chemicals run in the multichemical prevalidation study.
2. Establish interlaboratory variability using standard cytosol preparation.
 - a. Run saturation and competitive binding experiments in four participating laboratories and Battelle with R1881.
 - b. Run competitive binding experiments in four participating laboratories and Battelle with a strong and weak AR binder.
3. Establish interlaboratory variability using cytosol prepared in participating lab.
 - a. Run saturation and competitive binding experiments in four participating laboratories and Battelle with R1881.
 - b. Run competitive binding experiments in four participating laboratories and Battelle with a weak AR binder.

Validation Outline

1. Test eight to ten coded chemicals using the cytosol prepared in participating laboratories.
2. Do K_i experiments in 3 to 5 chemicals.
3. Do more chemicals in one lab to cross chemical classes.

Discussion/Clarifications

Mr. Timm asked the group to express any major concerns with the direction of the assay. No major concerns were raised by the members.

Mr. Timm clarified how the laboratories would be selected. Battelle solicits proposals for conducting the work, examines qualifications, and submits a list of selected candidates to EPA for final approval.

A member supported the concept of using K_i , but raised concerns about the difficulty of conducting the experiments and wondered whether it was necessary for all chemicals.

In response to a question about whether chemicals in one assay are looked at in the suite of tier 1 assays, Mr. Timm responded that EPA is seeking a suite of assays that includes a binding component to cross-check in vivo findings. Conclusions will be based on the total weight of evidence. Specifically, from an assay, EPA is looking for a set of chemicals that will provide

information on the results of the tier 1 assays, mode of action, and use in a pre-screening context to help select chemicals.

Mr. Timm asked the group for input on whether EPA is on the right track with the human receptor/primate receptor; or if the rat receptor is adequate. A member emphasized that if EPA uses a receptor in screening prior to tier 1, then it is important to discuss which preparations and receptors EPA would use, but this ultimately depends on the purpose and application of the assay. Mr. Timm clarified that a recombinant assay is intended for use as part of the screening battery, not as a pre-screen. Another member recommended that if EPA uses a recombinant assay, EPA should use the best surrogate, but noted that there could be a problem if primate receptors are used in the AR binding assay and rodents for all other assays. Several members suggested that EPA select a simple approach and use the simplest assay to get the information desired, such as identification of binders and the approximate affinity. They added that at this point, EPA should determine if compelling reasons exist not to use the rodent, that the interest in a human receptor is because it is recombinant, and that no substantial differences exist in results obtained from rats and human receptor binding. Another member stated that no compelling reasons exist to use primate or human receptors over rat receptors. An EPA staff member noted that ICCVAM has showed some preference for use of the human receptor, but recommends a recombinant purified receptor because the cell-free base avoids the use of animals. Mr. Timm suggested that EPA is open to considering several recombinant assays. He noted that in terms of sequencing, the committee is suggesting no particular advantage exists with the human receptor versus the rat receptor.

V. Update on Reference Chemicals

Mr. Timm explained that EPA seeks to focus the table of reference chemicals on a core set of chemicals to allow the agency to choose chemicals that will serve in comparing performance across assays. He noted that EPA expects to have enough chemicals in all the relevant assays to be able to compare androgenic/anti-androgenic activity and thyroid related activity, but may have insufficient chemicals for comparison of the ability of the adult male assay to detect estrogenicity. There also seems to be a deficiency of chemicals testing the ability of assays to interfere with the hypothalamus-pituitary-gonadal axis and aromatase inhibition. He noted that EPA is close to identifying four or five chemicals which the committee considered a reasonable number in the last discussion it had on this topic. Mr. Timm then highlighted changes in the table since it was distributed at the August meeting. He stated that choosing negative chemicals has been difficult and more could be added.

Discussion/Clarifications

One member asked how EPA plans to use the list of 78 chemicals listed in the ICCVAM report on ER/AR binding assays. Mr. Timm noted that EPA does not plan to use all 78 for all assays, but will run a large number of the chemicals in the ER and AR binding assays.

Members raised numerous comments on negative chemicals, emphasizing the importance of their selection. Some members indicated that negative chemicals should be negative for all modes of action and that if no across-the-board negative chemicals can be found, then the purpose of tier 1 screening is defeated. Another member stated that chemicals acting as positives or negatives are assay specific and EPA will need to accept a certain number of false positives. Several members supported EPA running some chemicals, such as nonspecific toxins, expected

to be negative across all assays. An EPA contractor representative suggested that possible across-the-board negative chemicals include Phenobarbital and carbon tetrachloride, while a member suggested that EPA look for negative chemicals in the NTP's database of chemicals.

Regarding specific chemicals listed, a member commented that there is not a need for both estradiol and ethynyl estradiol. Mr. Timm responded that both may not have been needed, but they could be used in different assays. One member suggested that octylphenol may be a better choice than nonylphenol. An EPA staff member responded that nonylphenol is more effective in vivo (when administered by diet), while octylphenol is not effective in vivo when administered by diet but is positive when administered by gavage. Another subcommittee member suggested, however, that nonylphenol is inactive in vivo. Another member suggested adding testosterone propionate in the Hershberger column as the standard used in that assay and ZM 189,154 in the uterotrophic assay.

Members also commented on dose levels, including concern that assays will need to be run at high doses to get negative response and that dose selection criteria are very important so that the chemicals do not cause overt toxicity. Another member suggested that EPA use range-finding studies and stay at doses below the MTD and toxicity to system activity to achieve the best response.

Suggestions for alterations to the table format included:

- Clearly indicate which chemicals are positive and which are negative.
- In the next version of the table, add a column for the 407 assay so the information can be used in an international forum.

VI. Reproductive Endpoints of the Pubertal Assay

Earl Gray, EPA, provided some interpretation of results from the studies on the pubertal male and female rat assays. (As indicated above, copies of slides from Dr. Gray's presentation may be obtained from the docket or EPA website.) He explained that he would not summarize all of the data from the studies but rather would focus on the results that indicate where the endpoints were useful, whether the expected results were achieved, and where the assays did or did not perform well.

Pubertal Female Assay

Dr. Gray first reviewed the female pubertal protocol and listed the published studies that have used the pubertal female assay and the chemicals studied this year in the assay at RTI and Therimmune. He then presented data and analysis on the estrogens, "antiestrogens" and inhibitors of steroidogenesis, CNS active chemicals, and PTU (antithyroid) in the pubertal female assay, noting that the study results were mostly as expected. He shared several summary observations on the pubertal female assay:

- The assay responds very well to estrogens.
- The assay responds very well to GnRH antagonist and potent aromatase inhibitors.
- The assay responds well to ketoconazole, but this chemical did not affect the pubertal landmarks.
- The assay gives reproducible responses to Atrazine.
- The assay gives reproducible responses to Phenobarbital.

- The assay was negative for Fenarimol, a weak aromatase inhibitor, at doses tested; need a study at the MTD with doses from dose-range finding, not guessing. The result was a surprise, though consistent with certain other studies.

Discussion/Clarifications

Members addressed three major issues in discussing the female pubertal assay: diet and environmental factors; vaginal opening; and the fundamental purpose of tier 1 screening and its role in the overall battery.

A member inquired about potential animal husbandry issues with running the assay, such as the fire at RTI, the presence of phytoestrogen in the feed, and housing conditions. Dr. Gray stated that the role of diet is a critical issue because of the effect on growth rates and age of puberty. He added that he has found no differences due to phytoestrogen in the diet, but at certain concentrations, it could affect estrogen landmarks. Animal bedding can also have an effect, such as corn cob bedding that contains anti-phytoestrogens and could impact puberty. Housing conditions for the assay involved 2-3 animals per cage to maintain social interaction. In response to other questions about the significance of diet, Dr. Gray responded that studies on diet, including OECD, Therimmune, RTI, Ashby and EPA labs have shown that dietary phytoestrogen does not have a strong effect. It is not a significant confounder, but researchers should keep aware of potential effects of diet on growth and development. One member emphasized the need for additional work on diet optimization. Another member added that other dietary factors include how much the animal is eating, but that he sees no compelling reason to change the diet based on the data. He referenced Dr. Julius Thigpen's (NIEHS) studies on metabolizable energy effects on puberty, which show an impact on puberty and that effects are dose-related.

A member inquired about the differences in data on vaginal opening of control animals in U.S. studies and one of J. Ashby's studies. Dr. Gray responded that the difference in timing is distinctive, but was unsure about the cause of the difference. Several members commented on the potential for variation in vaginal opening, which is critical in the female pubertal assay. A member recommended contacting John Ashby regarding what affects the day of vaginal opening. Dr. Gray suggested that variability issues can be addressed by documenting the onset and completion of the process, rather than only completion.

On the issue of toxicity, a member noted that fenarimol resulted in a negative and false positive in the assay and inquired whether any precedent existed for thyroid toxicity. Dr. Gray answered that thyroid toxicity is possible.

Another member emphasized that EPA should be aware of toxicity issues and should identify chemicals that have specific actions on the endocrine system, rather than secondary to general toxicity. Dr. Gray responded that the EDSTAC defined broader effects on hormones, the central nervous system, steroid synthesis, and metabolism. He recommended integrating toxicity data on animals and using available toxicological information.

A member asked for clarification on what constitutes a positive chemical in the assay. Dr. Gray answered that he would consider a statistically significant reproducible change a positive response, but that overall conclusions are based on the total weight of evidence and are taken in the context of results from the entire battery.

One member recommended developing a diagnostic profile for substances to help analyze data, including what is expected of the substance and how that compares to the results. Another member added that using a prediction model is very important in developing an in vitro test system and improving the predictability of the system.

Individual members stated support for several specific assay components, including the assay's ability to achieve a variety of responses and parameters, the use of Day 21 covariate, and the use of 10% reduction in body weight gain for Maximum Tolerated Dose (MTD).

Dr. Gray made the following additional clarifications in response to questions:

- The incidence of false positives in the assay is unknown.
- The assay did produce delays in estrogen-dependent endpoints.
- Fenarimol, an aromatase inhibitor might not have worked because of accumulation of testosterone before inhibition of estrogen levels.
- The recommendation for defining MTD as 10% decrease in body weight gain is reasonable; the definition should not be increased significantly.
- While relative organ weight was not examined in this assay, it can help inform mechanistic endpoints in some cases.
- Concerns exist about histopathology as an endpoint in the lack of consistent observations; guidance on observation is needed.
- Body weight is used as the endpoint to determine MTD and effects, in addition to cage-side observation.
- Some endpoints in the assay reproduce well, but others are inconsistent.

EPA staff noted that the agency intends to begin the validation before the next EDMVS meeting, so any additional comments should be submitted in the near future.

Pubertal Male Assay

Dr. Gray outlined the pubertal male protocol and reminded participants that the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the assay be developed and evaluated as a possible alternative assay in the tier 1 screening battery. He indicated the pubertal assay could replace the Hershberger assay in a modified battery. He commented that the questions to be answered are whether the pubertal male assay is as sensitive as the Hershberger assay, and if it is not as sensitive, whether it is sensitive enough. He noted that it is important to consider the detection ability of the entire battery.

Dr. Gray listed the published studies that have used the pubertal male assay and the chemicals studied this year in the assay at RTI and Therimmune. He then presented data and analysis on various endocrine active compounds in the assay. He shared summary observations as follows:

- The assay produced expected results for all chemicals.
- Both Sprague-Dawley and Long Evans strains responded significantly.
- The assay appears to detect all the activities as expected.
- The assay is more sensitive than the adult intact male assay.
- The assay is more sensitive than the Hershberger to DBP but not to the androgen receptor antagonists.

Discussion/Clarifications

A member inquired about the differences in sensitivity between the pubertal male and Hershberger assays. Dr. Gray explained that the Hershberger is more specific and easier to interpret, while the pubertal male is more apical and may detect a broader range of activities.

One member asked if results may have been confounded by the compound's ability to affect leydig cell differentiation. Dr. Gray answered that more information is needed on this issue, possibly through conducting a time series analysis and defining the mechanisms of action in the protocol.

In response to a question about responses with a specific 5-alpha-reductase inhibitor, Dr. Gray indicated he expects distinct differences would be seen in the sensitivity of tissues, including a delay in preputial separation.

VII. Pubertal Assay Thyroid Endpoints

Tammy Stoker, EPA, presented information on the response of the pubertal protocols to thyroid toxicants. (As indicated above, copies of slides from Dr. Stoker's presentation may be obtained from the docket or EPA website.) She said that two questions were examined:

- Are the male and female pubertal assays useful as screens for thyrotoxicants with different mechanisms of action?
- Are results reliable? What is the interlaboratory variability?

Dr. Stoker reviewed the mechanisms of altered thyroid homeostasis and then shared examples of chemicals with specific mechanisms. She explained that the pubertal assays identify three mechanisms (altered K/I symporter, altered thyroperoxidase activity, and altered clearance of T4), while other mechanisms have not yet been studied (binding proteins, deiodinase, and hypothalamic alterations).

Dr. Stoker shared the results from three contract studies. In summary she reported that all of the studies found alterations in thyroid parameters that were consistent with the chemical action. She also shared results from new EPA studies looking at other thyrotoxicants.

Dr. Stoker concluded with the following points on the pubertal assay thyroid endpoints:

- No failures have been observed so far.
- Three mechanisms have been examined.
- Do we need to do other mechanisms before moving on?
- Some possible disappointments encountered thus far: one contract laboratory missed the phenolbarbital effect on T4.
 - Was this a failure of the assay or the performer?
 - Thyroid effects were still observed.

Discussion/Clarifications

Members cautioned that results may vary with the use of different kits, differences in labs, and differences between male and female animals. Histopathology may bring variability if technicians have problems. Several member raised concerns with the thyroid histology variability in the assay with wide CVs for TSH and other assays such as the 407.

A member commented that a minimum of two sections of the thyroid are needed in pathology. Dr. Stoker explained that only two thyroid sections are used in pathology in the assay and results are consistent within sections.

Members emphasized the need to find the right assay to distinguish changes in thyroid function, including minor changes in hormone levels. EPA staff noted that this assay is not designed to identify chemicals that interact with receptors. One member asked how much TSH, T3, or T4 is needed to induce thyroid histological or growth effects. Dr. Stoker responded that an approximately 20-30% increase TSH caused changes in the thyroid. Another member commented that the assay should focus on hormone levels.

One member commented that the assay should be simple and effective to maintain costs and that glandular testing is not as important. Another member commented that glandular weight should be taken after fixation to minimize variability.

An EPA staff member added that industry can now purchase reliable TSH kits from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Dr. Stoker offered the following additional clarifications:

- Thyroid hormone levels did vary across kits, but the effects of the chemical were still evident.
- TSH kits were used.
- A chemical that elicits antithyroid activity by binding to the receptor should increase the clearance of T-4, but the effect on the thyroid hormones is unknown.
- PCBs were the only chemicals that interacted with the receptor.

VIII. Issues Associated with the Pubertal Assays and Interlaboratory Variability

Jim Kariya, EPA, presented issues, questions, and plans regarding the next steps in the validation of the pubertal assays. (As indicated above, copies of slides from Mr. Kariya's presentation may be obtained from the docket or EPA website.) He emphasized that in addition to comments at this meeting, written comments from members are particularly helpful. He reminded participants that the primary purpose of the interlaboratory validation study is to determine whether independent laboratories arrive at the same, correct conclusion about the ability of an unknown chemical to interact with the endocrine system when using the pubertal assays, in the absence of other information about the chemical. A secondary purpose is to refine the protocol, if necessary, by identifying specific areas of difficulty in performing the assay. Mr. Kariya explained that EPA is proposing a two-part study, with both parts done in parallel to expedite the process and avoid interference with the test of transferability. Part A will include dose setting done in each laboratory and provide the main test of transferability, and part B will include dose levels set centrally and identify performance problems, if any.

Mr. Kariya outlined the general considerations and the non-protocol/non-Good Laboratory Practices (GLP) deviations of the study. He noted some of the factors that will not be standardized in the protocol beyond what is already in the *Critical Reviews in Toxicology* protocol: strain, feed, water, caging, and bedding. He outlined the criteria for evaluation of the protocol:

- Did all labs arrive at the same conclusion about the ability of the weak chemical to interact with the endocrine system?
- Was the conclusion in line with expectations?
- Were any of the endpoints inconsistent across laboratories?

Mr. Kariya listed some of the clarifications to the protocols that were learned from three validation studies. He outlined the timeframe for validation, noting that the schedule is optimistic, with a goal of completion by December of 2005. As a final note, Mr. Kariya explained that dose setting is not realistic in this blinded study: usually some background information will be available. He commented that the criterion will be that the highest dose causes some decrease in body weight gain, but less than ten percent compared to controls, at time of necropsy.

Throughout the presentation Mr. Kariya posed questions to the EDMVS, as listed below with the summary of members' responses.

Discussion

1. Does the EDMVS agree that the pubertal assays show adequate sensitivity over a range of chemicals for use as a tier 1 screen? That is, are the pubertal assays ready for an interlaboratory validation study? If not, what specific areas need further prevalidation work?

Some members noted that further information on the protocol is needed. Some members raised concerns that the lack of standardization of the kit and histopathology problems weaken the sensitivity of the assay and encouraged EPA to examine these issues further before validation. Another member recommended that EPA conduct additional work on thyroid hormone measurement, but this does not prevent going forward with the assay.

2. Based on the currently available data, are there endpoints that should be dropped from the assay?

Members did not offer any comments on endpoints at this time.

3. Is there a better set or sequence of studies to perform for validation consistent with the timeframe?

Members offered a new sequence of studies for the validation. They recommended that EPA start with the proposed "Part B" to determine appropriate doses for each of the test chemicals and then tell the labs to test the chemicals at certain doses. Separately, they suggested EPA should determine the dose setting guidance for the future use of the assay and run a separate evaluation of that dose setting guidance, but not necessarily ahead of the actual validation. Labs would conduct range-finding studies, but not run the complete study in "Part A." Labs would only run the full protocol with the set doses in Part B, and no integrated test is necessary. Members generally agreed that this process is an appropriate validation of the pubertal assay.

4. Can the number of dose levels be reduced in this study?

Most EDMVS members concurred that the number of dose levels cannot be reduced from three doses in this study.

5. If doses do not reach, or exceed, the MTD, should the lab be allowed/required to repeat the study before interlaboratory results are compared?

Several members noted that this question is irrelevant given the new proposed study sequence, except for testing dose-setting studies.

6. Should laboratories be trained as part of the validation exercise?

Some members supported training laboratories, noting that this will reduce variability, particularly if observations are part of the study. They also emphasized the importance of clear standard operating procedures in addition to formal training. If in-person training cannot be conducted for all labs, then videos and/or dissection manuals with photographs and clear language that describe endpoints should be distributed. One member added that a criterion for lab selection should be experience running updated 2-generation studies, which would reduce the amount of training required.

Mr. Kariya clarified that the prime contractor will select the labs and that manuals and photographs are part of the study protocol.

Members also commented on the use of kits in the assay, noting that EPA should consider the weaknesses of kits and the lack of a standard kit before validation studies. The draft protocol should be very specific as to the kit for validation, time of day, handling, etc. to eliminate variability. The agency should also open the draft protocol for public comment.

7. Should positive controls be required in the protocol? In this study?

Most members concurred that positive controls were not needed in this study, but that some measure of lab capability is required in the protocol. Members also supported removing the three strong positive chemicals from the protocol. Some members commented that positive controls are needed to show that the test system works and may be needed for less-experienced labs, thus they are needed for dose setting in Part A. Other members noted that positive controls in other tests have shown limited overall utility and that it increases the number of animals used. One member suggested that controls could be run every two years rather than with every experiment, but others believed that this was not adequate.

8. Is there a toxic compound that is known to be endocrine inactive at MTD, which can serve as the “toxic negative” control?

One member asked for clarification of “negative control.” The member cautioned that toxic compound modes of action may not involve the endocrine systems or be an endocrine-active compound. Results below toxic dose levels may help verify specificity. Another member asked what is the relevance of tier one if no negative controls are used? One member suggested aspirin as one example and offered to send EPA a list of other possible negative controls.

Mr. Kariya invited members to send written responses and comments on the pubertal assays to EPA by mid-January.

IX. Aromatase Assays

Susan Laws, EPA, presented information on the in vitro aromatase assay prevalidation studies. (As indicated above, copies of slides from Dr. Laws's presentation may be obtained from the docket or EPA website.) She noted that the goals of the studies were to optimize the protocols, examine performance criteria, compare placental and recombinant microsomes, and prepare the protocol for multi-laboratory studies. She reviewed the radiometric method used in the assay. She explained that either estrone or tritiated water can be measured, but when human placental microsomes are used, estrone continues to metabolize, making it more difficult to measure than tritiated water. She noted that with recombinant microsomes, estrone appears to be more stable.

Dr. Laws outlined the indicators of an optimized protocol and then presented some of the data used to determine the optimized conditions for the human placental and human recombinant assays. She then reviewed the design and results of the experiment conducted to examine variability between assay day and technicians using the optimized protocols. She noted that the variability observed among some of the technicians indicates that some training may be necessary to perform the protocol well.

Dr. Laws outlined the experiment design and results of the comparison of test chemicals and shared the following conclusions:

- Variability between replicates is greater than expected for both assays.
- Technician error rather than inadequate protocol method is the likely cause of variability.
- Despite variability, both protocols correctly identified inhibitors.

In closing, Dr. Laws listed next steps and shared several summary comments:

Next Steps:

- Identify source of variability.
- Conduct additional experiments to evaluate day-to-day and technician variability.
- Rerun assays for test chemicals with incomplete curves.
- Evaluate the usefulness of estrone measurement rather than tritiated water for recombinant protocol.
- Prepare updated protocols for validation.

Summary:

- Protocols were optimized for placenta and recombinant assays.
- Assays produce similar data.
- Assays differ in advantages/disadvantages.
- High throughput assays:
 - KGN cell line
 - CYP19/Flourescent substrate (HTP) kit available

Discussion/Clarifications

One member commented that variability in the assay may relate to substrate concentration. Dr. Laws responded that she considered that source, but that a more likely cause is a mistake with the use of cold androstenedione.

X. Discussion of Aromatase Assays

Mr. Timm presented a series of questions on the aromatase assays and outlined the validation study plan. The EDMVS discussed several questions on the assay.

1. Does the EDMVS agree that the prevalidation studies demonstrate that both the placental and recombinant assays are effective in identifying chemicals that inhibit aromatase?

The EDMVS expressed general support that the assays are effective in identifying the chemicals. Some members noted the EPA should still consider the specificity of the assay in its ability to identify inhibitors and non-inhibitors and differentiate between weakly positive and non-active chemicals.

2. Pending the resolution of the issue regarding sources of variability observed in both assays, does the EDMVS believe that prevalidation will have been successfully completed for both assays? If not, what additional studies should be performed before beginning interlaboratory studies?

EDMVS members did not indicate any issues that would prevent completion of the assays or that additional studies need to be performed.

3. Should EPA continue to validate both the placental and recombinant assays?

Members that supported continuation of both types of assays noted that such an approach would provide a choice of sources and no compelling reasons exist not to do both.

Those in support of moving forward with the recombinant assay only commented that it is more practical, preparation is cleaner, and that the placental assay brings ethical issues. One member cautioned that if using the placental assay, EPA should be aware of potential mutations in human enzyme sequence, while recombinant assays use exactly the same enzyme. Another member noted that only a general level of detail is needed for the information that can be achieved adequately through the recombinant assay.

EPA staff clarified that the agency intends to develop performance criteria to allow for the use of other assays as long as those criteria are met. Another member encouraged EPA to use methods that enhance work on automation in preparation.

On other issues, two members commented that five labs may be unnecessary and actually increase logistical problems and recommended that EPA use three or four labs to simplify the process and save resources. EPA staff responded that they will consider the number of labs and look at the pre-validation data, but will have no more than five labs. A member recommended increasing the number of chemicals to provide more than one negative. Another member suggested that EPA explore several compounds in the area of very low solubility to see if they generate some false positives simply by physical interferences and things that are nonspecific.

One member raised a caveat for testing aromatase: the assay would detect compounds that interfere with enzyme activity, but might not detect compounds that alter aromatase activity by either decreasing the synthesis of the enzyme itself or increasing its degradation.

The EDMVS expressed general support for conducting both the placental and recombinant assays. Some members noted a limitation with the placental assay is that each lab may or may not be testing the same protein sequence from the placental tissue due to human variability. This is not the case with the recombinant assay. The EDMVS also supported the original proposed approach for rerunning studies on controls and chemicals for both centrally prepared microsomes and microsomes prepared in a participant lab, rather than either of the alternatives presented.

Mr. Timm summarized the key messages to EPA from the EDMVS:

- Support for recombinant assay.
- Some support for placental assay.
- Support for fewer labs and more chemicals, including more negative chemicals.

XI. **Preliminary Presentation on the 14-Day Adult Intact Male Assay**

John C. O'Connor, DuPont Haskell Laboratory, presented an overview of the adult intact male assay as an alternative tier 1 screening assay. (As indicated above, copies of slides from Dr. O'Connor's presentation may be obtained from the docket or EPA website.) He outlined the EDSTAC-recommended tier 1 screening battery and two alternate screening batteries, one including the adult intact male assay and the other including the pubertal male assay.

Dr. O'Connor reminded participants of the desirable attributes of a screening assay and noted that a benefit of the adult intact male assay is that it can provide hormonal information. He outlined the model, required endpoints, and optional endpoints of the intact male assay. He explained that the hormonal data collected from the assay can be compared to the "expected"

profile to identify the mode of action of an unknown compound. He then reviewed some of the data used to resolve several study design issues to determine the assay protocol.

Dr. O'Connor presented case studies with flutamide, ketoconazole, and finasteride. He reviewed the study data. He summarized that all three compounds decreased the androgen dependent tissue weights, so organ weights alone cannot be used to differentiate the modes of action; however, by using the hormonal profile in addition to the organ weights, one can differentiate the modes of action.

Dr. O'Connor presented data on the endocrine active compounds that have been examined in the intact male assay and compared the detection results of the intact male assay with those of the pubertal male, pubertal female, and Hershberger assays. He noted that the intact male assay detected most of the weak acting compounds, though not p,p'-DDE. He observed, however, that no assay will detect every compound with complete accuracy, which is why a battery of assays will be used for screening. Dr. O'Connor also noted that for an accurate assessment of detection ability and comparison among assays, the assays need to be run to limit dose levels, and all of the data from the adult intact male assay need to be considered, not just the organ weight data.

In closing, Dr. O'Connor listed the advantages of a tier 1 battery using the intact male assay:

- Comprehensive mode-of-action screen
 - Capable of evaluating several different modes of action in a single assay – by measuring mechanistic endpoints
 - Tier 1 with intact male provides mode-of-action profile to focus direction of any further testing
- Intact endocrine system
 - Design allows integration of new endpoints if desired
- Consider value of using intact male in tier 1
 - Need a more in-depth analysis – side-by-side comparison of tier 1 in vivo assays
 - Specificity and sensitivity of the alternative approaches should be directly assessed with common set of substances across different modes of action

Discussion/Clarifications

A member commented that because Tier 1 does not include a developmental assay, a strong need exists to identify the most sensitive screen to detect endocrine active compounds. Another member emphasized that relative sensitivity is the key issue, particularly in the utility of results at the lower end of the dose scale in determining whether the assay is more or less sensitive than other assays.

One member recommended that this assay not go forward as a high priority based on the results and lack of sensitivity. Another member commented that the intact male assay is valuable for its focus on specific endpoints as opposed to more apical ones. He added that this assay has several advantages compared to the pubertal assay and is worthy of consideration.

Another member stated that EPA should look at the specific hormone analysis in the intact male assay as a way to identify activity on the basis of hormones.

Dr. O'Connor offered the following responses to member's questions about the 14-day intact male assay:

- Researchers identified effects on thyroid weight at all doses, but the relative weights rather than absolute weights should be examined.
- Low levels of variability in the assay were achieved through resting the rats for an hour after dosing in the necropsy room before necropsy and blood draw to reduce variability and reduce stress hormones.
- The dose level could have influenced why apomorphine was not positive in the assay.
- Chemical was administered by oral gavage.

Mr. Timm noted that EPA plans to conduct a comparison of intact adult male and pubertal male and will test more compounds before that comparison. Regarding presenting the comparison, members noted that EPA should ensure that the assay is effective at detecting weak substances and that the dose used produces reliable effects, that tradeoffs or pros and cons are outlined and analyzed, and that the EDMVS is given sufficient time to consider the information.

XII. Update on OECD Ecotoxicity Activities

Les Touart, EPA, presented an update on OECD ecotoxicity activities and EPA in vitro fish efforts. (As indicated above, copies of slides from Dr. Touart's presentation may be obtained from the docket or EPA website.) He reported on the OECD fish gonadal histopathology consultation. The objectives of the consultation were to identify main features being used for evaluation of gonadal tissues by expert fish pathologists, evaluate the uniformity of assessments by expert fish pathologists, and develop guidance to standardize histopathological assessments of small fish gonad sections. Conclusions from the consultation included the following:

- Discrepancies were noted in evaluations due to differences in diagnostic criteria, differences in conduct of the tests, different strains of fish species, age and size of fish, inadequate histological techniques, and parasitism.
- Non-spawning status was a confounding factor.
- Histology results from phase 1A studies were not sensitive indicators of endocrine effects.
- Use of spawning fish may improve sensitivity.

Recommendations from the consultation were to develop a standardized atlas of fish gonad histopathology and glossary of terms and to allow spawning to occur in the assay.

Dr. Touart reported that the OECD Fish Drafting Group held a meeting and developed the following recommendations for phase 1B:

- Select one vitellogenin (VTG) method for each species; should be ELISA with homologous antibodies and VTG standard.
- Use mature fish and allow spawning to occur in assay; quantification of fecundity optional.
- Drop GSI as an endpoint (unreliable).
- Establish standard operating procedures for measuring secondary sex characteristics for each species.
- Provide detailed guidance for histopathology evaluation.
- Phase 1B should include three test chemicals and three positive controls.

Dr. Touart also shared recommendations from the OECD Invertebrate Expert Group meeting:

- Proceed to a phase 1 trial with the copepod assay.
- U.S. to prepare protocol suitable for phase 1 trials for mysid two-generation test.
- Encourage Japan proposal to revise Daphnia TG 211.

- Revise mysid detailed review paper (DRP) to include crustacean and aquatic insect methods.
- Encourage further work in developing methods for Prosobranch snails, earthworms, and exploration of methods with echinoderms.

Dr. Touart reviewed the objectives, approach, and progress of EPA in vitro fish research activities. Activities include work on the receptor binding assays, the reporter gene assay, and the trout liver slice assay. Dr. Touart also reported that the Office of Research and Development has begun a new computational toxicology research program. The initiative involves the application of mathematical and computer models together with molecular chemistry and biology approaches to the main toxicological issues faced by the regulatory program offices at EPA. More information on the program is available at www.epa.gov/comptox.

Discussion/Clarifications

A member noted that he sees much interest in Europe around in vitro assays, but controversy exists about the use of fish assays. Another member inquired about resolution of problems with histopathology described in the draft OECD report. Dr. Touart clarified that photomicrographs of tissues were distributed to help improve consistency in the interpretation of histopathology. Differences in descriptions and terminology were detected and the non-spawning status added some abnormalities. He noted that many of the discrepancies would be removed if the fish spawned and scientists agreed upon common terminology about lesions and other effects.

Another member disagreed that spawning fish improved sensitivity and noted that their use only answers different questions. The member expressed general support for the EPA fish assays, while also suggesting that the trout liver sliced assay has the same problems with the mammalian steroidogenesis assay, such as difficulty of evaluating the effect of chemicals on cell death. He concluded that this assay was not necessary. Dr. Touart noted that for purposes of observing developmental changes, with continuous spawners, the gonads are standard and responsive in development stages. In response to a question about the publication of a fish histological atlas, Dr. Touart noted that EPA would be involved with its preparation. A member suggested that atlas developers take into account the years of work on mammalian histology where commonalities exist.

Members asked for clarification on the specific Fish Drafting Group recommendations. A member asked why quantification of fecundity is an optional recommendation. Dr. Touart responded that during fish drafting group discussions, participants recognized that fecundity for zebrafish and medaka is needed, but all labs are not ready to quantify the data. As a compromise, the group left the recommendation optional. Another member inquired why GSI was dropped as an endpoint. Dr. Touart answered that for non-spawning fish, GSI was seen as unreliable with very high variability and that the group preferred to keep whole body measures. With spawning fish, female release of eggs was also viewed as a potential source of variation. Dr. Touart also clarified that Phase 1 B does not have an anti-estrogen endpoint, but this may be considered.

With regard to dose setting, Dr. Touart noted that the group did not foresee difficulties with dose setting. He added that a flow-through, rather than static, delivery is considered optimum.

Dr. Touart explained that more details on the OECD activities will be available when the meeting reports are finalized and made public (likely in January). EPA will post this information

on the website when available. He concluded by encouraging members to send comments on Phase 1 B as it is still in the planning phase.

XIII. Public Comment

At the conclusion of the deliberations on the first and second day of the meeting, members of the public were given the opportunity to provide comments. Mr. De Morgan encouraged all attendees to submit their comments in writing to Ms. Smith for inclusion in the EPA docket and posting on the website. Slides of some of the individuals' comments may be obtained from the docket or EPA website.

Wednesday, December 10, 2003

Richard Becker, American Chemistry Council (ACC)

Dr. Becker shared comments on the pubertal assays. He expressed concerns about the need for a predictive model, the specificity and sensitivity of the assays, and the value of the histopathology. He offered several recommendations:

- Develop a predictive model and apply systematically.
- Evaluate specificity and sensitivity.
- For dose setting, develop a standard procedure for unknowns.
- Evaluate results across alternative tier 1 assays.

Chris Borgert, Applied Pharmacology and Toxicology, Inc

Dr. Borgert shared information from a cost estimate survey conducted by APT. He presented summary tables of the number of animals consumed for each assay as well as estimated minimum, maximum, median, and mean costs. He noted that the estimates do not adjust for differences in number of dose groups among the assays. He also compared the estimated costs for the EDSTAC-recommended battery, an alternative battery using the adult intact male assay, and an alternative battery using the pubertal male assay, observing that the alternative with the adult intact male was estimated least costly in both financial terms and animal use. Dr. Borgert suggested that assay costs should be a consideration in the deliberations of the EDMVS and offered to provide members the full report from the survey.

Thursday, December 11, 2003

Richard Becker, American Chemistry Council (ACC)

Dr. Becker expressed concern that the EPA had not yet been able to identify “negative” compounds to run in the validation of the pubertal assays. He commented that if every substance tested at a MTD produces an effect in the assay, the assay is of no use for a tier I screen. He suggested that validation include not just one negative compound, but probably several negative compounds by different mechanisms of action to show that at the required test dose the assay can distinguish substances that have an effect by a primary hormonal mechanism of action from those that do not. He suggested several compounds that might be used as negative controls: classic hepatotoxic agents, inorganic phosphorus, bromobenzene, acetaminophen, ethanol, and carbon tetrachloride.

XIV. Next Steps

Mr. Merenda thanked members for their service on the EDMVS and announced that EPA will be contacting members about the nomination process and status of the new committee.

Members were thanked for their time and the meeting adjourned at 12:00 p.m.

EDMVS Members in Attendance

- Bill Benson, EPA
- Mildred Christian, Argus International
- Theo Colburn, World Wildlife Fund
- Bob Combes, Fund for Replacement of Animals in Medical Experiments (via teleconference)
- Rodger Curren, Institute for In Vitro Sciences, Inc.
- Peter deFur, Center for Environmental Studies, Virginia Commonwealth University
- David Hatten, Food and Drug Administration
- Bob Kavlock, EPA
- Tim Kubiak, US Fish and Wildlife Service
- Gerry LeBlanc, North Carolina State University
- Joe Merenda, EPA
- Ron Miller, The Dow Chemical Company
- Susan Nagel, University of Missouri-Columbia
- Willie Owens, The Proctor and Gamble Company
- Tom Potter, US Department of Agriculture
- Shane Snyder, Southern Nevada Water Authority
- Jim Stevens, Wake Forest University School of Medicine
- Bill Stokes, National Institute of Environmental Health Sciences
- Glen Van Der Kraak, University of Guelph
- Charles Eldridge, Wake Forest University School of Medicine
- Penelope A. Fenner-Crisp, International Life Sciences Institute
- William Kelce, Pharmacia
- Dorothy Bowers, NACEPT Council

Attachment: A. Supporting Materials for the EDMVS

Attachment A

Background Materials for the EDMVS

December 10 - 12, 2003 Meeting

Docket – OPPT-2003-0064

Website: <http://www.epa.gov/scipoly/oscpendo/>

1. General Procedural

- 1.1 Draft Agenda
- 1.2 June 5 – 6, 2003 EDMVS Meeting Summary – Final
- 1.3 August 18 – 20, 2003 EDMVS Draft Meeting Summary
- 1.4 EDMVS Work Plan

2. Androgen Receptor Binding Assay

- 2.1 Presentation: Update on AR Binding Assay

3. Reference Chemicals

- 3.1 Presentation: Update on Reference Chemicals

4. Pubertal Male and Female Assays

- 4.1 Assessment of Pubertal Development and Thyroid Function in Juvenile Male CD® (Sprague-Dawley) Rats After Exposure to Selected Chemicals Administered by Gavage on Postnatal Days 23 to 52/53
- 4.2 Assessment of Pubertal Development and Thyroid Function in Juvenile Female CD® (Sprague-Dawley) Rats After Exposure to Selected Chemicals Administered by Gavage on Postnatal Days 22 to 42/43
- 4.3 Draft Report – Pubertal Toxicity Study of Vinclozolin, Flutamide and Phenobarbital in Male Sprague Dawley Rats and Methoxychlor, Ethinyl Estradiol and Phenobarbital in Female Sprague Dawley Rats when Administered in Corn Oil by Oral Gavage
- 4.4 Questions on Pubertal Assays for the EDMVS

5. Aromatase Assays

- 5.1 Aromatase Optimization Supplementary Studies – Experiment #1
- 5.2 Estrogen Production in Human Placental Results
- 5.3 Figure 9 – Graphic Presentation of Control Means and Standard Deviations
- 5.4 Figure 10 – Placental Aromatase Response Curves
- 5.5 Figure 11 – Recombinant Aromatase Assay Response Curves
- 5.6 Aromatase Assay Questions for EDMVS

6. Adult Intact Male Assay

- 6.1 Presentation: Introduction to the 14-Day Adult Male Assay

7. Fish Screening Assays

- 7.1 Presentation: Update on OECD Ecotoxicity Activities