

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 July 23 - 24, 2002.

TO: Dorothy Bowers, Chair
National Advisory Council for Environmental Policy and Technology
Office of Cooperation and Environmental Management
And
Gwen Whitt, Designated Federal Official
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: William Benson, PhD., Co-Chair
Endocrine Disruptor Methods Validation Subcommittee
Director, Gulf Ecology Division, NHEERL, ORD

Please find attached the minutes of the NACEPT Endocrine Disruptor Methods Validation Subcommittee fifth open meeting held in Washington, D.C. from July 23-24, 2002. This meeting summary covers discussions on 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.

Information about NACEPT EDMVS meetings and activities can be obtained from the website at <http://www.epa.gov/scipoly/oscpendo> or the OPPT Docket, OPPT 2002-0029 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:

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Margaret Schneider, OPPT
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OPPT Docket 2002-0029

**REPORT and MEETING MINUTES
OF
ENDOCRINE DISRUPTOR METHODS VALIDATION SUBCOMMITTEE MEETING
A Subcommittee of the National Advisory Council for
Environmental Policy and Technology
JULY 23 – 24, 2002
AT
RESOLVE, 1255 23RD STREET, N.W. SUITE 275
WASHINGTON, D.C.**

This meeting covered criteria for screening methods, core chemicals for prevalidation, general dose setting, the amphibian metamorphosis and the Invertebrate DRPs, special studies on pubertal restricted feeding, and mammalian 2-generation PTU study, a status report on in vitro ER/AR assays, and public comment.

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

**William Benson, PhD., Co-Chair
Endocrine Disruptor Methods
Validation Subcommittee under
The National Advisory Council for
Environmental and Technology
Date: _____**

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EDMVS Members in Attendance at the July 2002 Meeting

William Benson, Ph.D., Vice Chair
U.S. EPA (by phone only on 7/24)

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

Theodora Colborn, Ph.D.
World Wildlife Fund (by phone)

Ron Miller, Ph.D.
The Dow Chemical Company

Robert D. Combes, Ph.D.
Scientific Director, FAME

Susan C. Nagel
U. Missouri - Columbia

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

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

Peter L. deFur, Ph.D.
Commonwealth University
Service

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

J. Charles Eldridge
Wake Forest U. school of Medicine
Authority

Shane A. Snyder, Ph.D.
Southern Nevada Water

Penelope A. Fenner-Crisp, Ph.D.
ILSI Risk Science Institute

James T. Stevens. Ph.D.
Syngenta

David Hattan, Ph.D.
Food and Drug Administration

William Stokes, D.V.M.
NIEHS

Robert J. Kavlock, Ph.D.
U.S. EPA (acting chair 7/23)

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

Nancy K. Kim, Ph.D.
NY State Department of Health

Valerie Wilson, Ph.D.
Tulane-Xavier Universities

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

James D. Yager, Jr., Ph.D.
Johns Hopkins University

Facilitator

Paul De Morgan
RESOLVE

Designated Federal Official

Jane Scott Smith
Office of Science Policy and Coordination

Presenters

In Order of Presentation

July 23, 2002

Jane Smith, DFO
EPA, OSCP

Gary Timm
EPA, OSCP

Dr. Tammy Stoker
EPA, ORD

Dr. William Stokes
NIH, NIEHS

Dr. Paul Foster
NIH, NIEHS

Dr. Jack Bishop
NIH, NIEHS

Dr. Gary Wolfe
TherImmune Research Corp.

July 24, 2002

Dr. Les Touart
EPA, OSCP

Joe Tiege
EPA, ORD

Dr. Charles McKenney
EPA, ORD

Oral Public Comment

In Order of Presentation:

July 23, 2002

Rick Becker, Ph.D.
American Chemistry Council

Christopher Borgert, Ph.D.
Applied Pharmacology and Toxicology, Inc.
And the University of Florida

Ellen Mihaich
Rhodia

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, their second meeting in December 2001, and their third meeting in March 2002. The fourth meeting of the EDMVS was conducted as an international teleconference in June 2002.

The July 23-24, 2002 open meeting of the EDMVS was announced in the Federal Register on July 10, 2002 (Volume 67, Number 132). 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 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.

**National Advisory Council for Environmental Policy and Technology (NACEPT)
Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Fourth Plenary Meeting
July 23-24, 2002
DRAFT Agenda**

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

Meeting Objectives:

- Review of criteria, recommended by EDSTAC and adopted by EDSP, for screens;
- Provide update on the NICEATM estrogen and androgen receptor binding efforts;
- Discuss and provide advice on general dose setting issues; and
- Provide comments and advice on:
 - Pubertals – Special Study: Restricted Feeding (Tier I)
 - Mammalian 2-Generation – Draft PTU Special Study (Tier II)
 - Amphibian Metamorphosis DRP (Tier I)
 - Invertebrate DRP (Tier II)

Tuesday, July 23, 2002

9:00 – 9:10 Welcome and Opening Comments

Dr. William Benson, EDMVS Vice-Chair/Acting Chair, Office of Research and Development (ORD), EPA

9:10 – 9:30 Introduction, Agenda Review, and Review of Previous Meeting Summary

Paul De Morgan, Facilitator, RESOLVE

9:30 – 10:00 Review of EDMVS Work Plan

Jane Smith, EDMVS Designated Federal Official, Office of Science Coordination and Policy (OSCP), EPA

10:00 – 10:45 Criteria for Screens – Review the EDSTAC Recommendations

Gary Timm, OSCP, EPA

10:45 – 11:00 Break

11:00 – 11:15 Update on Core Chemicals Selected by EDSP

Jim Kariya, OSCP, EPA

11:15 – 12:30 Pubertals – Special Study: Restricted Feeding (Tier I)

Dr. Tammy Stoker, Reproductive Toxicology Division, NHEERL, ORD, EPA

12:30 – 1:45 Lunch

- 1:45 – 2:45 NICEATM Status Report**
Dr. William Stokes, NICEATM, NIEHS
- 2:45 – 3:30 General Dose Setting Issues**
Dr. Paul Foster, NIEHS
- 3:30 – 3:45 Break**
- 3:45 – 4:45 Mammalian 2-Generation – Draft PTU Special Study (Tier II)**
Dr. Jack Bishop, NIEHS
Dr. Gary Wolfe, TherImmune Research Corp.
- 4:45 – 5:30 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.
- 5:30 – 5:45 Setting the Stage for Day Two**
- 5:45 Adjourn for the day**

Wednesday, July 24, 2002

- 8:30 – 8:45 Settling In**
- 8:45 – 10:40 Amphibian Metamorphosis DRP (Tier I)**
Dr. Les Touart, OSCP, EPA
Dr. Joe Tietge, Midcontinent Ecology Division, National Health and Environmental Effects Research Laboratory (NHEERL), ORD, EPA
- 10:40 – 10:55 Break**
- 10:55 – 11:55 Invertebrate DRP (Tier II)**
Dr. Les Touart, OSCP, EPA
Dr. Charles McKenney, Gulf Ecology Division, NHEERL, ORD, EPA
- 11:55 – 12:30 Next Steps and Agenda for December Meeting**
- 12:30 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. This 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.

Endocrine Disruptor Methods Validation Subcommittee (EDMVS)

Fifth Meeting

July 23-24, 2002

Meeting Summary

On July 23-24, 2002, the U.S. Environmental Protection Agency (EPA) convened the fifth meeting of the EDMVS. The meeting objectives included:

- Review of criteria, recommended by EDSTAC and adopted by EDSP, for screens;
- Provide update on the NICEATM estrogen and androgen receptor binding efforts;
- Discuss and provide advice on general dose setting issues; and
- Provide comments and advice on
 - Pubertals – Special Study: Restricted Feeding (Tier 1)
 - Mammalian 2-Generation – Draft PTU Special Study (Tier 2)
 - Amphibian Metamorphosis DRP (Tier 1)
 - Invertebrate DRP (Tier 2)

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/edmvs.htm>. EPA has established an administrative record for this meeting under docket control number OPPTS- 2002 – 0029. The docket is available for inspection in the TSCA Nonconfidential Information Center, 1201 Constitution Ave. N.W., Washington, DC. The center is open from noon to 4 p.m., Monday through Friday, excluding legal holidays. The telephone number of the center is (202) 566-0280.

Tuesday, July 23, 2002

I. Welcome and Opening Comments

Robert Kavlock, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory (NHEERL), Office of Research and Development (ORD), EPA, welcomed the EDMVS members and the public to the meeting. Dr. Kavlock explained to the EDMVS that the acting chair, Bill Benson, would not be available until the second day and that EPA had asked him to serve as interim chair for the day.

II. Introductions, Agenda Review and NACEPT Update

Paul De Morgan, senior mediator with RESOLVE, introduced himself and asked the EDMVS members to identify themselves and their organizations.

Jane Smith, designated federal official for the EDMVS, explained that the meeting was being held in accordance with the Federal Advisory Committees Act (FACA) and all materials distributed would be available through the docket and on the website. She invited anyone experiencing problems with the website or other concerns to contact her.

Mr. De Morgan gave an overview of the materials distributed to the members and reviewed the meeting agenda. He noted that time was allotted for public comment at the end of the first day of the meeting. Mr. De Morgan then reviewed the meeting ground rules.

Mr. De Morgan asked EDMVS members to share their thoughts on the June 11 steroidogenesis conference call. Members commented that conference calls are an effective way to address specific issues. One suggestion was to investigate using an internet-based service to make presentations if subcommittee members need to review data during a conference call. Another member suggested that conference calls could be useful for discussing papers between meetings.

Valerie Wilson, EDMVS member and NACEPT representative, gave an update on NACEPT's review of the subcommittee's interim recommendation. She reminded the subcommittee that NACEPT must review and approve all EDMVS products. The committee reviewed EDMVS' interim recommendation from December 10, 2001 regarding the development of a white paper on the effects of using different strains of rats particularly for the pubertal protocol. Dr. Wilson proposed this interim recommendation to NACEPT at their July 18-19 meeting in Washington, DC. The committee discussed the role of EDMVS and their activities and approved the interim recommendation. Ms. Smith then explained that EPA has already approved a work plan for the development of the white paper, and work will begin in August.

Sherry Sterling, acting Office Director for the Office of Science Coordination and Policy (OSCP) explained that the office is still looking for a co-chair to replace Vanessa Vu and

work with co-chair William Benson. She welcomed comments from subcommittee members.

III. Review of EDMVS Work Plan

Ms. Smith thanked subcommittee members and contractors for their work and progress. She then reviewed the status of individual assays in tier 1 and tier 2. (As indicated above, copies of slides from Ms. Smith's presentation, "The EDMVS' Draft Work Plan," may be obtained from the docket or EPA website.) She noted that future EDMVS meetings and topics need to be approved by EPA management. Ms. Smith will contact EDMVS as well as post further work plan information on the EDSP website as soon as it becomes available.

IV. Criteria for Screens – Review the EDSTAC Recommendations

Gary Timm, OSCP, EPA, presented the EPA approach to screening criteria, as adopted following the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). (As indicated above, copies of slides from Mr. Timm's presentation, "Criteria for Screens – Review of the EDSTAC Recommendations," may be obtained from the docket or EPA website.) He reviewed the purpose of tier 1, the criteria for screens, the advantages of *in vitro* and *in vivo* assays, and the biological activity on which each screen focuses. He outlined several parameters suggested by EDSTAC for weighing the evidence provided by various assays:

- *In vitro* assays cannot be gatekeepers.
- *In vitro* assays that assess activity with and without metabolic activation are worth more than those without that mechanism.
- Results from *in vivo* assays have more weight than *in vitro* assays.
- Results from apical *in vivo* assays are worth more than results from specific *in vivo* assays.

Mr. Timm explained the conclusion, as reached by EDSTAC and subsequent international bodies focused on the subject of endocrine disruptor screens, that a battery approach including both *in vitro* and *in vivo* screens is necessary since no single assay can, at this time, meet all of the criteria for a tier 1 screen.

A member asked for clarification as to how contrary results from different assays, such as the Hershberger and the uterotrophic, would be weighed; and how a choice would be made between, for example, a sensitive, specific assay and an assay that could screen for a number of mechanisms. Another member commented more broadly that the parameter statements for weighing the evidence may or may not hold true as more is learned from prevalidation and validation efforts. Mr. Timm commented that EPA expects to gain a better understanding of the role of each assay when validation is complete and specific chemicals are examined. He noted that the parameters were proposed based on available information at the time with the intent that they would be modified as necessary based on the results of the validation process.

Mr. Timm clarified what the EDSTAC's recommendations for screening criteria means for both EPA and the subcommittee. He noted that EPA proposed accepting EDSTAC's recommendations in 1998 as a starting point, recognizing that the science could change. While EPA can choose to take a different direction, there must be a valid scientific reason to do so. Thus, in assessing tier 1 screens, EDMVS should use the existing criteria. A subcommittee member suggested that criteria should be updated as new information becomes available. For example, *in vivo* assays should not always be given more weight than *in vitro* assays; an *in vitro* assay using a human receptor may be better than an *in vivo* rat assay.

A member also commented on redundancy across assays, requesting that EPA evaluate on a case-by-case basis whether redundancy is necessary. He said that the three R's should be applied at the outset of testing.

V. Update on Core Chemicals Selected by EDSP

Gary Timm, OSCP, EPA, updated the subcommittee on the decisions made regarding core chemicals since the March 2002 EDMVS meeting. He noted that EPA's goal in developing a list of core chemicals is to compare the performance of assays. EPA is still conducting this review, and a list will likely be completed by the end of summer. A list of chemicals recommended for testing in estrogen and androgen receptor binding and transcriptional activation assays from the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) work group may help inform the agency's decisions. EDMVS members suggested that this topic would be appropriate for a conference call in early fall.

VI. Pubertals – Special Study: Restricted Feeding (Tier 1)

Tammy Stoker, Reproductive Toxicology Division, NHEERL, ORD, EPA, presented the EDMVS with the study objectives, methods, results, background literature references, and conclusions of the tier 1 pubertals restricted feeding study. (As indicated above, copies of slides from Dr. Stoker's presentation, "Assessing the Impact of Body Weight on Male and Female Pubertal Development," and "Table 1: Summary and references for background literature addressing correlations between food restriction and pubertal development" may be obtained from the docket or EPA website.) Dr. Stoker noted that these data were only recently generated and a paper on these data is underway. Study conclusions included that a 10% reduction in body weight has no effect on pubertal female development. Males seem to be a little more sensitive but there is still no significant difference. Pubertal protocols detect a wide variety of endocrine disrupting chemicals apart from modest decreases in body weight. Dr. Stoker also cited two previous studies (Connor et al., 2000 and Chapin et al., 1993) that supported the conclusion of this special study.

Discussion

Members considered and gave comments on the following question requested by EPA:

Does the EDMVS agree that a decrease in terminal body weight gain up to 10% compared to controls has no significant effect on the endpoints measured in the pubertal assays?

A member commented that the question is too broad and the data from this study had not been made available to the EDMVS members for review prior to the meeting. He suggested that the endpoints themselves should be investigated and that it would also be helpful to review a detailed review paper.

The subcommittee discussed the use of pair-feeding. Some suggested that when a decrease of body weight gain of 10% or more is anticipated, a pair-fed control may be needed to aid in the interpretation of data and to cover the comparison of pubertal effects. Another suggestion was that EPA should conduct range-finding studies in advance in order to have an idea of what the response will be and whether pair-feeding is necessary.

One member voiced a concern about the variety and range of endpoints being proposed for the pubertal study. In particular, vaginal opening and preputial separation are of concern because these endpoints appear to be variable in some laboratories and have not been fully explored in terms of body weight and covariance of body weight. Another member expressed concern with the conclusion that a 10% decrease in body weight has no effect on pubertal development. She noted that the LOAEL was 12.1% body weight change for females and 12.5% for males, while the NOAEL was around 4%. She commented that the boundary lies somewhere between 10% and 4%, but where is unknown. A member commented that no single study would prove that a 10% decrease in body weight would have no significant effect on endpoints. He said his organization completed a restricted feeding study with a different strain of rat and found similar effects. He offered to share the findings of the study with the EDMVS for comparison to NHEERL's study. Another member requested that EPA, as they go forward with this and other assays, share the protocols with EDMVS members.

VII. Selection of Dose Levels for Mammalian *In Vivo* Tier 1 Assays

Paul Foster, National Institute of Environmental Health Sciences (NIEHS), presented the EDMVS with a summary of how dose levels for tier 1 mammalian *in vivo* assays might be selected. (As indicated above, copies of slides from Dr. Foster's presentation, "Selection of Dose Levels for Mammalian *In Vivo* Tier 1 Assays: Discussion Points," may be obtained from the docket or EPA website.) He said that the 10% reduction in body weight could be used to indicate the maximum tolerated dose (MTD) when other information is unavailable. However, the 10% level should not be used as an absolute in making determinations. All available data should be used, and the judgment of investigators is also a critical component in setting dose levels for individual chemicals. When the MTD is known, it should serve as the basis for the doses selected. Further, the MTD should not involve clinical signs that are indicative of pain or distress. Dr. Foster also briefly discussed the up-and-down approach to setting doses, which

involves modifying dose levels based on observed toxicity.

A member asked how these dose setting methods compared to dose selection for the Hershberger and uterotrophic studies. Dr. Foster explained that the uterotrophic and Hershberger studies were done using chemicals for which there was enough other information on which to base doses. The methods discussed by Dr. Foster are employed only when little or no information is known about the chemicals.

Earl Gray, NHEERL, ORD, EPA, presented slides on a dose range finding study conducted following the approach outlined by Dr. Foster for an *in utero* study. He explained that the original doses were chosen based on what was known about the chemical and others in its class. Doses at the lower end of the curve were chosen, with the intent of adjusting up or down as needed as the study progressed. Two animals were used for each dose level. Primary endpoints were body weight and overt toxicity. Dr. Gray shared the results of the study, noting that a highly statistically significant reduction in the weight gain over the dosing period was observed. He concluded that for this particular case, using two animals each in five-dose groups provided sufficient information to set doses for a real study. He noted concern about sample size and statistical significance but questioned what additional information could have been gained from using five or ten animals per dose rather than two. He remarked that the animals from the study will continue to be monitored for reproductive endpoints.

Discussion

A member reiterated Dr. Gray's point, encouraging EPA to make the maximum use of all animals engaged in the studies. He suggested that blood and tissues could be archived in addition to the uterus.

Members considered and gave comments on the following questions, as requested by EPA:

1. *Does the EDMVS agree that a 10% decrease in terminal body weight compared to controls is appropriate as a definition of Maximum Tolerated Dose (MTD) when evaluating Tier 1 in vivo studies of endocrine activity?*
 - There should be flexibility in using this approach.
 - This can be the default position, but if other effects are occurring, those effects should be used as the basis for determining MTD.
 - MTD should also be adjusted based on clinical signs of overt toxicity. Whatever dose is selected as the MTD, animals should not be subjected to pain or stress.
 - Regarding wildlife tests, studies should take into account the expected exposure.
2. *Does the EDMVS agree that when little or no other information is available from which to estimate appropriate doses for a Tier 1 in vivo study, doses should be set via a range-finding study lasting up to the length of the proposed study, in which dose groups are adjusted depending on the presence or absence of adverse effects as soon as the effects are observed?*

- Depending on the materials being worked with and the duration of the treatment planned, the method of adjusting dose groups works as a possible approach. However, it should not be mandated. If this procedure is not used, a justification must be given.
 - Publishing guidance on the use of this approach would be helpful.
 - There are concerns with adjusting doses for pubertal and pregnancy studies.
3. *Does the EDMVS agree that dose groups of 2 to 5 animals are appropriate for such a range-finding study?*
- The goal should be to use the least number of animals possible rather than specifying the size of dose groups to be used in the study.
4. Does the EDMVS agree that dose-setting should be validated separately from validation of the assay protocols?
- It is critical that dose-setting procedures be evaluated as part of the validation process. If sufficient background literature is available to suggest an appropriate dose level, only one dose and 2-5 animals may be needed.
 - The criteria for setting the maximum dose must be considered. These criteria will not be the same in each case. Due to unknowns, some doses may need to be worked out chemical by chemical.
 - All labs should conduct the dose-setting procedure, with the management team choosing the final dose for the validation study.

Mr. De Morgan reminded subcommittee members that EPA will continue to consider these dose setting approaches. EDMVS members can submit suggestions to Jane Smith, who will distribute comments to the appropriate EPA staff.

VIII. Report on the ICCVAM-NICEATM Expert Panel Meeting on In Vitro ER/AR Assays

Mr. Timm shared a few comments to provide some background to the presentation by William Stokes, National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). Mr. Timm recalled that when the validation activities began to get underway EDSP staff met with NIEHS staff, including Dr. Stokes, to discuss how some of the workload might be shared between the programs. NIEHS suggested that validating the *in vitro* assays would fit well with the ICCVAM mission, and EDSP concurred.

Mr. Timm observed that it was felt at the time that enough data existed for the estrogen receptor (ER) and androgen receptor (AR) binding assays to be able to do a retrospective validation. He noted that the EDSTAC suggested it would be best not to focus on a single protocol for some of the assays, especially the reporter gene assays. He said it was felt that because so many different constructs and cell types were possible, it would make more sense to give criteria by which one could judge the adequacy of assays. The focus for NICEATM, therefore, was to survey the literature and develop performance criteria.

Before beginning his presentation, Dr. Stokes introduced his colleagues who joined by phone from NICEATM, Barbara Shane, Ray Tice, and Christina Inhof. He also clarified that NICEATM is part of the NIEHS. Dr. Stokes then presented the results of an ICCVAM/NICEATM independent expert review panel that met in May, 2002, noting that several EDMVS members served on the panel. (As indicated above, copies of slides from Dr. Stokes's presentation, "Report on the ICCVAM/NICEATM Expert Panel Meeting on *In Vitro* ER/AR Assays," may be obtained from the docket or EPA website.) He cautioned that his presentation would include only selected conclusions and recommendations of the panel, that the report was still being finalized so wording might change slightly, and that the opinions presented would be those of the independent panel and would not necessarily represent the official positions of NIEHS or ICCVAM.

Dr. Stokes highlighted the key steps in the ICCVAM evaluation of ER and AR *in vitro* methods. In 2001 a background review document (BRD) was prepared on each of the four types of assays: ER binding, ER transcriptional activation, AR binding, and AR transcriptional activation. The BRDs found no standardized methods with completed validation studies. The charge to the expert panel in May was to review the BRDs and provide conclusions and recommendations on 1) assays that should be considered for further evaluation in validation studies, and their relative priority, 2) adequacy of the proposed minimum procedural standards for each of the four types of assays, 3) adequacy of available protocols for assays recommended for validation studies, and 4) adequacy and appropriateness of the substances recommended for validation studies.

Dr. Stokes summarized the panel's recommendations for assay development and validation for each of the four types of assays. He also outlined some of the recommended minimum procedural standards for the assays and listed the proposed test substances for validation studies. He shared the panel's conclusions on the adequacy of the various protocols reviewed in the BRDs and highlighted some of the panel's general recommendations.

Following the presentation, Mr. Timm provided a status update on related EDSP activities. He noted that EPA would have to wait for the final report of the expert panel to make several decisions, but some activities have begun.

- Significant progress has been made toward validating the ER rat uterine cytosol assay.
- Retesting is underway on the AR rat prostate cytosol assay.
- Commercial sources of the recombinant human estrogen receptors and primate androgen receptors are being investigated but patent issues (e.g., availability and cost of patent protections) are causing problems.
- An AR reporter gene assay has been developed.
- EPA is staying apprised of relevant international activities.

Mr. Timm then asked for EDMVS members' comments and reactions.

Discussion

In response to questions, Dr. Stokes clarified the remaining steps in the process: the panel will finalize its report; NIEHS will solicit public comment on the report; ICCVAM will prepare an expanded evaluation report including the panel's report and public comments on it; and then the entire package will be made available to the public and to agencies.

A member who had participated in the expert panel observed that there may be some confusion in regard to the recommendation that a metabolic activation system not be included in any of the assays at this time. He emphasized the key phrase "at this time," commenting that the panel felt that logistical difficulties and insufficient information made it difficult to determine how metabolic activation systems could be included and that more research should be done.

The member also reported that the panel recommended that some, if not all, chemicals used in the *in vivo* assays should be used in the *in vitro* assays as well to begin to examine the specificity and sensitivity of the *in vitro* assays.

A member asked why so few chemicals that are negative *in vivo* were listed in the BRDs. Dr. Shane explained that they had sought to include an equal distribution of strong, intermediate, and weak binders, and negatives. She said that they found, however, very few compounds had been negative in every single *in vivo* assay in which they were tested. Compounds that were positive in some assays and negative in others were considered positive in the BRDs. The member commented that when the standards are set for the analyzing the *in vitro* assays there might be some minimal amount of binding that could be considered a negative in the assay as far as triggering a tier 2 test.

In response to questions about what would happen with the recommendations, Mr. Timm said that EPA would review the final report and decide which recommendations to act upon and which not to act upon. He said the decisions would be reported to the EDMVS for comments, but EPA had not planned to involve the EDMVS in the details of the protocols. He commented that the Organization for Economic Cooperation and Development (OECD) is establishing an *in vitro* validation management group (VMG). He said EPA will need to determine for which assays there is international interest in developing protocols and procedures, as it would make sense to go forward with those assays through the OECD forum. Members commented that they would need sufficient information on the *in vitro* assays to allow them to compare all the assays to construct the tier 1 battery.

Asked whether NICEATM would ultimately form a recommendation on which assays to use, Dr. Stokes said that the original request was to develop performance standards for each of these four types of assays. He explained that this would be done in a follow-up expert meeting expected to take place in 2003. The end product would be a list of chemicals with the type of outcome that would need to be achieved by a test system in

order for that system to be considered an acceptable method. Mr. Timm added that EPA did not intend to validate a specific system or recommend specific methods, though these decisions might be revisited. He said the intent was to allow a great deal of freedom for people to use methods with which they were familiar and to avoid proprietary issues.

A member commented that the performance standards would have to include sensitivity criteria. Dr. Stokes commented that EPA will need to determine the necessary level of sensitivity, noting a potential trade-off between the most sensitive assay and a less sensitive assay with more favorable attributes lending itself to high throughput.

Members discussed whether it was necessary for EPA to validate one method for each of the four types of assays. One member commented that EPA should standardize and validate one method for each so that intralaboratory and interlaboratory variability have been properly demonstrated. He expressed concern with issuing performance standards when no method has been properly standardized and validated to demonstrate sensitivity, specificity, and reliability. Another member expressed a concern about time frame and delaying the process. He noted that some of the assays considered in the report have been used reliably for over twenty years and it would be unreasonable at this point to say they are inadequate. Dr. Stokes commented that although many of the methods have been in use for years, laboratories are not using a single standard protocol for each method. He noted, however, that many of the companies that submitted data to NICEATM now have the procedural details and are anxiously awaiting the chemical list so that they can run the substances through their test systems. He said that with a repository of chemicals to facilitate the effort, the tests could be run very rapidly. Mr. Timm indicated that was how the process was originally intended to work: given criteria and a test chemical set, laboratories would self-validate.

The subcommittee discussed whether the EPA should standardize ER and AR transcriptional activation assays. A member commented that it is necessary to validate at least one method to determine that the performance criteria work. Another member noted that the ER rat uterine cytosol assay would be validated. Mr. Timm added that the AR rat prostate cytosol assay likely would be validated as well. A member suggested that a decision should be made to proceed with some prevalidation of the transcriptional activation assays. He added that the validation work would provide data to the overall evaluation of tier 1. Mr. Timm stated that ORD has developed AR transcriptional activation assay and is working on an ER transcriptional activation assay. Another member commented that the assay EPA validates will be the assay that laboratories are likely to choose, regardless of whether the performance criteria allow for other methods.

A member complimented NICEATM and the expert panel on their impressive effort and important thought process. He said effort served as a model for examining performance criteria and testing protocols. He commented that he voiced these opinions in support of the thinking that assays should be largely performance specified rather than prescriptive in procedural detail. Another member commented that even if the system is ultimately performance based, the validation process is important.

A member commented that eventually there will have to be a hierarchical scheme for use of assays if all of the chemicals that need to be tested for endocrine disruption are in fact to be tested. He noted that the *in vitro* assays will act as a very important first stage, perhaps along with quantitative structure activity relationship (QSAR) models and the priority-setting database. He commented that, therefore, determining the main receptors involved in the endocrine process is an important objective. He voiced support for validating protocols, particularly for the transcriptional activation assays. He commented that the new OECD VMG would be an ideal forum to pursue the development of other receptor binding and gene activation assays involved in endocrine disruption.

A member commented that the chemicals used to validate the uterine cytosol assay should be made known so that they can be used in validation of other assays.

Members discussed the need for a common repository of chemicals from which companies could draw. A member pointed out that analytical chemistry would need to be done on each chemical in the repository to determine purity and composition. He noted that off the shelf chemicals often are mixtures.

A member requested that the panel recommendations be summarized in tabular form for each of the four types of assays.

Mr. Timm said that one next step in the process will be to reconvene the panel to review the data generated for EPA on the ER and AR binding assays. He acknowledged that the real question will be what to do about the reporter gene transcriptional activation assay. Dr. Stokes reiterated that the panel would be reconvened in 2003 to comment on the proposed performance criteria and make recommendations. He said the panel would have to consider at that point whether sufficient data were available to address performance criteria for the transcriptional activation assays; if not, the criteria could be addressed at a later date.

Dr. Stokes thanked the EDMVS members for their feedback. Mr. De Morgan thanked the NICEATM staff for participating from Research Triangle Park.

IX. Mammalian 2-Generation – Draft PTU Special Study (Tier 2)

Ms. Smith announced that the draft report included a large appendix with all of the data from the special study. She noted that copies of the appendix were available at the meeting for anyone interested in seeing the data.

Before beginning his presentation, Jack Bishop, National Institute of Environmental Health Sciences, introduced Gary Wolfe and Larissa Nehrebeckyj, TherImmune Research Corporation, noting that they would be available to assist him in answering questions. (As indicated above, copies of slides from Dr. Bishop's presentation, "Two-Generation Reproductive Toxicity of Propylthiouracil When Administered to Sprague-

Dawley Rats in the Drinking Water,” may be obtained from the docket or EPA website.) Dr. Bishop explained that the purpose of the special study was to evaluate the ability of a two-generation reproductive toxicity study, including some added thyroid toxicity endpoints, for its potential to detect thyroid toxicants. Propylthiouracil (PTU) was selected as the test agent because it is a known thyroid hormone synthesis inhibitor and used as an antithyroid agent for the treatment of hyperthyroidism. The high dose, set at 0.0015 percent weight per volume, was expected to decrease T4 and possibly T3 and increase Thyroid Stimulating Hormone (TSH), alter thyroid pathology, and decrease growth. The mid dose (0.0004 percent) was expected to have lesser effects, and the low dose (0.0001 percent) was expected to have no effects.

Dr. Bishop outlined the study design and noted that the high dose was shown to alter the estrus cycle of the parent generation (F0) females. He said that exposures continued throughout gestation and lactation and various measurements were made on the first and second generation (F1 and F2) pups. He commented that the bodyweights of the high-dosed F0 animals were significantly depressed in an interval prior to cohabitation and noted that food and water consumption also were depressed.

Dr. Bishop outlined other significant findings. As he progressed through the data he explained that all of the F1 high-dose pups were lost at weaning because of a developmental problem that was not anticipated; therefore, there are no post-weaning F1 data and no F2 data for the high-dose group.

- In the high-dose group, the number of F1 female pups per litter was significantly decreased, as was the combined number of male and female pups.
- The anogenital distance for F2 males was significantly decreased, but the effect was due mostly to bodyweight changes and essentially disappears with a relative measurement.
- Several parameters of sexual development were delayed in the F1 high- and mid-dose groups.
- The T4 levels were decreased and the TSH levels were increased in F0 high-dose males, while the TSH levels were increased in the F0 mid- and high-dose females. The T4s were decreased and the TSH was increased in the F1 mid-dose group.
- A number of organ weight changes were observed. Most of the organ weight changes, except for thyroid, were related to growth retardation, as evidenced by decreased bodyweight.
- For the F0 males and females, thyroid weights increased in the mid- and high-dose groups, both in absolute terms and relative to body weight. For the F1 males and females, absolute and relative thyroid weights increased in the high dose groups. Relative thyroid weight increased in the F1 mid-dose males.
- Thyroid follicular hyperplasia was observed in some of the F0 mid-dose animals, in all of the F0 high-dose animals, and in one of the F1 mid-dose males.
- An unexpected increase in number of animals experiencing degeneration of testicular germinal epithelium was observed all the way down to the low dose.

Dr. Bishop closed with a summary of the key points drawn from the study data:

- PTU at 0.0015% in drinking water is a reproductive toxicant based upon reduction in F1 litter size.
- Thyroid hormones (T4 and TSH) were altered at both 0.0004% and 0.0015% PTU.
- Thyroid hyperplasia was noted at mid- and high-dose levels and degeneration of testicular germinal epithelium was noted at all dose levels.
- Degeneration of the testicular germinal epithelium was the only observable effects of PTU at 0.0001%.

Discussion

In response to questions, Dr. Bishop and Dr. Wolfe said that brain histology was not done for the pups, but indicated they did a section through the skull and an analysis of the pattern of the tooth eruption. The description, for the high-dose animals only, was domed heads and shortened snout, and also their incisors failed to erupt. Dr. Bishop noted that the description of the rats parallels fairly closely to what is referred to as a toothless mouse phenotype. He said that brain pathology was not done but analysis of a section through the skull showed nothing abnormal other than delayed mandibular development. A member noted that examining the brain would be important in evaluating neurodevelopment. Another member expressed concern that brain histopathology was not done to evaluate delayed development effects. Dr. Bishop explained that brain pathology was not part of the initial target of the study and that the study achieved its purpose without doing brain pathology. He acknowledged, however, that in retrospect it would have been interesting to save and examine the brains. A member clarified that the key objectives of the study were 1) to verify that thyroid endpoints could be incorporated into a standard two-generation study and could be detected with a known thyroid toxicant and 2) to confirm previous work done with a number of developmental parameters, but not the body and reproductive parameters. The member commented that the study succeeded in these objectives.

A member asked whether the researchers had any explanation as to why there was no dose-response relationship for the degeneration of the germinal epithelium. Dr. Bishop responded that they did not.

A member suggested reviewing the literature more thoroughly to verify the consistencies and inconsistencies of the findings of this study in relation to previous work.

A member noted that the drinking water industry already has developed its first draft reference dose of a thyroid compound (ammonium perchlorate). He suggested that the documents prepared during this by the expert panel might be useful to EPA.

Dr. Kavlock noted that a member who could not attend the meeting submitted comments to EPA in writing, which were distributed to EDMVS members.

Members considered and gave comments on the following question, as requested by EPA:

Does the EDMVS agree that the thyroid endpoints tested are necessary and sufficient for characterizing the adverse effects of thyroid-active agents in tier 2 testing?

- The thyroid weight and histopathology are quite sensitive. The data set indicates that degeneration of the germinal epithelium would be the most sensitive index of exposure.
- There is no basis for continued focus on the date of eye opening as it appears not to be a very sensitive assessment.
- The validation process should include examination of the variability of the hormone measurements to determine the exact time that they should be taken.
- The standard two-generation assay is expensive, and adding hormone endpoints will raise the cost further. Therefore, a rationale is needed for any endpoints that are added. Something should not be included simply because it is technically feasible. The T3 measurements, for example, may not be worthwhile. The substantial responses achieved through thyroid weights and histopathology may obviate the need for any hormone measurements.
- The range of neurodevelopmental measurements was not wide enough to provide sufficient information for choosing the best endpoint. Additional studies may be useful to provide information to determine the effectiveness of other measurements, such as auditory function tests of the pups. A more thorough literature review should be done to examine some of neurodevelopmental, auditory, and ocular-related endpoints.
- T4 should be included as an endpoint given the link with hypothyroidism and IQ in children whose mothers were hypothyroid during pregnancy.

A member commented that if protocols were distributed to the EDMVS before studies began members could provide helpful advice on endpoints. Mr. Kariya noted that the protocol for this study was developed before the EDMVS was formed.

X. Public Comment

At the conclusion of the day's deliberations, members of the public attending the meeting were given the opportunity to provide comments. Mr. De Morgan indicated that each person's comments would not be captured verbatim in the meeting summary, but rather just briefly summarized. He encouraged all to submit their comments in writing to Ms. Smith for inclusion in the EPA docket and posting on the website. (As indicated above, copies of slides from Dr. Becker, Dr. Borgert, and Dr. Mahaich's presentations may be obtained from the docket or EPA website.)

Rick Becker, American Chemistry Council (ACC)

Dr. Becker shared comments on dose-setting issues and chemical selection. He discussed the importance of flexibility in the range-finding study and adapting studies based on information gathered, such as with the number of animals used and routes of information. He asked that the range-finding study be included within the test protocol for the assay to which it is being applied. He also highlighted the importance of investigating the effects of systemic toxicants and other confounding factors when

developing and evaluating data. Regarding chemical selection, Dr. Becker reiterated comments previously sent to EPA by the ACC. He supported the development of criteria for selecting substances, the use of readily available substances with high specificity and a wide range of activities focused on estrogen, androgen, and thyroid. He also encouraged some level of overlap and choosing chemicals to increase the numbers of negatives. Finally, Dr. Becker encouraged EPA to reflect on EDSTAC's recommendations regarding communication and to coordinate with OECD to ensure international standardization and harmonization.

Christopher Borgert, Applied Pharmacology and Toxicology, Inc. and University of Florida

Dr. Borgert underscored the importance of standardizing at least one *in vitro* assay per hormone and using assays that are readily available. He agreed that recombinant receptor proteins for binding assays should be promoted. Dr. Borgert encouraged going beyond performance-based criteria and using a single methodology for each receptor once an assay has been validated and standardized. He also highlighted the need for defining criteria for each assay, including coefficients of variations, cell systems, and acceptable levels of cytotoxicity.

Ellen Mahaich, Rhodia, Inc.

Dr. Mahaich spoke about the mysid and the amphibian detailed review plans and studies. Dr. Mahaich questioned whether the amphibian metamorphosis assay is necessary and useful, as there is already a screen, using rats, that is intended to identify thyroid-active agents. She stressed that the rat is a sensitive and widely accepted vertebrate species, and taxonomic diversity in testing is only needed when there are differences in metabolic pathways or receptors. She said that data is needed to demonstrate the relevance of proposed endpoints to the thyroid activity, and considerable basic research would be required before even prevalidation of that assay could occur. Regarding the mysid life cycle study, Dr. Mahaich said that, until the relevance to the endocrine systems of invertebrates and to estrogen, androgen, and thyroid is demonstrated, resources would be better spent in developing assays known to be relevant.

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Bill Benson, Office of Research and Development (ORD), EPA and EDMVS acting chair was able to participate by phone for the second day of the meeting.

XI. Amphibian Metamorphosis DRP (Tier 1)

Dr. Touart explained that the methods used to prepare the Amphibian Metamorphosis Assay Detailed Review Paper (DRP) included an online literature search, inquiries of several experts to identify additional documents, interviews with several experts, and an external and internal peer review. (As indicated above, copies of slides from Dr. Touart's presentation, "Amphibian Metamorphosis Assay Detailed Review Paper," may

be obtained from the docket or EPA website.) He outlined the scientific basis for the assay:

- Amphibian metamorphosis is well understood and generally involves morphological, biochemical, and molecular changes.
- These changes result in resorption, remodeling, and creation of new tissues.
- Thyroid axis control of metamorphosis in amphibians involves the central nervous system, hypothalamus, pituitary gland, thyroid gland, thyroid hormone transport proteins, thyroid hormone receptors, and transcriptional elements.
- Many aspects of the thyroid axis are conserved among chordates at both the morphological and molecular levels, enhancing the use of an amphibian as a general vertebrate model for evaluating thyroid disruption.
- The Amphibian Metamorphosis Assay will be useful in evaluating thyroid perturbation.

Dr. Touart described the key characteristics, strengths, and weaknesses of the test species considered in the DRP: *Xenopus laevis*, *Xenopus tropicalis*, *Rana pipiens*, *Hyperolius*, *Urodeles*. He commented that routes of exposure include aqueous, dietary, and parenteral, noting that aqueous is the most prominent. He outlined key considerations for the potential exposure periods: from premetamorphosis through metamorphic climax (35+ days); from prometamorphosis through metamorphic climax (28+ days); during prometamorphosis (about 14 days); and during metamorphic climax (about 14+ days).

Dr. Touart listed the morphological, histological, biochemical and molecular endpoints considered in the DRP. He described the techniques used to measure some of these endpoints. He noted that the molecular/biochemical endpoints are expected to be the most sensitive and the morphological endpoints are expected to be the least sensitive.

Dr. Touart outlined the species selection criteria and reported that only *Xenopus laevis* met the minimum criteria. Candidate protocols included *Xenopus* 28-day full metamorphosis assay (proposed by OECD, 2001), 14-day metamorphic climax assay (as described in Fort et al., 2000), and 14-day prometamorphosis assay (as described by Tietge et al., personal communication). Dr. Touart described the 14-day prometamorphosis assay recommended in the DRP and outlined the data gaps implementation considerations identified in the DRP.

Joe Tietge, Midcontinent Ecology Division, NHEERL, ORD, EPA, shared information on the development of an amphibian metamorphosis model for detecting thyroid axis disruption. (As indicated above, copies of slides from Mr. Tietge's presentation, "Development of an Amphibian Metamorphosis Model for Detecting Thyroid Axis Disruption," may be obtained from the docket or EPA website.) He outlined the frog metamorphosis assay proposed by the EDSTAC for a tier 1 screen, noting that the simplicity of the assay has merit. EDSP asked the Midcontinent Ecology Division (MED) to review and perform the assay. Based on a literature search and some contract work, MED concluded that the EDSTAC-proposed assay was not ready for use as a screening tool. MED undertook research to address some of the problems identified

with the EDSTAC-proposed assay. Mr. Tietge commented that the research objective was to exploit the *X. laevis* model to develop a streamlined approach that could be used to identify chemicals that disrupt the thyroid axis of vertebrates. He explained that the research pursued two paths simultaneously, seeking to develop both a protocol appropriate for a tier 1 screen and a diagnostic model of thyroid disruption. Life stage and exposure duration studies were conducted to test two hypotheses: 1) Larvae in pre- and pro-metamorphosis transition are more sensitive to thyroid inhibition than pro-metamorphic larvae, and 2) longer exposure (14 day) duration will be more effective than shorter (8 day) exposure duration.

Mr. Tietge explained that the studies tested two developmental stages (51 and 54) and four model compounds (methimazole, 6-propylthiouracil (6-PTU), sodium perchlorate, and lipoic acid). The endpoints tested included morphology, thyroid histology, multiple gene expression, and thyroid hormone and related compounds. Mr. Tietge shared findings on the thyroid histology, which showed significant hypertrophy in at least the top four stages and follicular hypertrophy in the bottom stages. The three inhibitors with different modes of action (methimazole, 6-PTU, and perchlorate) all reduced morphological development. Mr. Tietge commented that mode of action is important because it provides better grounds for extrapolating to mammals. He summarized the findings of the studies:

- *X. laevis* is sensitive to three thyroid inhibitors using the 14 day prometamorphosis protocol.
- Stage 54 is as sensitive as stage 51 (i.e., the expected advantages of pre-metamorphosis were not observed).
- The 14-day exposure period is more sensitive than the 8-day.
- Thyroid histology is an essential component of the assay as it is more sensitive, diagnostic, and rapid than development.

Mr. Tietge stated the research question examined in the gene expression analysis: can multiple gene expression experiments reveal patterns indicative of the mode of action of thyroid agonists and antagonists? He described the strategy for the analysis and listed the genes included in the array, noting that a second gene array will be available in August. He commented that typically, T3, T4, and TSH are used to indicate thyroid axis status, but these measurements provide no information regarding synthesis or degradation and, therefore, make it difficult to assess mode of action. He explained that a novel analytical method using liquid chromatography-inductively coupled plasma/mass spectrometry (LC-ICP/MS) provides information on the synthetic and degradation pathways and may allow for full analysis of iodine status with a single analytical run. Mr. Tietge commented that he was not necessarily advocating the use of gene arrays but did see value in selecting four to five genes known to affect mode of action.

Mr. Tietge commented that future studies at MED using the 14-day prometamorphosis protocol will develop response data for T3, T4, estrogen, androgen, UDPGT inducer, deiodinase inhibitor, and receptor antagonist. He noted that future efforts will work to

integrate the indicator approach and the diagnostic approach to combine an apical effects screen and a mode of action screen into a single protocol.

Discussion

Before the discussion began, Mr. De Morgan noted that Doug Fort, Environmental Laboratories, Inc., would also assist in answering questions.

In response to questions Mr. Tietge made the following clarifying points:

- All the compounds tested caused hypertrophy in all test animals. The effects were visible grossly at higher concentrations.
- The only thyroid active chemicals that have been run through the protocol are the three reported in the presentation, though those studies were repeated at the single concentration for each in order to obtain the time course samples for the gene expression work.
- The small sample size effect on statistical significance that appeared in some of the results would decrease as exposure continued because the controls would progress further, giving a greater difference to evaluate.
- There is more information on the thyroid axis than on androgen and estrogen because the process of metamorphosis has been studied so extensively. Several laboratories are examining the interplay of different hormonal systems with the thyroid system. MED will include at least one androgen and one estrogen in its study.

Several members commented that the study was well designed. One member commented that he supported the approach of looking at morphological change as well as changes in histology of the thyroid gland, noting that having two endpoints would give greater mechanistic confidence that thyroid disruption is being observed.

A member commented that because there are no known environmental chemicals that would serve as receptor agonists for thyroid, it would be appropriate to design the assay to give greater consideration to mechanisms with antithyroid effects. The member also cautioned against giving an exogenous hormone (T3 and T4) to the animal to examine the agonist effect as the organisms have mechanisms to regulate the exogenous forms. Mr. Tietge explained that he was aware of the complicating factors. The member commented that the key point is that with respect to the T3 and T4 experiments, a negative result does not necessarily reflect poorly on the assay; it may be due to the inadequacy of the test substances.

A member commented that in developing the protocol for a tier 1 screen EPA should focus on what is sufficient to provide the necessary information. Mr. Tietge said that they now have a basic protocol that relies on morphology and histology that can be done, though more studies are needed before EPA would consider it ready. Dr. Touart added that the protocol is still in the early prevalidation stage and that as it moves toward optimization EPA will seek input from the EDMVS as to what is sufficient.

A member asked EPA to clarify the target and purpose of the amphibian metamorphosis assay. Another member noted that the results of these studies and the PTU studies do not allow a comparison of sensitivity between the two assays. Dr. Touart commented that one purpose of the studies was to respond to the EDSTAC recommendation to have an assay that is somewhat thyroid specific. He said the amphibian metamorphosis assay was viewed as beneficial because it would capitalize and exploit the metamorphosis-like stage of amphibians and the knowledge of thyroid. He noted that aspects of metamorphosis could likely be exploited for evaluating developmental abnormalities and developmental constants but commented that EDSP's intent is to exploit the metamorphosis for its utility as a thyroid screen. He said when EPA begins validation of the battery as a whole, one question will be whether the amphibian metamorphosis assay is necessary or the best option for evaluating thyroid, or whether something like the pubertal assay would be adequate and sufficient. He noted that other considerations would be whether the amphibian assay could cover effects other than thyroid and whether it covers those effects more cost efficiently than other assays. He said that information to address these questions will be gathered as the various protocols are optimized and more chemical experience is gained. A member commented that the studies must show what value the amphibian assay can add to the battery that cannot be gained through the mammalian assays. He stressed that the goal is simple and cost effective assays.

A member expressed concern about including gene expression in a screening assay. Mr. Tietge commented that the primary purpose of the gene expression study is to get a better understanding of mode of action, with a goal of developing the relationships of the gene expressions to the endpoints. Dr. Touart commented that the information would help in identifying and narrowing the utility of the endpoints. He said that the information gained from the study may identify some particular genes that could be easily incorporated in the assay in some way to enhance its utility, or it may show that gene expression is too complex for a tier 1 screen. The member commented that he could see no way to include gene expression in the validation study in the foreseeable future. Mr. Tietge acknowledged the practical difficulties. Another member encouraged EPA to pursue gene expression data, at least from a research perspective. A third member added that it could prove to be a powerful tool for establishing mechanisms. He suggested also adding genes that might provide information on androgen and estrogen regulation. A member suggested including a vitamin panel to try to gain information from a long-term chronic perspective.

A member commented that some developmental toxicants that cause growth inhibition act by thyroid mechanisms while others do not. He suggested including some of the non-thyroid-mechanism chemicals in prevalidation studies to help determine whether they can be differentiated from those acting by thyroid mechanisms. Mr. Tietge said they had considered doing such a study and would welcome suggestions of specific chemicals to test. The member referred him to a background review document from a May 2000 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) expert panel meeting that lists chemicals that may cause growth inhibition.

A member asked whether there was a way to gauge thyroid effect other than through histology. Mr. Tietge said he had thought about that question and had experimented with various ideas but in the end decided that histology is the best semi-quantitative approach. Another member commented that histology looked promising and would be a good place to focus effort.

Members considered and gave comments on the following questions, as requested by EPA:

1. *Does the EDMVS agree that the Prometamorphosis Assay with Xenopus laevis is the appropriate amphibian assay to recommend for further implementation?*

A member noted that *X. laevis* would not be used for a chronic assay. He commented that if EPA intends to include a chronic assay the species chosen for the screening assay should be the same as the one chosen for the chronic assay to minimize husbandry challenges and costs. Dr. Touart responded that EPA is considering a chronic version of the assay and considering *Xenopus tropicalis* as the test species. He noted that *X. tropicalis* has a diploid gene and matures much more quickly than *X. laevis*. He said that hope is that *X. tropicalis* could be used in both assays, but the husbandry for *X. tropicalis* has not yet been sufficiently determined to use it in the studies underway at MED. Mr. Tietge confirmed that MED would likely switch to *X. tropicalis* in the long term and commented that progress is being made on feasibility, though some challenges remain. He noted also that MED is collaborating with the University of Victoria on native-species comparative studies. A member commented that an expert panel of the ICCVAM convened in 2000 to consider test species for a developmental toxicity screen recommended pursuing *X. tropicalis*. He said the top priority should be to resolve the husbandry and disease issues. He added that unless problems are identified in extrapolating from *X. tropicalis* to the North American species, using a nonnative species should not be an issue.

A member commented that the assay may be starting too late and suggested that EPA consider extending the study at the beginning to test for developmental effects. Mr. Tietge responded that it may be reasonable to test for developmental effects but the goal of the research was to examine how to evaluate chemicals that affect thyroid function. Noting that on a technical level there is probably no relationship between chemicals that affect thyroid development and chemicals that interfere with thyroid function, Mr. Tietge commented that evaluating developmental effects would probably require a completely different approach from evaluating functional effects. Another member commented that the tier 1 screens are intended to evaluate thyroid hormone activity, while developmental toxicity will be evaluated in tier 2.

A member commented that the rationale for using this animal model as a general screen for thyroid activity was not clearly demonstrated. He said that if the assay is intended only for redundancy, it is not justified. The rationale that EDSTAC used, the member recalled, was that this was purely going to be a redundant exercise. He requested that EPA decide soon on the relative merits of the assay compared to the

mammalian screens and suggested that this decision should be made before significant effort is expended on the diagnostic approaches, the biochemical approaches, or the gene arrays. Another member pointed out that EPA's statutory responsibilities include a wide variety of species. He noted also that studies may show that the amphibian assay is effective at evaluating androgen and estrogen effects thereby obviating the mammalian model. A member added that research might lead to a tool that is useful for ecotoxicity studies in the field, regardless of whether the assay is finally chosen for the battery.

A member referred EPA to the minutes and background review document of the 2000 ICCVAM expert panel meeting for comments on minimizing data variation for both the tier 1 screening protocol and tier 2 testing protocol.

2. *Does the EDMVS agree that prevalidation efforts should be phased as described in the DRP?*

A member commented that the program seems to be a long way from prevalidation and is rather in the stage of test development.

Mr. De Morgan noted that members did not explicitly comment on all the questions posed by EPA. He encouraged members to submit written comments if they wanted to make specific points on any of the questions.

XII. Invertebrate DRP (Tier 2)

Dr. Touart presented an overview of the Mysid Life Cycle Detailed Review Paper (DRP). (As indicated above, copies of slides from Dr. Touart's presentation, "Mysid Life Cycle Detailed Review Paper," may be obtained from the docket or EPA website.) He said that the DRP was based on an on-line literature search and underwent internal and external peer review. He summarized the scientific basis of the mysid life-cycle toxicity test:

- Estuaries, which are important ecosystems, are among the earliest recipients of endocrine disrupting chemicals (EDCs).
- Crustaceans are often among the most abundant and most sensitive organisms (particularly mysids) in estuaries and they form vital links in food webs.
- Many insecticides are considered putative EDCs. Certain insecticides formulated as IGRs adversely affect crustaceans by disrupting molting and metamorphosis.
- The endocrine system of an invertebrate differs from that of a vertebrate; therefore, the response of an invertebrate to an EDC could be expressed differently.
- Ecdysteroids, which are the molting hormones in mysids, are also involved in the control of reproduction and embryogenesis. Mysids show promise as a potential indicator for evaluating ecdysteroid and EDC interaction.

Dr. Touart summarized key characteristics, strengths, and weaknesses of the four test species: *Americamysis bahia*, *Holmesimysis costata*, *Mysidopsis intii*, and *Neomysis integer*. He noted that routes of administration of chemical exposure include aqueous, sediment, and dietary uptake. Potential measurements of endpoints include survival,

molting frequency, growth, measures of reproductive performance, and biochemical measures.

Dr. Touart outlined the four candidate protocols examined in the DRP: ASTM E1191, OPPTS 850.1350, Chapman, and an EPA-supported study by Langdon, et al. The protocol recommended in the DRP is a blending of the EPA and ASTM protocols using *Americamysis bahia* as the test species. It is a two-generation protocol that measures seven reproductive endpoints and potentially five biochemical endpoints.

Dr. Touart noted some of the data gaps identified in the DRP. He said that prevalidation studies will seek to determine specific information on longer testing duration and on incorporating a second generation. Efforts regarding biochemical measurement endpoints will include refining steroid metabolism endpoints or mysids, studying cytochrome P540 enzyme level, and conducting vitellogenin mechanistic studies to confirm endocrine disruption versus metabolic toxicity. Dr. Touart commented that the final goal is to determine whether specific endpoint responses can be linked to different classes of compounds affecting ecdysteroid, androgen, or other hormonal cycles.

Charles McKenney, Gulf Ecology Division, NHEERL, ORD, EPA, presented some of the advantages of a two-generation mysid protocol. (As indicated above, copies of slides from Dr. McKenney's presentation, "Advantages of a Two-Generation Mysid Protocol," may be obtained from the docket or EPA website.) He summarized the lifecycle of *Americamysis bahia*, noting that work has focused on this species. He commented that the most sensitive response to pesticide exposure has been decreased production of young. He noted that the present lifecycle exposure is postdevelopmental, beginning with juveniles, rather than developmental, beginning with embryos or larvae. The exposure therefore measures activational and reversible effects but not organizational and irreversible effects. A two-generation protocol would include developmental exposure.

Dr. McKenney explained that work on the crustaceans can take advantage of existing knowledge of the effects of juvenile hormone agonists on insects since the lifecycle process is similar in some crustaceans and involves some of the same chemicals. He noted also that the sensitivity of crustacean species to test substances is similar to that of mosquitoes and much higher than that of fish and rat species.

Dr. McKenney outlined the endocrine regulation of crustacean reproduction and growth. He noted that development of glands is associated with larval development and, therefore, any impact on glands would be expressed in the second generation and missed in the present standardized protocol. He reported that preliminary observations of the two-generation approach indicate that an altered sex ratio in the second generation is observed at concentrations below those for which effects are detected in the parents.

In closing, Dr. McKenney observed that crustaceans play a critical role in the ecosystem as energy producers for higher trophic levels. He commented that altered development,

growth, and reproduction of crustaceans by EDCs would restrict their role as dominant secondary producers.

Discussion

Several members commented that the mammalian tier 1 assays should not be relied upon to screen for invertebrate effects. Some questioned how relevant the program could be for ecotoxicological risk. One member argued that there is probably no rationale for extrapolating from invertebrates to other phyla. He added that if the tier 1 screens are not appropriate triggers, then those phyla are unprotected. He commented that large research gaps exist for invertebrates, noting in particular the mechanistic gaps to be filled in the invertebrate lipid tree. Dr. McKenney acknowledged data gaps but noted that there are fewer gaps for anthropods. Dr. Touart commented that the central question is what is the role of the mysid assay in tier 2. He indicated that one purpose is to gather information to allow a more comprehensive ecological risk assessment. He said it was thought that the mysid lifecycle protocol could satisfy this purpose, and EPA is now examining whether including a second generation adds valuable information and whether additional compounds should be tested. The member voiced support for trying to expand the assay to address the shortcomings of the present protocol. He repeated, however, that validating the battery requires scientific rationale, and there is no rationale for EAT assays applying to invertebrates.

Another member expressed concern with the inadequacy of the tier 1 assays to screen for hormone groups important to invertebrates. He suggested that it would be wise for EPA to consider a screening battery to cover a wide array of phyla rather than just including an invertebrate assay in tier 2 to test where EAT-active substances have other hormonal effects. Dr. McKenney suggested that the two-week crustacean metamorphosis assay could serve as a relatively quick and simple assay to cover invertebrate effects in tier 1. The member added that he previously submitted comments to EPA with suggestions for invertebrate assays.

A member commented that considering the several unique characteristics of invertebrates suggests a need to expand the tier 1 battery. He questioned, however, how all the phyla could be covered and what could be done with the information if they were. He suggested instead finding the most sensitive species and regulating based on them. Dr. McKenney suggested that the key factor to consider is the sensitivity of the functional role of the species in the ecosystem, such as crustaceans' critical role in ecosystem productivity. A member said that a year and a half ago the U.S. Fish and Wildlife Service and the National Marine Fisheries Service worked together to provide a list of 676 species for which EPA was going to address the 45 aquatic-life criteria of the Endangered Species Act. He noted that the list would be fairly representative of species at risk and could provide ideas of appropriate invertebrate groups and families for establishing priorities to examine.

A member commented that he failed to see the rationale in testing species for which hormones of interest are not known. He observed that the program being developed by

EPA is for EAT and commented that the mysid species does not have EAT relevance. He agreed that when invertebrate tests are invoked they need to have been triggered by appropriate screens but stressed that this is not the program for such screening and testing. Another member commented that a broader program is important because EPA has responsibilities with respect to other laws, including the Endangered Species Act. He noted that the EDSTAC assumed that EPA would make a policy jump in the EDSP to “functional equivalence,” looking at hormones in invertebrates that are functionally equivalent to EAT in vertebrates.

A member requested that EPA provide a table that lays out the potential tier 2 assays like the table Mr. Timm presented summarizing the potential tier 1 assays.

A member advised that as the assay progresses researchers should consider how often the organisms need to be monitored to catch the effects of antiectosteroids. He explained that they may delay molting by a matter of hours, so the effect could go undetected if the organisms are checked daily.

Mr. De Morgan referred members to the four questions EPA posed to them, noting that some of the points already raised in the discussion addressed aspects of the questions. He observed that in regard to the first question (Does the EDMVS agree that the two-generation method recommended with *Americanysis Bahía* is appropriate?), the comments thus far indicated that the proposed approach for the assay is generally sound but some members question the assay’s relevance to the EDSP purpose.

2. *Does the EDMVS agree that prevalidation should evaluate the increased sensitivity of a two-generation design over the existing one-generation standard practice?*

A member commented that it would be critical to evaluate the increased sensitivity and to demonstrate with data the value of a two-generation assay.

3. *Should EPA explore the feasibility and utility of biochemical endpoints, as described in the DRP, for possible addition to the recommended protocol?*

A member advised that biochemical endpoints should be a low priority due to the difficulties of interpreting the data and the possibility that the endpoints have nothing to do with endocrine disruption.

Another member commented that the DRP was clear that the endpoints could be observed, but it was not clear on the relevance of these endpoints. He asked that as the DRP is revised it be made as clear as possible about the justification for including the endpoints.

4. *Does the EDMVS have suggestions to improve the DRP?*

A member commented that the DRP should be augmented with current literature, including Dr. McKenney's work. Dr. McKenney acknowledged that there has been a recent "explosion" of information in this area and the DRP should perhaps be revisited.

A member suggested adding a chart to explain the constellation of hormonal activities.

XIII. Next Steps and Agenda for Third Meeting

Before the meeting adjourned on July 24, EPA staff presented a list summarizing the key points and potential action items they had drawn from the subcommittee's discussions. See attachment A.

Future Meeting Dates

- The next EDMVS meeting is scheduled for December 4-6, 2002.

XIV. Closing Remarks

Dr. Benson thanked the EDMVS members and the public for attending and thanked the speakers for their presentations.

Attachment:

Attachment A EPA Reflections

Slide 1:

Comments from EDMVS on restricted feeding study

- Difficult to evaluate the study without time to examine the data in advance, but generally no major problems with study.
- Effects at 12% reduction in BW gain (RBWG) make a conclusion of no effect at up to 10% questionable.
- Another unpublished study seems to confirm no RBWG effects on PPS, but there were other effects on reproductive organs (epididymal and SV weights). (VO not tested.) Study to be distributed to EDMVS.

Slide 2:

Restricted feeding study (2)

- Much discussion on need for pair-feeding:
 - Not needed if RBWG < 10%
 - Not clear how to interpret results if MTD is defined as 10% RBWG
 - Not a problem if EPA requires only one dose in the pubertal because controls can be pair-fed.

Slide 3:

Dose-setting issues

- MTD should not be based solely on RBWG. Clinical signs can be the basis. Stay below signs of pain and distress.
- Use minimum number of animals commensurate with need, usually in the range of 2 to 5.
- Flexibility in dose-setting methods is needed. Up/down procedure is fine for longer-term assays. Acute data may suffice for short-term assays.

Slide 4:

Dose-setting issues (2)

- Use all possible sources of data when guessing at initial dose (e.g., HPV data), and consider using in vitro data if no other info is available.
- Be aware of the PK and other differences that can arise by mandating gavage as route of exposure.
- “There is no substitute for good judgment.”

Slide 5:

Dose-setting issues (3)

- Dose-setting must be part of protocol validation, but there is also a need to know what happens when many labs use identical doses.
- For an unknown chemical in validation, it might be reasonable to have all labs do dose-setting independently, with a committee to consider all the results and decide on doses to test in the assay per se.

Slide 6:

Thyroid endpoints in mammalian 2-generation assay

- T3 not useful; thyroid weight useful
- T4 very useful vs. T4 not helpful beyond weight and histopath
- Consider other endpoints (e.g., auditory, ocular) for thyroid effects on neurodevelopment
- Look at what expert review for perchlorate RfD said were useful endpoints for thyroid.
- Follow up on the germinal epithelium effects

Slide 7:

Criteria for Screens

- In response to concerns expressed by the EDMVS it should be noted that:
 - The composition of the Tier 1 battery and weight-of-evidence guidelines will be updated based on experience with the assays in validation.
 - The desire for redundancy of endpoints in separate assays in the battery will be weighed against cost and animal welfare concerns.

Slide 8:

ER & AR Binding Assays

- EPA will proceed with the validation of the ER uterine cytosol and AR prostate cytosol binding assays developed by EPA taking note of the recommendations for challenge chemicals by the NIEHS expert panel.

Slide 9:

ER & AR Binding Assays

- Although EPA regards the ERTA and ARTA as assays in the research stage (the BRDs could not recommend a protocol or assay system for prevalidation studies) EPA is making substantial progress in the development of stably transfected assays for both ER and AR. EPA will standardize these assays and investigate options for their validation including partnerships with industry and other countries.

Attachment B
Supporting Materials for the EDMVS
July 23-24, 2002 Meeting
Docket – OPPT-2002-0029
Website: <http://www.epa.gov/scipoly/ospendo/edmvs.htm>

1. **General Procedural**
 - Proposed Agenda
 - March 25-27 2002 EDMVS Final Meeting Summary
 - EDMVS Work Plan, revised
 - EDMVS' Draft Work Plan (95 Kb Power Point File)
 - Criteria for Screens Presentation – Review the EDSTAC Recommendations
 - Criteria for Screens Presentation – Review the EDSTAC Recommendations (54 Kb Power Point File)

2. **Pubertals - Special Study: Restricted Feeding (Tier I)**
 - Pubertals - Special Study: Restricted Feeding Findings (Report will be made available upon finalization)
 - Pubertals – Special Study: Restricted Feeding Questions
 - Summary and References for Background Literature Addressing Correlations between food restriction and Pubertal development.
 - Assessing the Impact of Body Weight on Male and Female Pubertal Development (354 Kb Power Point File)

3. **ICCVAM-NICEATM - In Vitro ER/AR Assays**
 - Estrogen Receptor Binding Assays – Executive Summary
 - Androgen Receptor Binding Assays – Executive Summary
 - Estrogen Receptor Transcriptional Activation Assays – Executive Summary
 - Androgen Receptor Transcriptional Activation Assays – Executive Summary
 - ER & AR Assay: Initial Assumptions (14 Kb Power Point File)
 - Report on the ICCVAM / NICEATM Expert Panel Meeting on In Vitro ER/AR Assays (238 Kb Power Point File)

4. **General Dose Setting Issues**
 - Dose Setting Discussion Paper
 - General Dose Setting Questions
 - Selection of Dose Levels for Mammalian in Vivo Tier I Assays: Discussion Points (41 Kb Power Point File)

5. **Mammalian 2-Generation – PTU Special Study (Tier II)**
 - Mammalian 2-Generation – PTU Special Study Report
 - Mammalian 2-Generation – PTU Special Study Questions
 - Final Report: Two-Generation Reproduction Toxicity Study of PTU when Administered to Sprague-Dawley Rats in the Drinking Water (1.2 Mb PDF) (Note: this PTU Special Study has an appendix that is a 3 Mb Acrobat (PDF) file available upon request.)
 - Two-Generation Toxicity of PTU when Administered to Sprague-Dawley Rats in the Drinking Water (393 Kb Power Point File)

6. **Amphibian Metamorphosis Detailed Review Paper (Tier I)**
 - Revised Amphibian Metamorphosis Detailed Review Paper
 - Amphibian Metamorphosis Questions
 - Amphibian Metamorphosis Detailed Review Paper (95 Kb Power Point File)

- Detailed Review Paper: Amphibian Metamorphosis Assay for Endocrine Disruption (745 Kb Power Point File)
- Development of an Amphibian Metamorphosis Model for Detecting Thyroid Axis Disruption (45 Mb Power Point File)

7. Invertebrate Detailed Review Paper (Tier II)

- Invertebrate Detailed Review Paper
- Invertebrate Questions
- Advantages of a Two-Generation Mysid Protocol (10.5 Mb Power Point File)
- Mysid Life Cycle Toxicity Test (400 Kb Power Point File)