Summary Minutes

Peer Review Panel Public Meeting
***In Vitro* Methods for Estimating Starting Doses for Acute Systemic Toxicity Testing**
National Institutes of Health (NIH), Natcher Conference Center
Bethesda, MD

May 23, 2006
8:30 a.m. – 5:00 p.m.

Call to Order

Dr. Katherine Stitzel (Panel Chair) called the meeting to order at 8:30 a.m. and asked all Peer Panel members, National Toxicology Program Interagency Center for the Evaluation of Acute Toxicological Methods (NICEATM) staff, members of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the ICCVAM Acute Toxicity Working Group (ATWG) in attendance, and members of the public to state their name and affiliation for the record. She requested that all individuals identify themselves when they spoke and to use the provided microphones. She stated that two public comment periods would be held during the meeting and asked that individuals who wanted to speak, other than those who had pre-registered, to register at the registration table.

Welcome from the Director, NICEATM and Conflict of Interest Statements

Dr. Stitzel introduced Dr. William Stokes, the director of NICEATM. On behalf of the NIEHS and NICEATM, Dr. Stokes welcomed everyone and thanked the participants for agreeing to serve on the Panel. Dr. Stokes stated that he would serve as the Designated Federal Official for the public meeting. He stated that the meeting was being held in accordance with the Federal Advisory Committee Act (FACA) regulations and that the Panel is constituted under the NIH Special Emphasis Panel charter. Dr. Stokes read the conflict of interest statement and asked the Panel members to indicate if they had any conflicts and to recuse themselves from discussion and voting on any aspect of the meeting for they had any conflict. Dr. Daniel Wilson of the Dow Chemical Company stated that his company produces a number of chemicals used in the validation study, but that he did not consider this to constitute a conflict of interest.

Welcome from the ICCVAM Chair

Dr. Leonard Schechtman, U.S. Food and Drug Administration, Chairman of ICCVAM, welcomed everyone on behalf of ICCVAM. He expressed his appreciation for the Panel's willingness to participate in the peer review process and requested input from the Panel on *in vitro* methods for use in estimating the starting dose for acute toxicity testing. He thanked NICEATM staff and the ATWG, and other ICCVAM members for their efforts in developing the materials and draft recommendations being considered at this peer review meeting. He said that the Panel's report will used by ICCVAM in finalizing its recommendations.

Overview of the ICCVAM Test Method Evaluation Process and Charge to the Panel

Dr. Stitzel asked Dr. Stokes to provide an overview of the ICCVAM test method evaluation process. He stated that the international Panel was made up of 16 scientists from six countries

(United States, United Kingdom, Canada, Japan, Germany, and Italy). He described the 15 ICCVAM agencies and reviewed ICCVAM's history and development. Dr. Stokes summarized the preamble of the ICCVAM Authorization Act and detailed the purpose and duties of ICCVAM as prescribed by the Act. He noted that one of ICCVAM's duties is to review and evaluate new, revised and alternative test methods applicable to regulatory testing. Dr. Stokes described the role of NICEATM in conducting validation studies when funds are available. He stated that all of the reports produced by NICEATM are available from the ICCVAM-NICEATM website or directly from NICEATM.

Dr. Stokes stated that validation is performed to determine the usefulness and limitations of a test method for a specific purpose. He continued by stating that validation is defined by ICCVAM as the process by which the *reliability* and *relevance* of a procedure are established for a specific purpose and that adequate validation is a prerequisite for Federal regulatory acceptance. He listed the ICCVAM criteria for test method validation and acceptance. Dr. Stokes explained that acute toxicity testing was necessary to evaluate and classify the hazard potential of acute single exposures to substances. He stated that poisoning is the second leading cause of injury-related death in the United States.

Dr. Stokes briefly reviewed the ICCVAM International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, which was held in October 2000. The overall goal of the Workshop was to review the then current status of using *in vitro* testing for predicting acute oral toxicity. The workshop recommended that a near-term goal should be to reduce animal use for acute systemic toxicity assays by using *in vitro* methods to estimate starting doses. A long term goal should be to replace animal use with *in vitro* methods that can predict human acute systemic toxicity using human cells and tissues. In addition to a Workshop Report (ICCVAM. 2001. Report Of The International Workshop On In Vitro Methods For Assessing Acute Systemic Toxicity. NIH Publication No. 01-4499. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: http://iccvam.niehs.nih.gov/methods/invitro.htm.) A Guidance Document was also published (ICCVAM. 2001. Guidance Document On Using In Vitro Data To Estimate In Vivo Starting Doses For Acute Toxicity. NIH Publication No. 01-4500. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: http://iccvam.niehs.nih.gov/methods/invitro.htm.). This document also provided two standardized *in vitro* basal cytotoxicity protocols that were the basis for those used in the NICEATM/European Centre for the Validation of Alternative Methods (ECVAM) validation study. As a result of the workshop, ICCVAM made recommended that additional research and development should be conducted to develop the *in vitro* systems, in addition to basal cytotoxicity, that will be necessary to accurately predict acute toxicity without animals (e.g., those that can predict absorption, distribution, metabolism, excretion [ADME] and target organ toxicity). The ECVAM-sponsored A-Cute-Tox project is currently working to develop these *in vitro* test systems that will be necessary to develop this strategy.

Charge to the Panel

Dr. Stokes presented the timeline for conduct of the NICEATM/ECVAM validation study and he then reviewed the charge to the Panel: 1) review the BRD for omissions and errors; 2) evaluate the extent to which each of the applicable criteria for validation and acceptance

have been adequately addressed for the test methods and their specific proposed use; and 3) comment on the extent to which the draft ICCVAM test method recommendations are supported by the information provided in the BRD.

Dr. Stokes presented the rosters for the Peer Panel, ICCVAM, ATWG, and NICEATM and acknowledged the three laboratories that participated in the study: 1) U.S. Army Edgewood Chemical Biological Center, 2) Fund for the Replacement of Animals in Medical Experiments [FRAME] Alternatives Laboratory [FAL] and 3) Institute for *In Vitro* Sciences.

Overview of Acute Oral Toxicity Regulatory Testing Requirements, Hazard Classification Schemes, and the Current Acute Oral Toxicity Regulatory Testing Procedures

Dr. Amy Rispin presented the U.S. statutes and regulations requiring acute oral toxicity testing. She emphasized the use of the three Organization for Economic Cooperation and Development (OECD) Acute Oral Toxicity Test Guidelines (TG 425, TG 423, TG 420) that can be used to meet these test requirements. She stated that acute toxicity has been one of the longest standing areas of regulation in the United States and Europe. Regulatory applications include classification and labeling, risk assessment (key area emphasized by the U.S. Consumer Product Safety Commission [CPSC]), and risk management. Applications of acute toxicity testing have driven obligatory use of protective clothing and other improvements in safety with respect to potential chemical exposures. She stated that the United States is in an active transition period along with the rest of the world toward using the United Nations (UN) Globally Harmonized System (GHS) of Classification and Labelling of Chemicals for product labeling. Dr. Rispin described the current hazard classification systems of various regulatory authorities (i.e., U.S. Environmental Protection Agency [EPA], European Union [EU], U.S. CPSC, U.S. Department of Transportation [DOT], UN GHS).

With regard to test methods for acute toxicity testing, Dr. Rispin provided descriptions of the Up-and-Down Procedure (UDP) Limit test, the UDP Main test, the Acute Toxic Class (ATC) method, and the Fixed Dose Procedure (FDP). Dr. Rispin stated that the UDP has the greatest versatility and is the most commonly used method in the United States. The test uses the most sensitive gender of rat. She explained that the default dosing scheme for this method tends to yield a value lower than median LD_{50} value (i.e., the dose of a test substance that produces death in 50% of the animals tested), which provides the most conservative outcome with dosing of fewer animals. Each test method works better with a starting dose near the LD_{50} value. Background information on the test chemical is very helpful to determine the most appropriate starting dose for acute oral toxicity testing but a default starting dose is available for all methods if no other information is available.

Test Method Overview

Dr. Judy Strickland provided an overview of the NICEATM/ECVAM validation study. The study objectives were:

- Further standardize and optimize two *in vitro* neutral red uptake (NRU) cytotoxicity protocols to maximize intra- and inter-laboratory reproducibility
- Estimate the reduction and refinement in animal use from using *in vitro* basal cytotoxicity assays to identify starting doses for *in vivo* acute toxicity tests

- Assess the accuracy of the two standardized *in vitro* cytotoxicity test methods for estimating rodent oral LD_{50} values across the GHS categories of acute oral toxicity
- Generate high quality *in vivo* lethality and *in vitro* cytotoxicity databases that can be used to support the investigation of the other *in vitro* test methods necessary to accurately predict acute systemic toxicity

Dr. Strickland presented the prioritization criteria used for selection of the reference substances used in the validation study (e.g., substances needed human toxicity/exposure data, rodent toxicity data, and should be relatively nonvolatile). She then described the sequence of events involved in the testing of the reference substances. The reference substances were first tested using a solubility protocol and then tested in the *in vitro* NRU assays. She explained the test acceptance criteria used for ascertaining which tests were functioning optimally. A graphical presentation of an *in vitro* NRU dose-response curve was provided to illustrate how the IC_{50} values (i.e., the concentration of a test substance that reduces cell viability by 50%) were calculated. The IC_{50} values were then used in a linear regression equation to predict corresponding LD_{50} values and to estimate the starting doses for the UDP or ATC methods. Dr. Strickland explained that computer simulation modeling of *in vivo* testing was used to determine animal use with either the default starting dose or the NRU-based starting dose. She provided an example for the UDP method. She stated that testing chemicals with an LD_{50} > 300 mg/kg and using the NRU-based starting dose would save 1 – 2 animals per test, or about 11 to 20%.

Dr. Strickland acknowledged the members of the Study Management Team, the laboratories and study directors involved in the study, and other support personnel who assisted in the study.

PEER REVIEW PANEL EVALUATION:
(1) Background Review Document (BRD) for Completeness, Errors, and Omissions
(2) Validation Status of the Proposed Test Methods

Dr. Stitzel provided the following statement to the Panel prior to discussions of the BRD: "To ensure adherence to the Federal Peer Review requirements, the Panel is asked to determine the completeness of the BRD and identify any errors or omissions. Additionally, the Panel will: 1) evaluate the validation status of the proposed test methods, and 2) make a determination of whether the information provided in the BRD supports the draft ICCVAM recommendations."

Dr. Stitzel also stated that before the Panel finalized its conclusions and recommendations, there would be an opportunity for public comment. She introduced the relevant Panel Group Leaders for each BRD Section: (Dr. Marion Ehrich - Section 1, 2, and 11; Dr. Daniel Marsman - Section 3, 5, and 6; Dr. Eugene Elmore - Sections 7 and 8; Dr. Andrew Rowan - Sections 4, 9, and 10). The Group Leaders presented the draft responses to the Evaluation Guidance Questions for consideration by the entire Panel.

Proposed Panel Recommendations for the BRD

BRD Section 1
Introduction and Rationale for the Use of *In Vitro* Neutral Red Uptake Cytotoxicity Test Methods to Predict Starting Doses for *In Vivo* Acute Oral Systemic Toxicity Testing

Dr. Ehrich provided a brief summary of Section 1 and listed the group's draft recommended revisions to this section of the BRD.

- The major conclusions from the workshop presented in Seibert et al. 1996 (Acute Toxicity Testing In Vitro and the Classification and Labelling of Chemicals. The Report and Recommendations of ECVAM Workshop 16. Alternatives to Laboratory Animals 24:499-510) should be included.
- The possibility of using the NRU assays to determine the starting doses for the FDP acute toxicity test should be included.
- A better explanation of why the 3T3 and NHK cells were chosen for the study should be provided.
- The 3T3 and NHK cell doubling times should be included (as a range).

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 2
Test Method Protocol Components of the 3T3 and NHK *In Vitro* NRU Test Methods

Dr. Ehrich provided a brief summary of Section 2 and listed the groups draft recommended revisions to this section of the BRD.

- The rationale for not using heat-inactivated serum in the cell cultures should be presented.
- The rationale for not using 3T3 cells after approximately 18 passages in culture should be provided.
- The extent to which using different lots of NHK cells in different studies may affect test method variability should be discussed.
- The potential for NHK cells under confluence to differentiate should be discussed as this may affect their sensitivity to cytotoxic agents.
- The variability in the composition of the bovine pituitary extract added to the NHK culture medium should be discussed.
- The procedures for preparation of test chemical dilutions should be clarified.
- The extent to whether cells recover and/or divide should be discussed.
- The vehicle control NRU optical density at 540 nm (OD_{540}) ranges for each laboratory should be presented.
- A discussion should be provided as to whether something other than mechanism of action could have contributed to the unusual concentration-response curves.
- The reference substances that used the study's lowest acceptable test chemical dilution factor (i.e., 1.21) should be listed.

- Additional explanations as to how GraphPad Prism® software calculated the IC_{50} using the Hill function should be provided.
- Quantitative data and the extent of variability on the doubling times of the two cell types for all laboratories during initial cell seeding, after seeding the cells in 96 well plates, and during exposure should be included.

Dr. Stitzel asked for discussion and any other revisions from the Panel on this section of the BRD. No further revisions were proposed and the Panel agreed with the draft recommended revisions.

BRD Section 3
Reference Substances Used for Validation of the 3T3 and NHK NRU Test Methods

Dr. Marsman discussed Section 3. He was satisfied with the selection of the reference substances but questioned the selective removal of some reference substances (based on mechanism of action) from the analyses since there was an incomplete understanding of the mechanisms of action for all of the reference substances. He provided additional recommendations for this section and then Dr. Stitzel asked for comments from the Panel.

Dr. Ehrich asked if the outcome would change if more chemical classes were added. Dr. Marsman said that there was an adequate number of chemical classes tested. Dr. Hasso Seibert stated that characterization of the chemicals is important; however, it was not necessary to do a metabolic profile of each chemical in order to do testing but the information would be useful. Dr. Stokes said that it would be valuable to know if there is a standardized approach to getting such information and requested suggestions from the Panel. Dr. Seibert stated that he was unaware of any standardized methods. Dr. Elmore suggested adding octanol:water coefficients for the test substances if known.

Other recommended revisions to this section of the BRD included:

- The basis for the selection of reference substances appears to be well described and of generally high quality. A wide range of substances, belonging to many chemical classes, physical properties, and different types of toxicities have been included. However, there were no polycyclic aromatic hydrocarbons, catalysts, simple aldehydes, ketones, biocides, cosmetic ingredients, plant toxins or other natural compounds. Also, it would have been useful to include some mixtures similar to likely pesticide or household product formulations.
- The adequacy of the range of reference substances and their mechanisms of oral toxicity is difficult to judge as there is often very limited knowledge about their mechanisms of action. Specifically, there is little information about the reference substances to support that specific modes of action of acute systemic toxicity have been robustly explored.
- The molecular structure of each reference substance should be provided.
- The cytotoxicity endpoint for the assay is based on uptake of neutral red into lysosomes; no mention is made whether any of the references substances cause lysosomal swelling, which could cause artifacts. Within this context,

there may be some limited value in adding data from additional substances to improve precision, and inclusion of substances at the extremes of the GHS toxicity categories may be helpful.

- There is some concern that the potential for bias may exist if the reference substances were pre-selected based on best fit to a regression line plotting cytotoxicity versus *in vivo* LD_{50} to evaluate *in vitro* test methods to estimate the acute oral LD_{50}.
- To the extent possible, characterization of the metabolic profiles of the reference substances should be added.
- Several confounding factors have not been addressed in the selection or evaluation of materials. For example, the octanol:water coefficients and the surface-active potential (to the extent possible) should be characterized and this information incorporated into the assessment.

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 4
***In Vivo* Rodent Toxicity Reference Values Used to Assess the Accuracy of the 3T3 and NHK NRU Test Methods**

Dr. Rowan led the discussion on Section 4 and presented the following recommended revisions to this section of the BRD.

- In general, the *in vivo* acute oral toxicity data did not conform to modern standards of toxicity testing and hence their quality would be difficult to determine.
- The LD_{50} values from the Registry of Toxic Effects for Chemical Substances (RTECS®) used in the Registry of Cytotoxicity (RC) linear regression model may not be the "gold standard" values. Extreme values may be unreliable and could lead to a misleading model of the desired linear relationship.

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 5
3T3 and NHK NRU Test Method Data and Results

Dr. Marsman presented the recommended revisions to the Panel and then Dr. Stitzel asked for comments from the Panel.

The Panel suggested performing a comparison of cell types, with respect to sensitivity to the individual chemicals, by normalizing the IC_{50} values to the IC_{50} of the positive control (PC). The comparative response of each cell type might elucidate whether an individual chemical is an outlier (with respect to prediction of GHS classification). The concordance of IC_{50} values for the two test methods is basically good since only 3% of the reference substances differed by two orders of magnitude and 3% of the reference substances differed by greater

than five orders of magnitude. It is important to know how these cell types respond to the different chemical classes. This relates to the precision of the test in relation to the GHS classification. A 10-fold difference in IC_{50} values between 3T3 and NHK cells may not pose a problem since biology is not exact, but it is important to know the biological differences since this will help in understanding how the systems work.

Other recommended revisions to the BRD included:

- Explanations, if available, should be added as to why carbon tetrachloride and a few other reference substances could not be adequately tested by all laboratories.
- An explanation, if available, for the considerably higher sensitivity of the NHK versus 3T3 cells to the positive control should be provided.
- Further discussion is needed exploring the biological significance of and possible reasons for the differences in sensitivity and selectivity between the two cell lines; this may be useful for selecting the appropriate cell line(s).
- A descriptive summary of the IC_{50} values and orders of magnitude that includes the fraction that were within specific IC_{50} ranges should be provided.
- The Hill function slope data and LD_{50} slope data should be provided for potential comparisons of IC_{50} slopes to LD_{50} slopes.
- A discussion about why the IC_{50} values for aminopterin and digoxin differ by five orders of magnitude when comparing 3T3 and NHK values should be provided. Information about organic anionic transporters should be included.
- The relative IC_{50} ratios between the reference substances and the positive control (at the level of the individual lab) should be used to compare materials across assays.

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 6
Accuracy of the 3T3 and NHK NRU Test Methods

Dr. Marsman led the discussion of Section 6.

The Panel was not sure if it is important to separate rat and mouse LD_{50} data but recommends separation because it is more scientifically acceptable. The animal data already has much variability (e.g., age, gender, etc.) and additional variability such as combining data from different species should be avoided.

The Panel addressed the use of corrosive chemicals in the study. A caveat should be added to the BRD that *in vivo* testing of corrosives is neither advocated nor recommended. If, however, historical *in vivo* data on such chemicals exist, the data should used and analyzed in conjunction with *in vitro* data.

There was a consensus that adequate data were generated to draw conclusions about the accuracy and reproducibility of the two test methods. The statistical approaches adopted to

analyze the data enabled accurate and scientifically robust analyses of test method accuracy. The information presented in this section of BRD appears sufficient with the following exceptions, which the Panel recommended as revisions to this section of the BRD:

- The overall accuracy is modest, and enhancement of accuracy through material selection (modular approach), model refinement, or tiered testing strategy should be pursued.
- The basis for the orders of magnitude difference in IC_{50} values for numerous reference substances between 3T3 and NHK cells should be explained (i.e., is the difference a consequence of cell-specific cytotoxicity or differences in exposure conditions or something else?).
- Chemicals in the RC database that showed underprediction of toxicity were deemed to have mechanisms of toxicity that could not be detected in the 3T3 and NHK NRU assays. These mechanisms included neurotoxic and cardiotoxic mechanisms, interference with energy utilization, and alkylation of macromolecules. The Panel indicated that interference with energy metabolism and alkylation of proteins and DNA represent important mechanisms of cytotoxic action. Thus, the rationale for excluding the substances from the RC database with "specific mechanisms of action" appears very questionable (i.e., all chemicals should remain in the regression).
- Given that a mode of action is unlikely to be known about a random material, a modular approach based upon mechanism is not a viable option. A better approach would be one based on chemical class, implying similar mode of action.
- Use of metabolically competent systems is recommended as one approach to improve the accuracy of *in vitro* predictions of acute toxicity and should be explored in the future.
- Corrosivity was one of the exclusionary criteria that was originally attempted to be applied to the reference substances. However, corrosive materials as a class were not deleted from calculation of the regression lines.
- Graphs should be added to compare the responses of the 58 RC substances to the same agents with the 3T3 and NHK NRU tests.
- The criterion for removal of some substances to arrive at the best regression is of limited merit; however, without removal, the 26% accuracy for prediction of GHS class is poor although better than a random selection using the 72 chemicals (1/6 accuracy).
- As a future task, the properties of the cell lines (e.g., metabolism, receptors, transporters) that are important for basal cytotoxicity should be better characterized. These properties could be used in performance standards.
- The proprietary nature of the composition of the NHK culture medium makes it impossible to assess the role differences in media composition may have had on the results.
- It would be informative to show comparisons of the regressions (using RC IC_{50} and LD_{50} data) for the selected agents used in this study versus the individual lab responses for each test instead of the data shown in Figures 6-6 to 6-8 of the BRD, which compares the *in vitro* responses to the overall RC millimolar (mM) regression.

- Protein binding should be taken into account in additional analyses (i.e., to the extent possible, consider the free fraction in serum that corresponds to the LD_{50} dose).
- The Hill function slope data and LD_{50} slope data should be compared.
- Graphing of IC_{50} values ± the standard deviation (SD) and rat LD_{50} values ± SD should provide a better comparison of variation in the two sets of values.

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 7
Reliability of the 3T3 and NHK NRU Test Methods

Dr. Elmore led the discussion of Section 7 in regard to the draft recommended revisions to this section of the BRD.

- Additional consideration as to the underlying reasons for the variability between the laboratories would be helpful. Everyone participating in these studies should be adequately trained in the basics of cell and tissue culture and sound scientific methods.
- This section adequately elucidated associations between intra- or inter-laboratory reproducibility and chemical classes, chemical properties, and potency categories; there were no clear associations between any of these parameters and coefficient of variation (CV values). However, the reproducibility of both methods depends on the laboratory performing the measurements. A discussion of the possible reasons for this laboratory-specific reproducibility would be helpful.
- IC_{50} values do not indicate the steepness of the concentration-response curve. IC_{20} (i.e., the concentration of a test substances that reduces cell viability by 20%) and IC_{80} (i.e., the concentration of a test substances that reduces cell viability by 80%) values were collected, but not used. For some reference substances, there was only one point between 0 and 100% viability.
- The reference substances failing to yield IC_{50} values were mostly solvents (e.g., carbon tetrachloride, methanol, xylene, trichloroethane). An explanation should be provided.
- The Panel questioned the utility of the analysis of variance analysis (ANOVA) for addressing the issue of intra- and inter-laboratory reproducibility. Depending upon the sample size and intralaboratory variation, a significant difference could correspond to a very small variation between laboratories or a nonsignificant difference could correspond to a very large difference between laboratories. The content of Table 7-4 should be examined to assure that the correct data are included.
- Based on the ANOVA analysis performed, FAL reported significantly different results from the two other laboratories for 20 substances (3T3 NRU assay) and for 18 of these substances FAL reported the highest values. The BRD should explain this phenomenon.

- Independent of the statistical method used, there were more reference substances with deviating results between laboratories for the 3T3 NRU assay than for the NHK NRU assay. The BRD should explain this.
- The BRD should explain why some laboratories failed to obtain IC_{50} results for some reference substances.
- It might be helpful to look at ratios of the maximum IC_{50} values to minimum IC_{50} values to see how they compare vs. rodent LD_{50} values. Those chemicals having ratios ≥ 3.0 should be presented in a separate table together with their calculated ratios and the names of the labs that delivered the corresponding IC_{50} values.

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 8
3T3 and NHK NRU Test Method Data Quality

Dr. Elmore led the discussion of Section 8. The Panel did not recommend any revisions to this section of the BRD. Dr. Stitzel asked for comments from the Panel; the Panel accepted the draft decision to not recommend revisions to Section 8.

BRD Section 9
Other Scientific Reports and Reviews of *In Vitro* Cytotoxicity Test Methods and the Ability of These Test Methods to Predict Acute Systemic Toxicity

Dr. Rowan led the discussion of Section 9 on the following draft recommended revisions to this section of the BRD.

- Additional discussion from the published literature about the advantages and limitations of using various supplemental metabolizing systems in cell culture for cytotoxicity testing could be included.
- Based on the Perloux et al. (1992) study, a discussion about whether the relatively good predictive value is a result of the route of exposure (intravenous [iv] and intraperitoneal [ip]), as well as information on the range of chemical types and the range of toxicities should be included. The poorer correlations for the oral route, along with the better correlations for the iv route, should be included. The correlation of different routes of exposure and the reflected kinetic variation should be discussed.
- The results of the workshop presented in Seibert et al. 1996 (Acute Toxicity Testing *In vitro* and the Classification and Labelling of Chemicals. The Report and Recommendations of ECVAM Workshop 16. Alternatives to Laboratory Animals 24:499-510) should be included.
- It would be useful to compare the range of *in vivo* toxicities and modes of action represented in these other studies reported in Section 9 with the present ICCVAM study.

- Clarification about the percentage reduction of animal use as referenced in the ICCVAM 2001a report should be included (i.e., what is the likely basis for the difference between then and now).

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 10
Animal Welfare Considerations (Refinement, Reduction, and Replacement)

Dr. Rowan led the discussion of Section 10.
All supplemental data and information provided to the Panel via the NICEATM restricted website will be added to the final BRD.

Dr. Strickland stated that when the evaluation was performed with all of the reference substances, the RC millimole regression provided the best animal savings results, especially for substances with high toxicity. The Panel reviewed Table 1 from the AnimalUse.doc file provided on the restricted website. The biostatisticians questioned the difference in animal use for the default starting dose between the RC millimole regression and the other two regressions.

The Panel discussed whether or not a millimolar or a weight regression should be used to estimate the starting dose for acute oral toxicity tests. They recommended that if the molecular weight (MW) is unknown, the mg/kg regression should be used. If MW is known, they recommended using the mM regression since this would be more appropriate biologically. A decision tree may be needed to determine which regressions should be used for a test chemical. Other recommended revisions to this section of the BRD included:

- A substantial percent of the time the toxicity of "highly toxic" molecules *in vivo* was predicted to be less toxic using the cytotoxicity assays. In these instances, animals would be lost and subjected to untoward toxicities by using the higher predicted starting doses. Thus, the Panel recommended that the cytotoxicity tests only be used in a weight-of-evidence approach to determining starting doses for acute oral toxicity test methods.
- Although the accuracy appears to be low, it is still better than starting at the default starting dose if no other information is available.
- Based on existing data, where molecular weight information is available for a relatively pure test substance, the mM regression should be used; in the absence of such data, the mg/kg regression should be used.
- The possibility of using the NRU assays to determine the starting dose for the FDP acute toxicity test should be more carefully evaluated.
- Animal savings should take into account, to the extent possible, prevalence (i.e., the chemical distribution within the various GHS classifications).

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

BRD Section 11
Practical Considerations
Dr. Ehrich provided a brief summary of Section 11 and listed the recommended revisions for the BRD.

The Panel agreed that extra efforts such as better education for laboratory technicians are needed for transferability of the test methods. Laboratories have their own ways of doing things and it is understandable to have differences in data. The protocols should have better detail to make sure everyone does the same thing during a test. The ICCVAM recommended performance standards and protocols should emphasize what education and proficiency is needed.

The Panel concluded that it is difficult to compare the value of the *in vitro* NRU assay per chemical to achieve an IC_{50} versus an animal test to achieve a LD_{50}. However, given that, the information presented in this section of BRD appears sufficient, with the following exceptions.

- It appears that transferability was not as easy as was stated. Minor protocol differences can have profound effects.
- Adequate training must be conducted prior to the initiation of the study.
- The costs for equipment and working time needed to perform the assays and a cost-benefit analysis should be included.
- NRU assays are not for replacement but for reduction. It would be appropriate to describe the reduction in the number of animals used.
- The time needed to prescreen NHK culture medium should be described.

Dr. Stitzel asked for comments from the Panel on this section of the BRD. Since no comments were provided, the Panel agreed upon the draft recommended revisions.

PUBLIC COMMENTS (Session 1)

Dr. Manfred Liebsch - Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) - Germany
Dr. Liebsch stated that he represented the ECVAM Scientific Advisory Committee (ESAC) Shadow Panel on the ICCVAM Peer Review of *In Vitro* Acute Toxicity Test Methods. The Shadow Panel's purpose is to facilitate a transparent communication process between ICCVAM and ECVAM. He provided the following comments:

- Why were the following recommendations from the ICCVAM *In vitro* Workshop of 2000 not adequately considered: (1) immediate implementation of the ZEBET Registry of Cytotoxicity approach to estimate acute toxicity starting doses, and (2) development of a 2-3 year validation study using *in vitro* methods to replace rodent acute oral toxicity testing
- The study's objectives were partly conflicting in regard to validation of the RC prediction model
- The selection of test chemicals was inappropriate to achieve the main study goal

- The *in vitro* data on intralaboratory and interlaboratory variations should be related to other multi-centre studies using NRU assays
- Take into account the influence of variability of both *in vitro* and *in vivo* data (in particular in the very toxic range) on the accuracy of predictions obtained
- Explain the poor fits of the data to the combined laboratory 3T3 and NHK regressions
- Appropriately discuss the study outcome in relation to other studies
- Take into account the prevalence chemicals, with respect to toxicity, for calculations of animal savings (not predictive power)

Ms Jessica Sandler – People for the Ethical Treatment of Animals (PETA)

Ms Sandler spoke of her involvement in the 1990s with the EPA and The Johns Hopkins Center for Alternatives to Animal Testing to impress upon the organizations that Dr. Bjorn Ekwall's methodology using cell death was an alternative to animal testing. She expressed dismay in the lack of interest by both groups in following this avenue. She also stated that toxicity tests should apply to the species of concern and that animal tests do not protect humans. She was critical of ICCVAM for not following the ICCVAM *In Vitro* Workshop 2000 recommendations on accepting non-animal testing as replacements. She stated that she believes ICCVAM's congressional mandate requires it to focus on the replacement of animals in lethal dose testing. Ms Sandler's public statement is available on the ICCVAM/NICEATM website in pdf format at the URL link provided (http://iccvam.niehs.nih.gov/methods/invidocs/brdcomm.htm).

Dr. Rodger Curren – Institute for *In vitro* Sciences (IIVS)

Dr. Curren thanked the Panel for their reviews and enthusiasm. He provided the following comments:

- A more accurate assessment of the "accuracy" of the method would be to model the results using a chemical set which more closely matched the original Halle chemical distribution in the RC regression. The current set of chemicals is biased toward outliers.
- The calculations for "animals saved" would be more informative if the data used for modeling was more representative of the original Halle chemical distribution in the RC regression
- It would be more logical to use the closest default dose to the estimated LD_{50} as the starting dose than to follow the OECD protocols which say to use the next lower dose (of a set of predetermined doses) to the value estimated by the cytotoxicity assay
- Minor comments included: the human response to digoxin is much higher than the animal response; information on most components of the keratinocyte growth medium should be available to researchers; the difference in SLS sensitivity between the two cell types may be influenced by the presence or absence of serum in the medium; the variability between labs should be examined more carefully to determine whether it is biologically significant

Final Review of the BRD

Dr. Stitzel asked the Panel to review the recommended revisions for each BRD section, taking into account the public comments, and to decide if additional changes are necessary. If no changes were recommended, then the recommendations for that section of the BRD were considered to as final.

No changes were made to the draft recommendations for Sections 1, 2, 4, 5, 7, 8, 9, and 11.

Section 3: The Panel asked for additional discussion of and reaction to the public comments from Dr. Manfred Liebsch. Dr. Stokes stated that the validation study tried to maximize the use of chemicals that had human and rat toxicity data. ECVAM is reviewing the human lethal serum/blood concentrations (LC) data for future use. Despite Dr. Liebsch's assertions, validation of the RC regression was not an objective of the NICEATM/ECVAM validation study. Dr. Stokes said that these clarifications would be in the final report. No other comments were made and the draft recommendations for this section were accepted by the Panel.

Section 6: The Panel asked for additional discussion of and reaction to the public comments from Dr. Rodger Curren. Dr. Seibert indicated the test methods should be so reliable that they could be done around the world, but there is no established and accepted criterion for reliability. Dr. Elmore suggested a graphical analysis in which the data from each individual laboratory is compared with the laboratory mean to determine whether one laboratory is different from the others. Dr. Stokes said this analysis could be added to the final report. No other comments were made and the draft recommendations for this section were accepted as final by the Panel.

Section 10: The Panel recommended the addition of prevalence data based on the reference from Dr. Liebsch. The accuracy number needs to be corrected in the BRD so that it reflects the right regression (i.e., the RC). No other comments were made and the draft recommendations for this section were considered to be final.

Validation Status

Dr. Stitzel asked the Panel whether the test methods are valid and supported by the data. The Panel agreed that the test methods are valid as a weight-of-evidence approach for estimating starting dose. Although the test methods are useful, they are not necessary and should not be made obligatory. Additional clarity is needed on how to use the weight-of-evidence approach, but this may require additional data.

The Panel agreed to the following statement on the validation aspect of the test methods.
The Panel agrees that the applicable validation criteria have been adequately addressed for using these in vitro test methods in a weight-of-evidence approach to determine the starting dose for acute oral in vivo toxicity protocols.

DRAFT ICCVAM RECOMMENDATIONS FOR *IN VITRO* ACUTE TOXICITY TEST METHODS

Presentation of Draft ICCVAM Recommendations

Dr. Marilyn Wind presented the draft ICCVAM recommendations for test method use and future studies. ICCVAM draft recommendations are now presented at peer review meetings due to OMB requirements for peer review of the scientific information used as the basis for the recommendations. Dr. Stitzel reminded the Panel that the discussion was to determine whether the scientific data and information in the BRD supports the ICCVAM recommendations.

Are the Draft ICCVAM Recommendations on Proposed Usefulness/Limitations Supported by the BRD?

Dr. Marsman led the discussion. The Panel agreed to the following statements in response to the ICCVAM recommendations.

(1) *"The 3T3 and NHK NRU test methods are not sufficiently accurate to predict the acute oral toxicity of substances for the purposes of hazard classification (see Section 6 of BRD)."*
- The Panel agrees with this statement in that neither of the two basal cytotoxicity tests can be used as alternatives for the *in vivo* acute oral toxicity test for the purposes of hazard classification.
- In the BRD, the rat data was not all generated in accordance with Good Laboratory Practice (GLP) standards

(2) "*For the purposes of acute oral toxicity testing, the 3T3 and NHK NRU test methods may be used in a weight-of-evidence approach to determine the starting dose for the current acute oral in vivo toxicity protocols (i.e., the UDP and ATC)."*
- The Panel agrees that the *in vitro* test methods may be useful in a weight-of-evidence approach to determine the starting dose for acute oral *in vivo* toxicity protocols.
- Given its limited predictive capacity, however, it is unclear whether it will provide substantial weight in that decision.
- The overall accuracy is modest, and enhancement of accuracy through material selection (modular approach), model refinement, or tiered testing strategy should be pursued.

(3) *"Consistent with the U.S. Government Principles on the Use of Animals in Research, Testing, and Education (National Research Council 1996), and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002)[1], in vitro basal cytotoxicity test methods as part of a weight-of-evidence approach to estimate the starting dose for acute oral in vivo toxicity test methods should be considered and used where appropriate before testing is conducted using animals. For some types of substances, this approach will reduce the number of animals needed. In some testing*

situations, the approach may also reduce the numbers of animals that die or need to be humanely killed.“

- The Panel agrees.

(4) *“Substances with specific toxic mechanisms that are not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic, cardiotoxic, interfere with energy utilization, or alkylate proteins and other macromolecules) will likely be underpredicted by these in vitro basal cytotoxicity test methods. Therefore, until such time as a more predictive testing approach is developed, the results from basal cytotoxicity testing with such substances may not be appropriate.”*

- The Panel disagrees with elements of this statement; specific toxic mechanisms that the BRD stated are not expected to be active in 3T3 and NHK cells, such as “interference with energy utilization and alkylation of proteins and other macromolecules”, are mechanisms of cytotoxic action and should be detectable with 3T3 and NHK cells.

(5) *“The regression formula used to determine starting doses should be the RC regression line [with IC_{50} values in µg/mL and LD_{50} values in mg/kg] developed with the RC chemicals using rat LD_{50} data only and excluding chemicals with mechanisms of action that are not expected to be active in in vitro basal cytotoxicity test methods.”*

- The Panel does not agree with this statement.
- There was consensus among the Panel that the data contained within the BRD or the open literature were not sufficient to justify the exclusion of materials based on mechanism.
- It is not justified to (retrospectively) exclude substances because of assumed modes of toxic action *in vivo* and/or possible involvement of biotransformation reactions.
- The Panel recommends that ICCVAM consider convening a work group to explore mechanisms of action of acute toxicity, and how acquiring additional information on acute toxic mechanisms might be put into practice under acute toxicity testing.
- Although a modular approach to use of the model looks like it may be more reliable, the database is likely too small for most mechanisms of action to draw sound conclusions regarding strengths and limitations of the test methods with respect to chemical classes, mechanisms of toxicity, or physico-chemical properties. Given that it is likely that mode of action for a random source material would be unknown, it is unlikely that a modular approach based upon mechanism is a viable option. A better approach to validation would be one based on chemical class, implying similar mode of action.
- *The Panel recommends moving the last two comments to the ICCVAM recommended future studies section.*

(6) *"The performance of other in vitro basal cytotoxicity test methods that are based on similar scientific principles and that measure or predict the same biological response (i.e., basal cytotoxicity and the rat acute oral LD_{50} value, respectively) should be demonstrated to meet or exceed the accuracy and reliability of the 3T3 and NHK NRU test methods."*

- The Panel agrees with this statement although the reliability of the methods in this study was not quite satisfying (e.g., interlaboratory reproducibility), the reproducibility of these methods (e.g., intralaboratory reproducibility) are modest, and the accuracy of these methods are poor.

(7) *"Compared to the NHK NRU test method, the 3T3 NRU test method appears to be less labor intensive and less expensive to conduct; therefore, the 3T3 NRU cytotoxicity test method is recommended for general use."*

- Some Panel members agreed in a general sense, but cautioned that one model may be preferred over the other based upon specific knowledge regarding known mechanisms of action (e.g., the rationale for the disparate results observed with aminopterin and digoxin). Other Panel members agreed with this statement because the use of continuous cell lines is more efficient, especially since the overall animal savings were relatively low.
- One Panel member noted that NHK NRU IC_{50} data have shown a better correlation with human LC_{50} values (R^2=0.62) than do rodent 3T3 NRU IC_{50} data (R^2=0.51) and better than rodent LD_{50} data correlates with human LC_{50} values (R^2=0.56) as reported by S. Casati et al. at the 5th World Congress in Berlin, 2005. It is important to remember that hazard assessment relates to the safety of humans, not rats.
- Based on costs of commercial keratinocytes, the NHK NRU assay may be cost-prohibitive.
- The proprietary nature of the composition of the NHK culture medium makes it impossible to assess the role differences in media composition may have had on the results.

Draft Recommended Test Method Limitations

The Panel recommended adding the following verbiage to the draft report.

- Colored substances (besides red substances) may absorb light in the optical density range of the NRU assay and would affect the test system.
- The BRD indicates that optimization to allow for testing of mixtures was being undertaken, yet no mixtures were used in fitting the regression curve. Given the limitations of the assays in accurately predicting materials of known or uncertain mechanisms, the testing of mixtures seems highly controversial.

Dr. Stitzel asked for comments from the Panel on these draft ICCVAM recommendations as to the proposed usefulness and limitations of the two *in vitro* cytotoxicity test methods. No additional comments were provided and the Panel agreed unanimously with the draft revisions to the ICCVAM recommendations.

Are the Draft ICCVAM Recommended Standardized Protocols Supported by the BRD?

Dr. Ehrich led the discussion on the protocols. The Panel agreed that the protocols are generally quite detailed and laboratory technicians should be able follow the procedures. The Panel recommended the following clarifications be added to the 3T3 and NHK NRU test method protocols:

Protocol Recommendations

- The rationale for testing the positive control on separate plates rather than on the test plates should be provided.
- The number of definitive tests that should be performed for a test substance should be specified.
- The range of linearity of the microplate reader should be confirmed (as per in-house Standard Operation Procedures [SOPs]) for the recommended optical density (OD_{540}) and stated.
- Maximum absorbance values needed by a spectrophotometric plate reader should be provided for application to the NRU assays.
- The test method protocols should be streamlined. (Undefined as to how this should be accomplished.)
- Guidance for using methods other than the Hill function to determine IC_{50} values should be provided.
- The lowest acceptable test substance dilution factor (i.e., 1.21) should be reduced rather than accepting only one cytotoxicity point between 0 and 100% viability on a steep dose-response curve to use for determination of the IC_{50} value.
- Study directors and quality assurance units are necessary only if testing is performed under GLP.
- Good cell culture practices (e.g., Hartung et al. 2002) must be followed.
- Whether or not a prequalification test of new keratinocytes should be performed by the laboratory prior to actual testing should be stated.
- A recommendation that keratinocytes should be procured only through commercial sources and not by preparing primary cultures from donated tissue should be included.
- Additional guidance to the solubility step-wise procedure should be added (i.e., ensure that test substance solution preparation procedures can be easily understood by laboratory technicians).
- The need for training of laboratory technicians so they may be able to better understand solvent and solubility determinations should be included.
- Additional guidance as to the use of a microscope to assist in determining solubility of a test substance should be added.
- Test substances that may etch plastic or “film out” in medium should be identified (the importance of detecting such compounds by the laboratory technicians should be emphasized).

- The protocols should recommend the use of a solvent (e.g., dimethyl sulfoxide [DMSO], ethanol) at its lowest possible concentration at each test substance concentration level.
- There is concern about the differences in solvent selection between laboratories as compared to the BioReliance solvent information. The variability between laboratories in the selection of solvent points out a possible flaw in the solvent determination protocol. This should be evaluated for future studies.

Dr. Stitzel asked for comments from the Panel on these draft ICCVAM standardized protocols for the two *in vitro* cytotoxicity test methods. Since no additional comments were provided. The Panel agreed unanimously with the draft recommended revisions to the draft ICCVAM standardized protocols.

Are the Draft ICCVAM Recommended Test Method Performance Standards Supported by the BRD?

Dr. Elmore presented the Panel comments on whether the ICCVAM draft recommended test method performance standards were supported by the BRD.

The available data from this study appear to support the validity of the recommended performance standards for the test methods. The usefulness and limitations are well covered, and if validated, the methods may be a worthwhile option. However, there may be some cause for concern if use of the methods is made compulsory for regulatory purposes.

- Recommendations made in section 2.3.2 (Application of the Test Substances), section 2.3.3 (Control Substances), and section 2.3.4 (Viability Measurements) are acceptable.
- A discussion is needed about whether or not the NRU assays are recommended for use with unknown substances and mixtures.
- The significance of the secondary chemical subset to be used for "investigational purposes" should be better elucidated in the document.

Dr. Stitzel asked for discussion from the Panel on whether the draft ICCVAM recommended performance standards for the two *in vitro* cytotoxicity test methods were supported by the BRD. No additional comments were provided. The Panel agreed unanimously with the draft recommended revisions to the ICCVAM recommendations.

Are the Draft ICCVAM Recommended Future Studies Supported by the BRD?

Dr. Rowan presented the Panel comments on whether the ICCVAM draft recommendations on the recommended future studies were supported by the BRD. He stated that efforts should be made to collect GLP LD_{50} data from industry for use in *in vitro/in vivo* databases. The ICCVAM recommendations were discussed and the bullets below represent the Panel's responses.

(1) ICVAM draft recommendation: *"Additional data should be collected using the 3T3 and/or the NHK NRU test methods to evaluate their usefulness for predicting the in vivo acute oral toxicity of chemical mixtures."*

- The Panel generally agrees that this is a good recommendation, although collecting data could be difficult and doing correlation with *in vivo* data would be even more difficult. It may be useful to suggest that such data only be collected with the 3T3 NRU test method, and that it would be necessary to clarify the reasons for the interlaboratory variations for future use of the method.

(2) ICVAM draft recommendation: *"Additional high quality comparative in vitro basal cytotoxicity data should be collected in tandem with in vivo rat acute oral toxicity test results to supplement the high quality validation database started by this study. Periodic evaluations of the expanded database should be conducted to further characterize the usefulness and limitations of using in vitro cytotoxicity data as part of a weight-of-evidence approach to estimate starting doses."*

- The Panel believes this could be valuable under certain conditions, especially if NRU data are collected as acute toxicity testing is conducted.
- However, no panel member wants *in vivo* testing conducted solely to collect data to assess the usefulness of the NRU test, particularly given that the savings in animal numbers that arise from the use of the NRU test to determine the starting dose for the ATC method or UDP are fairly modest.

(3) ICVAM draft recommendation: *"Additional efforts should be conducted to identify additional in vitro tests and other methods necessary to achieve accurate acute oral hazard classification; specifically, studies should be conducted to investigate the potential use of in vitro cell-based test methods that incorporate mechanisms of action and evaluations of ADME to provide improved estimates of acute toxicity hazard categories."*

- The Panel agrees with this statement and adds that there should be additional effort towards development of alternative methods to adequately predict the *in vivo* acute toxicity of chemicals for the purposes of hazard classification.
- An additional statement to include could be, "and the development of methods to extrapolate from *in vitro* toxic concentrations to equivalent doses *in vivo*."

(4) ICVAM draft recommendation: *"The in vivo database of reference substances used in this validation study should be used to evaluate the utility of other non-animal approaches to estimate starting doses for acute oral systemic toxicity tests (e.g., widely available software that uses quantitative structure-activity relationships [QSAR])."*

- The Panel agreed with this recommendation.

(5) ICVAM draft recommendation: *"Standardized procedures to collect information pertinent to an understanding of the mechanisms of lethality should be included in future in vivo rat acute oral toxicity studies. Such*

information will likely be necessary to support the further development of predictive mechanism-based in vitro methods."

- The Panel agrees with this recommendation; this is really important and could further the development of non-animal alternatives in the future.
- To facilitate comparisons and model development, future studies should incorporate high quality animal data for required testing of new agents, (where possible) blood levels from animals (LC_{50}), and high quality *in vitro* data from the same agents.
- The Panel recommends that ICCVAM consider convening a work group to identify the appropriate *in vivo* endpoints to assess during acute toxicity testing so as to generate information on mechanisms of acute toxicity.
- Although a modular approach to use of the model looks like it may be more reliable, the data base is likely too small for most mechanisms of action to draw sound conclusions regarding strengths and limitations of the test methods with respect to chemical classes, mechanisms of toxicity, or physico-chemical properties. Given that it is likely that a mode of action is unlikely to be known about a random source material, it is unlikely that a modular approach based upon mechanism is often going to be a viable option. A more likely approach to validation would be one based on chemical class, implying similar mode of action.

(6) ICVAM draft recommendation: *"An expanded list of reference substances with estimated rat LD_{50} values substantiated by high quality in vivo data should be developed for use in future in vitro test method development and validation studies."*

- The Panel agrees with this recommendation; there should be a concerted effort to collect proprietary data.

Dr. Stitzel asked for comments from the Panel on these draft ICCVAM recommendations for future studies. Since no additional comments were provided, the Panel agrees with the draft revisions to the ICCVAM recommendations.

PUBLIC COMMENTS (Session 2)

<u>Ms Kristie Stoick - Physicians Committee for Responsible Medicine (PCRM)</u>

Ms Stoick introduced herself as a representative of the PCRM and requested that a full replacement of *in vivo* testing be sought. She appreciates the progress toward reduction and refinement of animal use in acute toxicity evaluations, but suggests that total replacement, rather than reduction and refinement, is the solution to poor concordance. She faulted ICCVAM for not following up on the research and development recommendations from the ICCVAM *In Vitro* Workshop in 2000. She expressed hope that the appropriate government agencies will implement any validated reduction and refinement measures and urges the implementation of a dedicated mechanism to collect all data generated from these tests for evaluation and determination of its usefulness in replacing *in vivo* acute toxicity tests.

Final Review of the Draft ICCVAM Recommendations

Dr. Stitzel asked if any Panel member wanted make any changes to the comments of the Panel regarding the draft ICCVAM test method recommendations. No further changes were requested. Dr. Stitzel affirmed that the Panel unanimously concurred with all of the above comments. The Panel agreed also that the statement on validation of the test methods was acceptable.

Concluding Remarks

Drs. Stitzel and Stokes thanked the Panel members for their time and effort.

Adjournment

The meeting was adjourned at 5:23 p.m.

William S. Stokes, D.V.M.
NIEHS
P.O. Box 12233
MD-EC17
Research Triangle Park, NC 27709

Dear Dr. Stokes:

The Meeting Summary, Peer Review Panel Public Meeting, *In Vitro* Methods for Estimating Starting Doses for Acute Systemic Toxicity Testing, dated May 23, 2006, accurately summarizes the Peer Review Panel Public meeting of May 23, 2006, in Bethesda, MD.

Sincerely,

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Signature	Printed Name	Date