

**National Toxicology Program
Board of Scientific Counselors**

June 11-12, 2008

National Institute of Environmental Health Sciences
Research Triangle Park, NC

Summary Minutes

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I. Attendees

Members in Attendance:

Christopher Bradfield, University of Wisconsin
Tracie Bunton, Eicarte LLC
Russell Cattley, Amgen
Kenny Crump, Louisiana Technical University
Katharine Hammond, University of California Berkeley
William Janzen, Independent Consultant
Nancy Kerkvliet, Oregon State University
Gail McCarver, Medical College of Wisconsin (chair)
Jon Mirsalis, SRI International
Raymond Novak, Wayne State University
Michael Pino, Sanofi-Aventis
Kenneth Portier, American Cancer Society
Jim Riviere, North Carolina State University
Diane Robins, University of Michigan Medical School
Keith Soper, Merck & Company

Members not in attendance:

Edward Carney, The Dow Chemical Company
George Friedman-Jimenez, New York University School of Medicine
David Wegman, University of Massachusetts, Lowell

Ad Hoc Members

Michael Baum, Boston University
Kim Boekelheide, Brown University (via teleconference)
Robert Cardiff, University of California, Davis (via teleconference)
Gregory Kedderis, Independent Consultant
Max Costa, New York University School of Medicine
J. Steven Leeder, Children's Mercy Hospitals and Clinics
Ruthann Rudel, Silent Spring Institute
Richard Sharpe, The University of Edinburgh Academic Centre
Barry Timms, The University of South Dakota
Jorma Toppari, University of Turku

National Institute for Environmental Health Sciences (NIEHS) Staff

Eddie Ball	Barbara Shane
Chad Blystone	Michael Shelby
John Bucher	Diane Spencer
Rajendra Chhabra	Stan Stasiewicz
Helen Cunny	William Stokes
Christine Flowers	Matthew Stout
Paul Foster	William Suk
John French	Kristina Thayer
Dori Germolec	Julius Thigpen

Tammy Hardee	Raymond Tice
Michelle Hooth	Molly Vallant
Richard Irwin	Suramya Waidyanatha
Gloria Jahnke	Nigel Walker
Ruth Lunn	Lori White
Robin Mackar	Samuel Wilson
David Malarkey	Kristine Witt
Colette Malone	Mary Wolfe
Scott Masten	Michael Wyde
Ronald Melnick	
Retha Newbold	
Sheila Newton	

Other Federal Agency Staff

Norris Anderson, Food and Drug Administration (FDA)
Barry Delclos, FDA/National Center for Toxicological Research (NCTR)
Dan Doerge, NCTR/FDA
Goncolao Gamboa Da Costa, NCTR/FDA
Andrew Hotchkiss, Environmental Protection Agency (EPA)
Paul Howard, NCTR/FDA
Michelle Twaroski, FDA
Mark Toraason, National Institute for Occupational Safety and Health (NIOSH)
Richard Wang, Centers for Disease Control (CDC)

Public

Nena Baker, North Point Press
Jonathan Brania, Underwriters Laboratories
Raymond David, BASF Corporation
Sandrine Deglin, Exponent
Sanford Garner, Constella Group
Tom Goldsworthy, Integrated Laboratory Systems (ILS)
Claudine A. Gregorio, ILS
Steven Hengtes, American Chemistry Council
Marc Jackson, ILS
Michelle Lancaster, N.A. Metal Packaging Alliance
Joseph Manuppello, People for the Ethical Treatment of Animals
Catherine Price, RTI International
Leslie Recio, ILS
Neville Shaw, Westend
Shelley Tyl, RTI International

II. Introductions and Welcome

The National Toxicology Program (NTP) Board of Scientific Counselors (BSC) met on June 11-12, 2008, at the Radisson Hotel Research Triangle Park, Research Triangle Park, North Carolina. Dr. Gail McCarver, Chair, welcomed everyone to the meeting and asked the BSC members and attendees to introduce themselves. Dr. John Bucher, NTP Associate Director, NIEHS, welcomed and thanked the BSC members for their attendance and service to the NTP.

Dr. Gail McCarver extended a special welcome to the *ad hoc* BSC members. Dr. Barbara Shane made a few announcements and read the conflict of interest statement. She noted that *ad hoc* reviewers would not vote and no conflicts of interest were identified.

Dr. Samuel Wilson, Acting Director of the NIEHS and NTP, welcomed the BSC members and expressed his gratitude to them for their attendance at the meeting and for their advice to the NTP on its activities.

Dr. Bucher briefly highlighted NTP activities since the December 2007 BSC meeting. In January 2008, he authored an editorial in Environmental Health Perspectives outlining a realignment of NTP within NIEHS and new NTP initiatives. On February 14, the NIEHS announced the signing of a memorandum of understanding (MOU) between the NIEHS/NTP, the NIH Chemical Genomics Center, and the U.S. Environmental Protection Agency (EPA) Office of Research and Development for a cooperative program to leverage the strengths of each group in a new toxicity testing agreement to use high-speed, automated screening robots to test suspected toxicants using cells and isolated molecular targets. These data will be used to set priorities for chemical testing activities with the aim of trying to replace some of the *in vivo* approaches currently in use with *in vitro* studies. An article in Science published February 15 outlined the MOU and the plans for the research program, which addresses the vision for a new toxicity testing paradigm laid out in the National Research Council Report, "Toxicity Testing in the 21st Century: A Vision and a Strategy."

On February 5, 2008 the NTP celebrated the tenth anniversary of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). At that meeting, the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM unveiled their five-year plan to reduce, refine, and replace animal use, while maintaining or improving the protection of human and animal health and the environment. A workshop, "Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations," was held the following day.

He then highlighted some of the topics on the 2-day agenda for this meeting. He pointed out that an important activity for this meeting would be peer review of the draft NTP Brief on Bisphenol A (BPA). The draft brief was prepared by the Center for the Evaluation of Risks to Human Reproduction (CERHR) and released for public comment on April 15, 2008. The draft brief provides the NTP's opinion on the potential for BPA

to cause harm to human reproduction or development at current human exposures. CERHR was created in 1998 and about 20 chemicals have been reviewed for their ability to affect human reproduction and development. The goal of CERHR is to provide an unbiased and complete evaluation of animal and human literature on a compound, but not to develop a quantitative risk assessment.

III. Review of the Draft Brief on Bisphenol A

Dr. Michael Shelby, NIEHS, provided background information on the process used by CERHR to prepare monographs and presented an overview of the draft NTP Brief on Bisphenol A (BPA).

He outlined the format for the discussion on the draft brief: (1) Dr. Shelby would describe the CERHR process and present an overview of the draft NTP Brief; (2) Dr. Richard Wang from the Centers for Disease Control and Prevention (CDC) would present their findings on the biomonitoring of BPA in human tissues; (3) the BSC would give general comments; and (4) public comments would be presented. Next, Dr. Kristina Thayer, NIEHS, would present the scientific evidence supporting the NTP's conclusion on each of the topic areas covered in the draft brief (metabolism and route of administration, exposure, brain and behavior, puberty, mammary gland, prostate gland). Each presentation would be followed by *ad hoc* reviewers' comments and BSC discussion. After all the topics were discussed, the BSC would vote on the NTP's draft recommendations on BPA and as appropriate, make recommended changes. The peer review comments and discussion would be compiled into a report that would be appended to the minutes.

a. CERHR Process

CERHR considers several factors when determining whether to evaluate a substance for its potential as a reproductive and/or developmental hazard for humans: production volume, extent of relevant animal and/or human literature, human exposure, and public concern. Dr. Shelby said the evaluation occurs in three phases: nomination and selection of a substance, preparation of the expert panel report, and preparation of the NTP Brief and NTP-CERHR Monograph. CERHR convened an external scientific panel to prepare an independent report on BPA; the report was released in November 2007. The expert panel report served as the primary supporting document for preparing the draft NTP brief; however, the NTP also considered the public comments and new relevant literature published since the expert panel report was completed. Peer review of draft NTP Briefs can occur by one of two mechanisms: letter review by mail or public peer review. There is scientific controversy regarding whether exposure to BPA adversely affects human health; therefore, the NTP chose to have the BSC, supplemented with *ad hoc* experts, peer review the draft brief in a public forum.

The draft NTP Brief on Bisphenol A was released for public comment on April 15, 2008. Following the peer review today, the peer review comments, BSC's recommendations, and public comments will be carefully considered as the NTP finalizes the brief and

prepares the NTP-CERHR Monograph on Bisphenol A for publication. The monograph will contain the NTP brief, the expert panel report, and public comments on that report.

b. Background Information on BPA

The estimated U.S. production of BPA in 2004 was ~ 2.3 billion pounds. BPA is used in the production of polycarbonate plastics used in the manufacture of various products including food and drink containers, baby bottles, water bottles, and plastic tableware and in epoxy resins used to coat metal food cans and in some dental sealants or composites.

People are exposed to BPA primarily through the diet. According to the CDC, human exposure to BPA is widespread. The 2003 National Health and Nutrition Examination Survey (NHANES) detected BPA in the urine of 93% of the samples collected from ~2500 people six years of age or older in the United States. In smaller studies, BPA has been detected in human blood and breast milk. The estimated daily intake is highest in infants and children; 1 - 13 µg/kg/day in formula-fed infants, 0.2 - 1 µg/kg/day in breastfed infants, and < 0.300 µg/kg/day in adults.

c. Human Studies

Only a small number of studies on BPA have been conducted in humans. Their use in assessing whether BPA is a hazard for humans is limited by small sample size, cross-sectional design, lack of large variations in exposure, and/or lack of adjustment for potential confounders.

The NTP concurs with the CERHR Expert Panel that exposure to BPA may alter the levels of the reproductive hormones testosterone and follicle-stimulating hormone in men exposed occupationally. There is *insufficient evidence* that BPA causes adverse developmental or reproductive effects in humans based on epidemiological studies.

d. Laboratory Animal Studies

For developmental effects reported in animal studies, the literature was separated into “high” dose (> 5 mg/kg/day) and “low” dose (≤ 5 mg/kg/day).

The literature provides *clear evidence* of adverse effects on development at high doses.

There is *limited evidence* of effects on development at low doses with adverse effects being noted on the brain and behavior, the prostate and mammary glands, and early onset of puberty in females.

There is *some evidence* of effects on reproduction in animals exposed only during adulthood including decreased fertility, altered estrous cycling and testicular effects, but the studies are not divided into high and low doses.

e. Review of the Literature

Dr. Shelby noted that the scientific literature on BPA includes Good Laboratory Practices (GLP) compliant “guideline” studies required by regulatory agencies and studies

conducted in academic laboratories. The GLP-compliant studies were multigenerational studies in rats and mice, the sample size usually exceeded 20 animals/group, standardized protocols were used, and record keeping was according to GLP. Oral administration was usually the route of exposure to BPA. In contrast, the academic studies addressed specific experimental questions, each treatment group usually consisted of 10 or fewer animals, and the experimental design varied. Exposure to BPA was via the oral or subcutaneous route. Endpoints evaluated in academic and guideline studies often differed because of differences in the experimental design used in the two types of studies.

The NTP brief focuses on the effects of BPA highlighted by the CERHR Expert Panel Report and other recent publications and evaluates whether the *in vivo* effects are biologically plausible. Consideration was given to whether the *in vivo* effects represent adverse health findings in laboratory animals or in humans, and whether these effects had been reproduced by the same group or in similar investigations by different authors. Limitations in the experimental design that could have had a potential impact on the outcome were also considered.

The NTP did not establish a minimal acceptable sample size, but considered studies with small sample sizes in the context of other studies assessing similar endpoints. Inadequate control for litter effects was considered a significant design flaw. Positive controls were considered helpful, but were not required.

Data from the biomonitoring studies on BPA suggest that current human exposure to BPA is high enough to raise concern. The estimated exposures in infants and children are similar to levels of BPA associated with several “low” dose laboratory animal findings.

f. Draft NTP Conclusions

Dr. Shelby presented the draft NTP conclusions on BPA.

The NTP concurs with the CERHR Expert Panel on BPA that there is *negligible* concern that exposure to BPA causes reproductive effects in non-occupationally exposed adults and *minimal* concern for workers exposed to higher levels in occupational settings. Data from studies in humans are not sufficient to determine if BPA *adversely* affects reproduction; however, there is a suggestion of a possible effect on reproductive hormones, especially in men exposed to higher levels of BPA in the workplace. Effects on reproduction in animal studies are reported only at exposure levels that far exceed those experienced by the general population.

The NTP has *negligible* concern that exposure of pregnant women to BPA will result in fetal or neonatal mortality, birth defects or reduced birth weight and growth in their offspring. This conclusion is based on laboratory studies that provide “clear” evidence of adverse effects on the development in animals exposed during perinatal life, but only at exposure levels that are far in excess of those experienced by humans.

The NTP concurs with the conclusion of the CERHR Expert Panel that there is *some*

concern for neural and behavioral effects in fetuses, infants, and children at current human exposure to BPA.

The NTP has *some* concern for BPA exposure in fetuses, infants, and children based on effects in the prostate gland, mammary gland, and an earlier age for puberty in females. This conclusion is based on laboratory animal studies that provide “limited” evidence for effects on these endpoints at levels of exposure similar to those experienced by infants and children.

Dr. Shelby emphasized that the highest level of concern, “some concern,” is based on limited evidence of developmental effects and that there is uncertainty regarding the “adverse” nature of the effects reported, the long-term health consequences of these effects in animals, and how these effects might be translated to effects in humans. None of the low dose studies in animals measured urinary or blood levels of BPA.

The peer review charge is to determine whether the scientific information cited in the draft NTP Brief on Bisphenol A is technically correct, clearly stated, and supports the NTP’s conclusions regarding the potential for BPA to cause adverse reproductive and developmental effects in exposed humans.

B. Biomonitoring of Bisphenol A in Human Samples

a. Presentation

Dr. Richard Wang, CDC, made the presentation on behalf of his two colleagues, Dr. Needham and Dr. Calafat, who could not be present. He defined biomonitoring as the measurement of the level of a chemical in a biological fluid or tissue. The quality of the data depends on the method used to measure the chemical, its accuracy and precision, as well as the collection and handling of the specimen. On each day that measurements are made, the appropriate reference material is included in the analysis.

Enzyme linked immunosorbent assay (ELISA) and isotopic dilution mass spectrometry (IDMS) are two methods to measure BPA. CDC uses IDMS because of the potential problems with ELISA relating to the specificity of antibody or antigen recognition, and cross reactivity. With IDMS, BPA itself is measured and not a byproduct. Since BPA often exists as a glucuronide or sulfate conjugate in biological tissues, the first step is to deconjugate BPA using an enzyme preparation. Subsequent steps include solid phase extraction, chromatographic separation and quantification using IDMS. One concern is contamination of the biological specimen, especially if the level of the chemical in question is low or the chemical is ubiquitous. Field and laboratory blanks using saline or deionized water are used to evaluate the amount of contamination.

Dr. Wang described the measurement of BPA in specimens from two National Health and Nutrition Examination Survey (NHANES) studies conducted in collaboration with CDC’s National Center for Health Statistics. The NHANES III study evaluated the concentration of BPA in archived urine specimens collected from a nonrepresentative group of adults sampled from 1988 to 1994. BPA was detected in 95% of the specimens

with a median concentration of 1.3 µg/L and a 95th percentile of 5.2 µg/L; the limit of detection (LOD) was 0.1 µg/L.

The second study evaluated the concentration of BPA in specimens collected in 2003 and 2004 from 2,500 people participating in NHANES. The data were representative of the general U.S. population based on age, gender, and race/ethnicity. The specimens were stratified by age: children (6-11 years), adolescents (12-19 years), and adults (20 years and older). BPA was detected in about 93% of the specimens with a median concentration of 2.7 µg/L and a 95th percentile of 15.9 µg/L; the LOD was 0.4 µg/L. This is an important finding as a high prevalence of exposure was found in a representative sample of the U.S. population. Demographically, females had higher levels than males, children had higher levels than adolescents or adults, and non-Hispanic whites' levels were similar to non-Hispanic blacks but higher than Mexican Americans.

Dr. Wang reported on three special studies. The first measured the distribution of free BPA and estimated its conjugates in urine specimens from 30 adults. The frequency of detection of BPA glucuronide was 90%, which comprised about 70% of the total BPA. The significance of this finding is that of the total BPA in human urine, the majority exists as BPA glucuronide and free BPA was only found in about 10% of the urine samples.

In the second study, the levels of total (free plus conjugated) BPA were measured in blood bank serum specimens from 15 adults. Only one specimen contained free BPA with no conjugates at detectable levels (LOD = 0.3 µg/L) in these adults.

The third study evaluated the concentration of BPA in milk from four women. BPA was detected (LOD = 0.3 µg/L) in its free form in all four specimens making up 50% of two specimens and nearly 100% of the remaining two specimens.

The NHANES data and the additional studies suggest that there is a high prevalence of exposure to BPA in the general U.S. population. Bisphenol A is primarily excreted in the urine as the glucuronide conjugate, and free BPA may distribute to breast milk.

Dr. Wang discussed the conditions that need to be considered when measuring an analyte as opposed to using the analyte as an exposure biomarker. In both situations, a validated method using an analytical standard and inclusion of laboratory blanks are essential as well as quality assurance and quality control. Knowledge of the pharmacokinetics including bioavailability is important if the analyte is being used as an exposure biomarker. Other concerns are the stability of the analyte in body fluids, such as BPA glucuronide in the urine, and the inclusion of appropriate field blanks when sampling.

In conclusion, Dr. Wang reiterated the high prevalence of exposure to BPA in the general U.S. population. He identified the need for increased attention to sampling protocols used in the field. He noted the importance of obtaining biomonitoring data on BPA exposure from vulnerable populations as well as toxicology studies for understanding whether exposure is harmful for human health.

b. BSC Discussion

Dr. Katharine Hammond asked Dr. Wang to comment on the apparent differences in the BPA concentrations in the large NHANES study and the blood bank specimens. In the former case, 10% of the BPA was in the free form in the urine whereas in some of the blood bank specimens and in the human milk specimens 50% to 100% of BPA was in the free form. Dr. Wang replied that he did not know why the percentage of free and conjugated BPA were different in urine and milk samples, but it could be due to the solubility of the conjugates in an aqueous solution and free BPA in lipids. However, this possible explanation has not been substantiated, as this was one of the first studies to measure the concentration of BPA in human milk specimens.

A discussion ensued on the inclusion of data where the concentration of BPA was less than the LOD. Dr. Wang replied that there are different ways to handle data points that are reported as being less than the LOD. These include the use of fixed variables, the detection level itself, or the LOD divided by 2 or by the square root of 2. CDC uses the LOD divided by the square root of 2. Ms. Ruthann Rudel said there is no indication that CDC counted a sample with a non-detectable level as a detectable one and Dr. Wang is not imputing or using the LOD to calculate the percentage of samples with a detectable level. Dr. Wang added that the percentage of samples with detectable levels of BPA is based on the concentration that was actually measured. He added that if the level of BPA is below the LOD, one cannot state whether there is or is not BPA only that the level was below the LOD.

Dr. Mark Toraason, NIOSH, asked whether the 10% of samples with free BPA were from a specific demographic, or if this result was perhaps a false negative. Dr. Wang replied that none of the data points had been linked to a demographic because the specimens were collected anonymously.

Dr. Jon Mirsalis asked whether an attempt was made to correlate the results of the urinary BPA concentrations with possible exposure sources, such as plastic water bottles, or whether the analyses were done on random samples. Dr. Hammond thought that NHANES collected a great deal of dietary data and that CDC might evaluate relationships between BPA levels and diet. Dr. Wang replied that NHANES and the special studies were not designed to relate BPA levels to food, dietary information, or water intake. The NHANES analyses only aim to evaluate the prevalence and magnitude of detection of specific environmental chemicals, such as BPA, in a representative sample of the general U.S. population. Dietary information was collected but not with assessment of BPA in mind.

Dr. Barry Timms noted that the LOD was different in the first and second studies namely 0.1 µg/L and 0.4 µg/L, respectively. Dr. Wang replied that two different methods were used in these respective studies; with the earlier study (NHANES III) a gas chromatography–isotope dilution–mass spectrometry method was used and the later study (NHANES 2003-2004) used a high performance liquid chromatography–isotope dilution–tandem mass spectrometry method.

Dr. Paul Howard, FDA, said the agency wished to acknowledge the tremendous effort by CERHR in this evaluation in which over 850 manuscripts were reviewed. The CERHR report is an important step in the evaluation of the safety of BPA. The brief is an environmental health resource for the public and health regulatory agencies. As noted in the brief, it is a hazard identification document and not a quantitative risk assessment. The principal regulatory agency for BPA is the FDA and the agency will use the NTP brief in its evaluation of BPA. Dr. Howard outlined FDA activities on BPA. Early in 2007, the FDA Center for Food Safety and Applied Nutrition initiated a formal reexamination of the safety of BPA. In April 2008, the FDA Commissioner formed an agency-wide task force to (1) review current research and new information on BPA, (2) assemble an inventory of FDA products that may contain BPA, (3) explore what is known about the safety of BPA, and (4) to generate a report. On June 6, 2008, the FDA Commissioner announced subcommittee of the FDA Science Board would hold a public meeting in late summer to review the draft FDA task force report and report its findings to the FDA Science Board in the fall.

C. Public Comments on the NTP Draft Brief

a. People for the Ethical Treatment of Animals

Mr. Joseph Manuppello, PETA, said PETA supports the BPA-free Kids Act of 2008, which would prohibit the use of BPA in children's products and require the CDC to study the health effects of BPA exposure in all age groups and pregnant women. PETA maintains that too much attention is placed on animal testing at the detriment of epidemiological studies. He referred to the animal studies in which cigarette smoke, benzene, and asbestos were tested that showed that animals were refractory to these compounds although they have been known to be human carcinogens for many decades. He said CERHR was too reliant on animal tests for BPA even though the CERHR expert panel stated that animal tests are problematic because the animal's food and water may be contaminated with phytoestrogens and BPA, respectively, which would interfere with the measurement of endocrine mediated end points. Different animal species and strains demonstrate different sensitivity to estrogens with BPA causing neural and behavioral effects in some strains and abnormalities in the mammary and prostate glands in others. The expert panel was uncertain how to interpret animal studies using low dose levels of BPA when no health effects were reported in some studies but adverse effects in others. He referenced a two-generational study published in 2008 that showed no adverse reproductive effects in mice exposed to BPA while shorter studies have shown effects. He questioned the relevance of animal studies since humans conjugate BPA to the harmless BPA glucuronide three times faster than rats. He suggested that the BPA developmental effects be addressed by precautionary regulation, and it is unlikely that further animal tests would provide more useful information.

b. Polycarbonate/BPA Global group

Dr. Steven Hentges, Polycarbonate/BPA Global group, provided comments. Since the brief will be used by the public and government agencies, he believed it is critical that the conclusions are scientifically sound and supported by reliable scientific data. He thought

that the CERHR expert panel review process was reliable and scientifically sound because the panel was consistent in its published guidelines for selection of the studies for evaluation. Although CERHR used the expert panel report as its supporting information in preparing the NTP brief, the conclusions of the brief deviate from the conclusions of the CERHR expert panel report in ways that suggest the NTP did not apply the same or equivalent criteria in their selection of the studies for review. In his opinion, this has resulted in an inconsistent evaluation of the scientific quality and validity of the studies and, thus, the conclusions are based on inadequate studies.

He disagreed with the conclusions in the draft NTP brief regarding “some concern” for early onset of puberty in females because the evidence cited refers to two studies determined by the CERHR expert panel to be of limited utility based on the measurement used to determine the onset of puberty in one study and the use of subcutaneous injection of BPA in the other. Based on the weight of evidence, he believed there is insufficient evidence for a conclusion.

He disagreed with conclusion in the draft NTP brief regarding “some concern” of adverse effects for the mammary gland. There is limited evidence for the two cited studies. He said the NTP's reliance on the two studies that used subcutaneous injection does not satisfy the NIH commitment to apply rigorous scientific standards to ensure the accuracy, reliability, and reproducibility of the research results. In his opinion, a more appropriate conclusion would be insufficient evidence for a conclusion on the mammary gland.

He disagreed with conclusion in the draft NTP brief regarding “some concern” of adverse effects for the prostate gland. He questioned NTP's reliance on two studies as support for its conclusions. One study that found effects in fetal mice but not in adults, has not been replicated or corroborated, and, thus, is not adequate to support a conclusion of some concern. The CERHR expert panel said the second study was of limited utility because neonatal rats received a single subcutaneous injection. The acceptance of the data of this study by NTP is based on a recent, flawed study that reported no differences in blood concentration of free or conjugated BPA as a function of route of exposure. Based on this limited evidence of available data the more appropriate conclusion for the prostate gland would be insufficient evidence.

c. Environmental Health Sciences

Dr. Peter Myers, Environmental Health Sciences, provided comments on the issue of risk for adult exposure to BPA. NTP currently discounts these risks because of a mistaken conclusion that adult exposure to the active form of BPA is insignificant, yet available scientific data show this conclusion is incorrect. More sensitive assays show repeatedly that the median levels of unconjugated BPA in humans are above those that cause a wide range of effects in animal experiments including lowered adult sperm count and development of insulin resistance, a pre-condition to type II diabetes. Low levels of BPA cause changes in gene expression in normal human breast cells, which subsequently develop characteristics of highly aggressive breast cancer cells. The BPA-induced gene expression profile in breast cancer cells results in decreased patient survival, large tumor size, and high histological grade.

d. Dr. Frederick vom Saal

Dr. Frederick vom Saal, University of Missouri, discussed the dichotomy between academic, governmental, and standardized toxicological studies. The latter studies are based on paradigms developed in the last century while academic and government studies use state-of-the-art approaches. The panel needs to consider whether new methods are deemed less valuable because they are not using standard approaches. The details of the traditional pathological approaches that were used to study the prostate were crude and could not have revealed the effects observed in more recent studies using state-of-the-art methodologies. He said he has published relevant studies on the effect of estrogen and estrogen disrupting chemicals on the prostate and has shown that hyperplastic cells within the developing prostate are progenitor cells. These studies have been replicated and are predictive of stimulation of the prostate and an increase of androgen receptors in adulthood. The standardized GLP studies did not replicate these findings, possibly because they used the CDS rat and CD1 mouse that required massive positive control doses to show an effect and thus were insensitive to a low dose of BPA. He believed the guideline studies are designed specifically to be insensitive and raise doubt, and he is frustrated when the newer techniques are criticized.

e. Natural Resources Defense Council

Dr. Sarah Janssen, Natural Resources Defense Council, said it is extremely important that the BPA report be of the highest scientific quality. NTP evaluations are an invaluable resource for regulatory agencies and serve an important role in insuring that public health takes precedence over economic interest. She was pleased that the NTP expanded the level of concern to developmental outcomes in fetuses and sensitive children. NRDC believes that NTP would be justified in raising the weight of evidence for developmental toxicity at low doses of BPA in fetuses and children. She also thought more weight should be put on the significance of precancerous lesions in the prostate and mammary gland although the progression to cancer has not yet been observed. One study found that normal human breast tissue exposed *in vitro* to environmentally relevant levels of BPA resulted in gene expression changes consistent with those of a highly aggressive type of breast cancer that is associated with poor survival. Perinatal exposure to BPA has been shown to alter prostate development and predispose animals to preneoplastic lesions and the development of high-grade preneoplastic intraperitoneal neoplasia (PIN) lesions that are highly predictive for the development of cancer in men. The relevance of animal models and the significance of precancerous lesions for human disease are clear and warrant a stronger level of concern from the NTP. She encouraged the rapid compilation of the monograph so that government agencies could use it for guidance when considering approaches to limit exposure to BPA.

f. Environment California Research and Policy Center

Ms. Rachel Gibson, the Environment California Research and Policy Center, said that according to a recent evaluation by CDC, roughly 93% of Americans have detectable levels of BPA in their bodies, and because of its short half-life in people, this suggests that most Americans are exposed continuously to the chemical via multiple sources. Alarming, many Americans are exposed to levels above the current safety level set by

US EPA, which is significantly higher than the levels shown more recently to cause a wide array of health effects in animals. BPA is leached from polycarbonate baby bottles and coated cans of infant formula at levels ranging from 5-10 ppb and higher. Further, a breast-fed baby is exposed to BPA commonly found in breast milk, and those fed infant formula are also exposed during the first year of life. Infants are exposed prenatally to BPA and this is serious and a good reason to be concerned.

The Canadian government recently classified BPA as toxic according to the Canadian Environmental Protection Act, which will trigger the ban on BPA in baby bottles in Canada. The US EPA recently announced it would review its regulatory policy of BPA as a result of recent scientific studies on the chemical and the Canadian action. Legislation has been introduced in Congress and in a dozen or more states to prohibit the use of BPA in products used by infants and children. Many retailers, including Wal-Mart and Toys 'R' Us, announced they would phase out the sale of BPA-containing bottles, and leading manufacturers of baby bottles and water bottles will stop selling products containing BPA. It is hoped that the NTP will take a strong position on BPA given the scientific evidence suggesting its potential harm to infants and children.

g. Dr. Maricel Maffini

Dr. Maricel Maffini, Tufts University Medical School, said his research group is funded through the NIEHS, but he has no conflict of interest or financial disclosure to reveal.

He is very pleased that the conclusion of “some concern” for BPA exposure in fetuses, infants and children is based on effects observed in the rodent mammary gland, but he would be more satisfied if the qualifier “some” was withdrawn.

Exposure of pregnant mice to BPA in his group’s studies is through osmotic pumps surgically implanted under the skin. Although this route of administration is controversial, it is important to note that the fetus is exposed through the internal milieu of the mother regardless of how a chemical is administered to the dam. They have found both mammary ductal hyperplasias and carcinoma *in situ* in the developing adult rat exposed during the perinatal period to BPA. These findings are important because the sequential steps in the natural history of breast cancer are: ductal hyperplasia, atypical ductal hyperplasia, carcinoma *in situ*, and invasive carcinoma. The mammary carcinomas *in situ* were of the cribriform type, a common type of tumor observed in women.

Based on the results obtained using laboratory animals exposed perinatally to various doses of BPA, he is confident that the conclusions of the study are relevant to the human population at large. Considering how accurately the rodent animal model for DES exposure predicted the outcomes observed in humans over 20 years later, he feels that the level of concern for BPA should be higher.

h. Environmental Working Group

Dr. Sonya Lunder, Environmental Working Group (EWG), said the EWG supports NTP’s assessment and concurs with the conclusion that BPA exposures pose concern for

human reproduction and development based on BPA's toxicity, its detection in about 93% of the US population, and the levels to which people are exposed are harmful to laboratory animals. NTP's draft assessment is an improvement over the expert panel report. The EWG is pleased that the NTP correctly considered the studies using subcutaneous exposures. However, the EWG believes the draft brief does not fully reflect the magnitude of health risks faced by children and other vulnerable populations. NTP used findings from 12 low dose studies to conclude "limited" evidence of developmental toxicity, but these studies are part of a larger body of literature that consistently reports evidence of harm at low doses and, thus, the EWG recommends that NTP increase the weight of evidence for developmental toxicity to "some" or "clear" evidence. The doses to which animals were exposed in these 12 studies overlaps with the daily intake of infants and children particularly within the first year of life. Since these studies showed effects to the developing brain, reproductive organs, and timing of puberty, the EWG believes that the NTP is justified in increasing the level of concern for fetuses, infants and children from "some concern" to "serious concern."

i. Making Our Milk Safe (MOMS)

Ms. Mary Brune, Making Our Milk Safe (MOMS), said her group is grateful that NTP reviewed the science relating to BPA and that the draft report discusses the potential for developmental harm to fetuses, infants and children. The demand for baby bottles that are manufactured without BPA is increasing. This is a prudent move as infant's bodies, brains, and endocrine system are still developing at this critical time of development. Her organization feels that an increase in the level of concern is warranted along with further study of the potential risk to children during development. It is necessary to ensure that chemicals linked to reproduction and developmental toxicity are studied thoroughly and, when necessary, are removed from products that pose the greatest risk of exposure to the most vulnerable populations.

j. Dr. Rochelle Tyl

Dr. Rochelle Tyl, Research Triangle Institute, said she was the study director for a three-generation rat study and a two-generation mouse study on dietary BPA that were published in 2002 and 2008, respectively. She challenged the statement from Dr. vom Saal that the testing guidelines are antiquated. The rat study began in 1998 and the most current guidelines for studies with rats with enhancements were used. The mouse study began in 2001 and used the OECD test guidelines for mice and included enhancements such as positive controls and close examination of the prostate and mammary glands.

k. Ms. Retha Newbold

Ms. Retha Newbold, NIEHS, said her statement reflected her own opinion, and not that of the NIEHS. She is pleased with the revised draft, but was disappointed that one of her studies, published in Reproductive Toxicology in 2007, was excluded by the NTP in arriving at its conclusion on the potential risk of BPA for humans. This study documents the long-term adverse effects of neonatal BPA exposure in an experimental animal model and showed a statistically significant increase in ovarian cysts, cystic endometrial hyperplasia of the uterus, as well as more serious uterine pathologies including adenomyosis, fibroids, atypical hyperplasia, and stromal polyps. Additional changes in

the oviduct and uterus as well as remnants of the Wolffian duct, associated with exposure to estrogenic substances, were seen in all dose groups exposed to BPA. She asked the NTP to reexamine her study and revise the current draft to include this supporting scientific evidence for long-term effects of BPA on the mammary gland, uterus and the ovary.

D. Presentation on the Major topics of the NTP Draft Brief

Dr. Kristina Thayer, NIEHS, presented information on the metabolism of BPA and its effect on behavior, puberty, the mammary gland, and the prostate gland. The slides from her presentation are available at <http://ntp.niehs.nih.gov/go/9741>. She briefly outlined both academic studies and multigenerational GLP compliant studies on which the NTP's decisions were based. She described the reasons for the NTP's decision on assigning a level of evidence for each end point. They ranged from "clear evidence of no adverse effects" to "clear evidence of adverse effects." Following each section of her presentation, the *ad hoc* and BSC members provided comments on that topic in the draft NTP Brief. Upon completion of the discussion on all topics, the BSC voted on the proposed conclusions in the draft brief. The discussions are assembled into a separate document, which was reviewed by the *ad hoc* members and all the BSC attendees and approved for publication and is appended to the minutes as the "Peer Review Report for the Draft NTP Brief on Bisphenol A."

a. Metabolism

Dr. Thayer discussed metabolism in relation to age of exposure and how this impacts consideration of studies that use a non-oral route of administration. The unconjugated or "free" form of BPA is considered to be the biologically active form, and oral studies are considered the most relevant for human risk because human exposure is mostly dietary. BPA undergoes glucuronidation in the gut and the liver and sulfation in the liver to more water-soluble compounds that are excreted in the urine.

Adult rodents conjugate BPA to water-soluble and less toxic compounds more rapidly following oral administration compared to non-oral routes of exposure such as subcutaneous injection. For this reason, studies using non-oral exposure regimens have been considered less meaningful in health evaluations of BPA. However, in the NTP Brief, the NTP considered studies that used subcutaneous administration to neonatal rodents to be useful in the evaluation. Studies that used subcutaneous injection in adult animals were only considered useful for hazard identification.

Neonatal rats metabolize BPA less efficiently than adult rats due to the immaturity of the relevant enzyme systems. This difference has not been addressed specifically for humans with BPA exposure, but there are indications of a similar immaturity of the same enzymes in fetuses and young children. Based upon studies in rodents, the NTP concluded that first pass metabolism is less efficient in younger animals. Metabolism of BPA in neonates at doses to which humans are exposed might be more efficient, but at present this is unknown.

The route of exposure affects the rate of metabolism of BPA in adult rodents with free BPA blood concentrations being higher in adult animals exposed subcutaneously, compared to exposure to the same oral dose. NTP concluded that the data from studies where non-oral routes were used are not particularly useful to interpret potential health risks from dietary exposures because of the rather high serum levels of free BPA compared to oral dosing. The implication of less efficient first pass metabolism in neonates is that the route of administration in young animals is less important. The NTP considered studies where BPA was administered by the subcutaneous route useful in assessing BPA health effects in rodents exposed as neonates.

b. Brain and behavioral effects

Dr. Thayer discussed the more controversial “low dose” effects and NTP’s strategy in arriving at the level of evidence. She discussed issues relating to replication, data limitations, and NTP’s weight of evidence for concluding that there was limited evidence for adverse behavioral effects to BPA.

Reviewing the literature was challenging as there was a lack of reproducibility of methodologies, strategies, and the specific tests investigators used. Collectively, the literature indicated a loss of sexual dimorphism. Effects highlighted in the academic studies would not have been detected in the GLP-compliant multigenerational studies, as behavior was not assessed in the latter.

The NTP concluded that there is “limited” evidence of adverse effects based on a number of well-conducted studies that reported effects in the low dose range of less than 1 mg/kg of BPA. A higher level of evidence than some concern could not be proposed as there was uncertainty regarding adversity and human relevance.

c. Puberty

Dr. Thayer discussed the data on sexual maturation from mice and rats separately. In mice, the literature was inconsistent with three positive studies and three negative studies. In rats, one out of eight studies was positive and the remainder was negative.

In the rat, vaginal opening and the first estrus tend to occur at the same time, thus vaginal opening is an accepted surrogate of puberty. In mice, the most consistent difference between the “positive” and “negative” studies was the endpoint used to measure puberty: time of first estrous or vaginal opening. In rodents, first estrous is considered to be the indicator of puberty in females and, in contrast to the rat, this event in mice does not necessarily occur coincident with vaginal opening. There was some measure of replication of the three positive mouse studies relating to age at first estrus and the interval between vaginal opening and first estrus. Since vaginal opening and first estrous are not tightly coupled in the mouse, these endpoints may be providing different information about sexual maturation, thus there might be unique drivers to these events.

One possibility for variable responses in rats and mice might be species differences and/or strain responsiveness. There is some indication that puberty in mice can be perturbed by external factors such as exposure to a mature male, a finding that is less

robust in the rat. Not finding effects on vaginal opening or time of first estrus in rats limits NTP's confidence of these findings, but it does not negate the positive mouse studies.

The NTP thought these findings present "limited" evidence of adverse effects based on the consistency in the mouse studies on the measure of first estrus. The NTP did not believe that a higher level of evidence was warranted because of the lack of effect in the rat and that an interpretable end point, such as age at first estrus, would be more robust than the interval between vaginal opening and age at first estrus.

d. Mammary Gland

Dr. Thayer discussed several studies in rats (Wistar, Wistar-Furth and Spague Dawley) and one strain of mice (CD1) in which pre-neoplastic lesions or other effects were reported in mammary gland tissue following low dose exposure of fetuses to BPA while *in utero*. These reports of pre-neoplastic lesions are relevant to humans, as similar changes have been described as risk factors for invasive breast cancer in women.

The NTP's conclusion for the mammary gland is "limited" evidence for adverse effects as opposed to "insufficient" because several studies identified the mammary gland as the target, and the types of effects were consistent with those that predispose mammary tissue to disease later in life. In addition, the authors clarified during the public comment process that litter effects were controlled for by using only one animal per litter.

The draft brief did not propose a higher level of evidence because (1) there was no evidence of the progression of the pre-neoplastic lesions to tumors, (2) the small sample size that detected carcinoma *in situ*, (3) none of the studies reported statistically significant increases in incidence, (4) the use of the subcutaneous mini-pump for administration of BPA to the pregnant dam, and (5) the inclusion of greater than 50% of DMSO in the mini-pump, although the NTP was not convinced that the use of 100% DMSO accounted for the reported effect because of the consistency of the types of lesions reported when 100% or 50% DMSO was used.

e. Prostate Gland

Dr. Thayer discussed three studies in which developing embryos were exposed to low levels of BPA that resulted in lesions in the prostate gland. One study reported preneoplastic intraperitoneal neoplasia (PIN) and a second morphometric effects. Rodents are normally resistant to developing PIN lesions and tumors, thus researchers use either genetically modified animals or hormonal manipulation by injecting estrogen and testosterone into adult animals to induce these lesions.

The NTP reached a conclusion of "some concern," because the study in BALB/c mice confirmed that the prostate gland was a target tissue and because the other two studies reported effects at 10µg/kg, which overlaps with the range of intake estimated for infants. In addition, the NTP felt additional data published in a third study following the expert panel meeting supported consideration of the findings from subcutaneous injection

studies in neonatal animals.

The NTP thought that the effects on the prostate presented “limited” evidence of an adverse effect because there were two key studies and one supplemental study that identified the prostate gland as a target organ. The study on the morphometric changes was considered of high utility by the expert panel. “Limited” evidence seemed most appropriate since there is no information whether PIN lesions progress to a tumor or the long-term implications of the morphometric findings.

f. BSC Recommendations

The charge to the BSC and *ad hoc* members was to determine whether the scientific information cited in the draft NTP Brief on Bisphenol A is technically correct, clearly stated and supports the NTP’s conclusions regarding the potential for BPA to cause adverse reproduction and developmental effects in exposed humans.

The BSC recommendations on the conclusions in the draft NTP brief are detailed in the peer review report (see Appendix A).

Dr. Bucher said he truly appreciated the extraordinary discussions on BPA and thanked the BSC and *ad hoc* reviewers for the time and dedication they gave to the peer review. He expressed his gratitude to the CERHR staff for their outstanding effort in compiling the information on BPA. He thanked Dr. McCarver for the exemplary manner in which she directed the peer review.

Dr. Shelby expressed his thanks to the BSC and the *ad hoc* reviewers for active participation in the peer review. He appreciated Dr. Bucher’s support in getting the document completed, and Dr. Thayer who mastered the massive literature on BPA.

June 12

The meeting resumed on June 12, 2008 at the Radisson Hotel Research Triangle Park, Research Triangle Park, North Carolina at 8:30 a.m. Two additional *ad hoc* members of the BSC were in attendance: Max Costa, NYU Medical School and Gregory Kedderis, private consultant.

Dr. Bucher welcomed those who were not in attendance at the meeting on June 11, 2008. He briefly outlined the agenda for the day which would include the discussion of the concepts on NTP nominations to the testing program, a concept review on an NTP and NIEHS Investigative Research Support Contract, a report on the Technical Report Review Subcommittee meeting held in February 2008, the introduction of an activity by Dr. Paul Foster in which the NTP will propose criteria for evaluating outcomes in reproductive, developmental and immunotoxicity studies, and a report on the host susceptibility program.

IV. Nominations to the Testing Program

Dr. Scott Masten, NIEHS, provided background information on the source of the

nominations and how they are incorporated into the NTP testing program. Nominations arise from many parties, primarily from other federal agencies and NIEHS and NTP staff. A federal interagency group reviews the nominations and public comments are sought through a Federal Register notice. Substances are selected for study based on their known or anticipated human exposure, production level, suspicion of toxicity based on structure or existing health effects data, availability of adequate toxicological data, and public concern. Research concepts are prepared by NTP study scientists and reviewed internally before presentation to the BSC. The BSC is asked to advise the NTP on the merit and priority of the studies outlined in the research concepts. The NTP revises the research concepts based on the BSC's input. The NTP Executive Committee reviews the revised research concepts before the NTP begins the proposed study programs.

There are six proposed research programs for review: 2,2'-dimorpholinodiethyl ether (DMDEE), an industrial chemical; 2-ethylhexyl *p*-methoxycinnamate (EHMC), a sunscreen constituent; tetravalent and pentavalent vanadium compounds, drinking water contaminants and dietary supplements; melamine and cyanuric acid and furan, food contaminants that have been previously evaluated by the program for which there are outstanding issues that need to be addressed; and 4,7,10-trioxatridecane-1,13-diamine, an industrial chemical that is recommended for a limited set of studies.

The charge to the BSC is to determine whether sufficient justification is provided for the use of the NTP's testing program resources to carry out these proposed research projects as outlined in the draft research concept documents. Each document outlines key issues, data gaps, and hypotheses and/or specific aims that the program plans to address. The BSC is asked to comment on the clarity and validity of the rationale for the proposed research program, the merit of the program relative to the goals of the NTP, the scope of the proposed program and its appropriateness relative to the public health importance of the issue under study, and the priority of the proposed research program.

Dr. Toraason asked about the name change and the approach now being used for the review of NTP projects compared to chemical nominations that was used in the past. Dr. Masten replied that very little has changed except more information regarding proposed studies is being provided to the BSC and public for review. Dr. Bucher added that the purpose of the review has not changed. This is the third meeting that the NTP has brought proposed research projects to the BSC and to the public. The primary purpose is to allow the public access to the initial thoughts of the program.

A. 2,2-Dimorpholinodiethyl ether (DMDEE)

a. Presentation

Dr. Richard Irwin, NIEHS, discussed the concept for DMDEE, which was nominated by the National Cancer Institute because current production exceeds one million pounds and DMDEE is not included in the EPA High Production Volume (HPV) program. There is significant potential for occupational exposure by inhalation and dermally, but virtually no data are available on this compound. It is used as a catalyst in the manufacture of polyurethane foam and hot-melt urethane adhesives. Due to the high temperatures used

in production, there is an opportunity for the catalyst to escape. There is also the possibility of exposure to unreacted catalyst during the machining of foams into the finished product such as slabstock foam products used in furniture and seat cushions. Based on its structure, there is a potential for the formation of N-nitrosomorpholine, a known rodent carcinogen during metabolism.

The Material Safety Data Sheet (MSDS) lists DMDEE as a skin, eye, and respiratory irritant. It has a very low vapor pressure, is miscible with water, and has a pH of 10.4.

The first aim is to examine the absorption, distribution, metabolism, and elimination (ADME) of DMDEE and to specifically monitor for the formation of N-nitrosomorpholine or N-nitroso(2-hydroxyethyl)glycine during metabolism. The second specific aim is to examine its DNA reactivity using bacterial mutagenicity studies, the formation of DNA adducts, and its activity in the Comet assay. These studies will provide basic information to determine whether additional studies are warranted.

b. BSC Discussion

Mr. William Janzen, a BSC reviewer, thought the research program was clear but not completely developed. Some predictive software programs have shown species and sex differences of toxicity and this information should be reviewed. The concept is well aligned with the goals of the NTP and evaluates a possible hazard that did not fit within the EPA's scope of testing. He thought the program should be assigned a moderate priority for study.

Dr. Michael Pino, a BSC reviewer, thought the TOPKAT structure activity relationships showing high predictability of carcinogenic activity should be included in the document. He thought the program has merit relating to the NTP's goal of providing information on potentially hazardous substances. He agreed with the approach in the specific aims and assigned the program a moderate to high priority.

Dr. Irwin responded that routinely the NTP tests both sexes of rodents, and will monitor for the parent compounds and its metabolites.

Dr. Christopher Bradfield asked whether cancer studies would be performed if N-nitrosomorpholine was identified in the ADME studies. Dr. Irwin replied that generally when a nomination is received, specific tests are not requested. Once the metabolites are known, the program will then decide whether additional studies are warranted. If the potentially carcinogenic metabolite was not found, NTP would contact NCI to inquire whether they have more specific concerns about DMDEE. Since it is a high-production compound with a certain potential for human exposure, the NTP might proceed with a toxicological evaluation and possibly a two-year study, depending on the risk of exposure and NCI's potential interests.

Dr. Mirsalis asked why the micronucleus assay is not included in the genetic toxicology battery and wondered if there is a technical reason for omitting it. Dr. Irwin responded that this assay is usually included as part of a prechronic study and currently there are no

plans for a prechronic study.

Dr. Toraason thought that an *in vivo* Comet assay should be performed as it is used as a biomarker for human exposure. Dr. Irwin replied that the Comet assay would be done *in vivo*.

B. 2-Ethylhexyl *p*-methoxycinnamate (EHMC)

a. Presentation

Dr. Michael Wyde, NIEHS, discussed 2-ethylhexyl *p*-methoxycinnamate (EHMC), a common ingredient used in over 2300 sunscreens and personal care products as an ultraviolet filter for UVB radiation.

EHMC serves as a protective barrier in the stratum corneum against long-term exposure to UV radiation that could result in skin cancer. EHMC was nominated by NCI for toxicological evaluation because limited data exist in the published literature. EHMC is weakly estrogenic, stimulates proliferation of MCF-7 cells, elicits a positive response in *in vitro* transactivation assays, and has reproductive effects. It is not a skin irritant or a photosensitizer, is not mutagenic in bacteria, and has a high oral LD50 in mice. Human data are primarily limited to transdermal studies and in animals less than 3% is absorbed. One study showed a decrease in serum T4 levels and an increase in TSH levels only at low doses of EHMC.

A two-generational, reproduction study concluded there is no influence on sexual landmarks of pups and the authors estimated a NOAEL of 450 mg/kg. The authors cited a slight decrease in implantation rates in the F1 and F2 generations at doses of 1,000mg/kg. Dr. Wyde said he disagreed with the interpretation of this study, as there was a statistically significant decrease in the number of implantation sites, which was concordant with reduced litter size. Also there was an increase in the number of stillborn pups, a reduced viability index, and reduced pup weight gain in both generations. There were mixed results in two uterotrophic assays with one positive and one negative outcome.

The 2-ethylhexyl moiety in EHMC may indicate potential carcinogenicity as the 2-ethylhexyl diester of phthalic acid and adipic acids were carcinogenic in NCI/NTP bioassays. It is unknown whether the metabolism of EHMC results in the formation of 2-ethylhexanol or 2-ethylhexanoic acid, two known developmental toxicants.

The research program proposes to evaluate the differential ADME and metabolic profiles comparing the dermal and oral routes of exposure and to determine if 2-ethylhexanol or 2-ethylhexanoic acid is formed. These studies will be followed with subacute, subchronic, and chronic *in utero* studies in rats and mice to characterize toxicity and measure the levels of male and female sex hormones and thyroid hormones. Photodegradation and photoisomerization products will also be identified. This would be followed by a robust multigenerational study in the rat incorporating estrogen-sensitive end points.

The use of EMHC is widespread and chronic. The metabolism and clearance of EHMC in children is unknown. Likewise, it is not known whether the stratum corneum is as protective in children as in adults. The data generated from these studies would increase the scientific basis on which regulatory agencies could interpret a toxic effect associated with exposure to sunscreens that contain EHMC.

b. BSC Discussion

Dr. Kerkvliet, a BSC reviewer, found this concept difficult to evaluate, although the rationale of the potential issues was clearly developed. This may have been due to the conflicting results from oral exposure and a concern regarding the adverse effects after dermal exposure. She thought the proposed studies comparing the two routes and the possibility of an age effect were important and that more information is necessary before multigenerational studies are implemented. She asked whether EMHC could be brought back to the BSC after the ADME studies are completed for the BSC's input into future studies.

Dr. Boekelheide, an *ad hoc* reviewer, provided input by telephone. He agreed that an extensive study of EHMC is justified based on its widespread use, life-long exposure, and the potential for endocrine disruption especially in children, who may have lower metabolic capacity than adults. The background material provided is out of date, and fails to consider the potential effect of nanoparticle formulations in sunscreen on the absorption and photodegradation of EHMC. New literature on the stability and toxicity of EMHC should be considered in the BSC's evaluation.

He agreed with the comparison of studies using the dermal and oral routes. He acknowledged the difficulties of designing a robust study covering a wide dose range, but this is important in terms of metabolism when different routes of exposure are used. He suggested that the subcutaneous route be considered and thought that a multigenerational study is warranted.

The issue of nanoparticles, photoproducts, and susceptible subpopulations needs to be considered in the concept's development. Nanoparticles may either inhibit or enhance absorption, and although little is known of this possibility, it ought to be considered in the design of the studies. Photoisomerization could substantially alter the chemical nature of EHMC, and some of the newer publications suggest this may be the case. Thus, he suggested the inclusion of an additional group of animals that would receive a combined UV plus EMHC exposure.

He said further discussion of the potential metabolism of EHMC to known developmental toxicants is warranted including a calculation of the concentration of the metabolites that pose a hazard. He gave this concept a moderate priority.

Dr. Wyde said the NTP would take a tiered approach to the ADME studies. The use of oral or dermal exposures would be dependent on the toxicokinetic data. The two-generational study would, of necessity, be through oral exposure. Study scientists do an

exhaustive literature search for new publications and all the latest relevant literature were incorporated into the concept. Recent data suggest that nano capsules increase the time of contact with the skin, and the testing of nano emulsions or nano capsules will be considered. The suggestion of using a subcutaneous route is interesting and might be used for two-year studies.

c. Public comments

(i) BASF Corporation

Dr. Raymond David, BASF Corporation, said his company manufactures OMC, the name for EMHC in the market place. Less than 1% of EMHC penetrates through human skin, and sunscreen partitions primarily in the stratum corneum. Since nano encapsulation increases the residence time in the skin, which is the reason for including it in sunscreens, a nano preparation may not be the best choice for testing. BASF completed uterotrophic and Hershberger assays and both were negative, thus OMC does not appear to be an endocrine disruptor. In one study, animals received 900 mg/kg for four weeks and no relevant endocrine or hormonal effects were noted. A continuous breeding study over a couple of generations would not provide any in-depth information, and he did not believe it is warranted.

EMHC is hydrolyzed to 2-ethylhexanol and cinnamic acid; the former is a weak carcinogen, but cinnamic acid is not. OMC has been tested in photocarcinogenicity studies, and it delays the onset of skin cancer, an advantageous outcome for a UV absorber in sunscreen. This finding contradicts an earlier study using skin painting where papillomas were found on 1 or 2 animals following 40 weeks of exposure. The European Union reviewed EMHC and found minor changes in clinical pathology in subchronic studies. He thought that another long-term study is not warranted.

(ii) People for the Ethical Treatment of Animals

Mr. Joseph Manuppello speaking on behalf of PETA said his comments were prepared by their scientific consultant, Dr. Nancy Douglas. The NCI based its nomination of EMHC on its concerns for human exposure and its epigenetic and reproductive effects.

PETA found a number of relevant publications after the supporting document was completed. He asked the BSC to conduct a thorough review of all the existing data before making a decision regarding the need for extensive new studies.

He questioned why the NTP is proposing oral studies since human exposure is expected to be exclusively dermal. He was unsure why Dr. Wyde disagreed with the interpretation of the multigenerational study, although a recent study using oral exposure reported no adverse effects on a wide-range of reproductive parameters. However, general toxicity, manifest as reduced food intake, liver effects, and stomach erosion interfered with the interpretation of the effects of EMHC on the implantation rate and onset of puberty. Additional oral studies are unlikely to provide useful information for an assessment of

EMHC toxicity. The proposed animal tests do not differ significantly from previous studies on the estrogenic and carcinogenic potential of EMHC. As a result, they will not address the ambiguities of the existing data that prompted this nomination.

He enquired as to why epidemiological approaches have not been considered despite EHMC's widespread use for a number of years. Several large-scale international studies have already begun to assess the health effects of sunscreens containing EHMC. He asked why the NTP has not considered standard *in vitro* tests including phototoxicity assays in yeast, 3T3 cells, photomutagenicity studies in bacteria and CHO cells, and mitochondrial gene expression assays in cultured human cells. He said the extensive proposed studies in both rats and mice without consideration of relevant *in vitro* and epidemiological approaches is not in keeping with the principals of the three Rs. He urged the NTP to reconsider the large number of animals that will be used for the proposed extensive animal testing and instead to utilize appropriate *in vitro* assays.

BSC Discussion (continued)

Dr. Mirsalis asked Dr. David whether the reproductive studies were conducted under GLP guidelines and whether BASF would provide copies of the studies to the NTP for consideration to ascertain if additional tests are in fact necessary. Dr. David replied that BASF conducted the studies in their GLP laboratory. His company has offered the studies to regulatory agencies for risk assessment purposes. BASF would certainly provide robust summaries of the reports, but due to the uncertainty of REACH legislation, he is unsure if his company would provide the reports unless they are maintained as confidential.

Dr. Jim Riviere said *in vitro* assessments are needed on the formulations to ascertain their absorption properties before one embarks on a large study. Most of the encapsulated formulations are designed to increase the residence time on the skin surface but not on penetration. He agreed that photoisomerization is an expanding area. He would be very cautious about studies using the subcutaneous route because it would completely abrogate the ability of the stratum corneum to selectively absorb the test article.

Dr. Howard, FDA, said EHMC falls under the regulatory purview of the U.S. Food and Drug Administration. The FDA has evaluated this nomination and the available literature and concurs with the proposed dermal and oral toxicokinetic studies as well as a robust multigenerational study. Dr. Howard agreed with the comments regarding formulation. There are overwhelming statistical difficulties when evaluating the photoactivation of a compound while dosing an animal with a robust amount of ultraviolet light, and there are technical difficulties in ascertaining any underlying mechanism(s). People have been exposed to nano-formulated EMHC only recently, but any adverse effects from prior exposure to EMHC should not be discounted.

Dr. McCarver was concerned about the exposure of babies and young children at the beach to EMHC. Young mothers, aware that sunburn increases the risk of skin cancer, are vigilant in applying sunscreen to young children. In addition, the elderly who have thin skin should be considered in the design of these studies.

Dr. Tracie Bunton had concerns about the relevance of the data that would be collected from the animal studies and the use of NTP resources. More preliminary studies are needed before the NTP embarks on a large-scale animal-testing program. She asked whether any epidemiology studies have indicated that EMHC or its metabolites pose a risk, and whether human toxicokinetic data exist to relate back to the information that will be collected from the animals.

Dr. Wyde said the human study in which transdermal penetration was measured was very small, and only excretion was measured, not the distribution of EMHC. The NTP considered these studies, but the information was inadequate.

Dr. Howard said he did not think that epidemiological studies would provide much information, and it would be statistically impossible to track the onset of a small increase in skin cancer. Sunscreens are efficacious in reducing solar-induced skin cancer, but vitamin D is becoming more and more of an issue in skin maintenance. Sunscreens do reduce the incidence of basal and squamous cell carcinoma of the skin. For an epidemiological study, the appropriate control group would be one who applies sunscreen in the absence of light, but such a group is unlikely to be included in a study.

Dr. Kenny Crump agreed with Dr. Button's recommendation to proceed slowly in pursuing the long-term studies. He did not believe that the animal studies would provide an answer to the questions posed. There is an opportunity for obtaining skin penetration data in humans, as well as epidemiological information. His recommendation was not to initiate a large animal study until more information is available.

Dr. Nigel Walker said Dr. Wyde referred to the human skin painting studies where two standard emulsions, nano emulsions and nano encapsulations, were investigated. Nanoparticles are designed to increase the residence time in the skin and hence another human skin painting study will not add to the understanding of penetration.

Dr. Pino asked whether FDA receives animal data submissions on ingredients coming to market in new formulations such as sunscreens. Dr. Howard replied that Dr. David pointed out that this is proprietary information that cannot be released by the FDA to the public.

Dr. Kerkvliet was concerned that the same issues will be raised regarding EMHC as were discussed the previous day regarding bisphenol A if there is an underlying concern that EMHC causes developmental or endocrine-related effects. The first priority should be to obtain ADME data before embarking on any toxicological assessments.

C. Tetravalent and Pentavalent Vanadium Compounds

a. Presentation

Dr. Michelle Hooth, NIEHS, discussed the background and proposed study for tetravalent and pentavalent vanadium compounds, which were nominated by the NIEHS and EPA

for toxicological characterization, chronic toxicity studies by the oral route, and a multi-generational reproductive toxicity study. Vanadium was nominated due to its use as a dietary supplement in the tetravalent form (18.6 mg vanadium/day), its presence in foods, and the occurrence of the pentavalent form in drinking water (5 µg/L to 100 µg/L). Vanadium is thought to have anti-diabetic effects and antilipidemic properties. NTP conducted a two-year inhalation study of vanadium pentoxide where lung tumors were found in male rats and in male and female mice. However, there are insufficient data to assess the human health risks from oral exposure.

The chemistry of vanadium is complicated because it exists in a number of oxidation states from -1 to +5. The penta- and tetravalent forms are the most stable oxidation states and the reduction potential favors the reduction of the pentavalent to the tetravalent state. There is *in vivo* evidence of the oxidation of the tetravalent form to the pentavalent form. There may also be interconversion of the pentavalent forms (i.e. metavanadate to orthovanadate); therefore, it is essential that the speciation and stability of these compounds be understood before any *in vivo* study is initiated.

Pentavalent vanadium is poorly absorbed in the gastrointestinal (GI) tract, but three times more effectively than tetravalent vanadium. This may be due to the active transport of pentavalent compounds, which are then reduced in the cell to the tetravalent form. Vanadium compounds are distributed to multiple tissues. Absorbed vanadium is excreted relatively rapidly in the urine and unabsorbed vanadium is excreted in the feces. Despite its low absorption, it may be toxic as was found with chromium compounds, which are poorly absorbed. Ingested hexavalent chromium is toxic and carcinogenic.

Vanadium compounds generally do not induce gene mutations in bacteria and mammalian cells, but both the tetra- and pentavalent forms cause aneuploidy, polyploidy, and endoreduplication, the duplication of the genome without mitosis, *in vitro* and *in vivo*. There is some evidence that vanadium compounds cause DNA damage via reactive oxygen species, and clastogenic effects including chromosomal aberrations and micronuclei.

The overall goal of this proposed research program is to investigate the potential for water-soluble vanadium compounds to cause systemic toxicity and carcinogenicity. The literature states that toxicity increases with valence; therefore, the pentavalent compounds are thought to be more toxic, although this hypothesis has not been rigorously tested. The NTP proposes to test the water-soluble vanadyl sulfate as the tetravalent compound, but an appropriate pentavalent compound has not been selected.

Prior to any animal studies, the NTP proposes to conduct *in vitro* tests to evaluate the speciation and stability of the compounds in dosing solutions exposed to air. Studies to identify and characterize the vanadium species formed *in vivo* under various physiological conditions in the GI tract by treating vanadium salts with reducing and oxidizing agents under different pH conditions will follow.

Based on the results of the *in vitro* studies, the NTP will select appropriate compounds

for the subchronic studies in rats and mice. Chronic studies will follow in which the speciation of vanadium compounds in tissues will be measured to link with any histopathological lesions that are found. Additional end points will be considered in the experimental design including clinical pathology, enzyme inhibition, cardiotoxicity, neurotoxicity, and immunotoxicity.

Developmental and reproductive toxicity studies will be conducted because limited studies in the literature, often where only a single dose was used, suggest decreased fertility and litter size, decreased survival rate to weaning, and maternal and paternal toxicity. The data obtained from the proposed studies would allow the determination of an upper acceptable intake level for sensitive populations including pregnant and lactating women, children, and infants.

Vanadium is listed on the US EPA Candidate Contaminant List as a priority contaminant. The data from these studies could be used to determine if drinking water regulations and standards are needed and to calculate estimates for tolerable intake levels of vanadium in food.

b. BSC discussion

Dr. Mirsalis, a BSC reviewer, thought the rationale clear and appropriate, and the significant human exposure via drinking water and dietary supplements warranted a better understanding of the oral toxicity of vanadium. Also, adverse effects seen in inhalation studies of vanadium pentoxide in rodents as well as questions of genotoxicity suggest that further study is needed. The study fits within the goals of the NTP and addresses a potential health risk of a compound with a significant human exposure.

He agreed that preliminary studies on the stability and speciation are necessary before any biological studies are initiated. He cited the past experience with chromium, where an understanding of the chemistry of hexavalent and trivalent chromium was critical to data interpretation. Perinatal subchronic studies, including toxicokinetics and tissue distribution, are very important. Oral administration is the appropriate route. The genotoxicity literature is very confusing, thus properly conducted GLP genotoxicity studies across the standard battery are needed. Developmental toxicity studies are important and an evaluation of vanadium in milk from lactating dams should be included, if feasible. He gave the program a moderate priority based on potential human exposure.

Dr. Max Costa, an *ad hoc* reviewer, agreed with Dr. Hooth regarding the differences between chromium and vanadium compounds. Hexavalent chromium is reduced to the trivalent form, which is not oxidized back to the hexavalent form in the cell while vanadium can cycle back and forth. He believed that both chromium and vanadium are nutritional supplements, although they have been called essential nutrients. He stated that there are limited data showing how vanadium functions as a nutritional supplement.

Metals such as arsenic and nickel have been studied extensively *in vitro* and *in vivo* but this is not the case for vanadium; the mechanism of its nutrient or toxicant properties is unknown. Because both nickel and chromium affect signal transduction and cause large

epigenetic effects, he recommended that the mechanism of vanadium be investigated using tissue culture studies. A major problem with vanadium is its ubiquitous presence in cells and media, which needs to be considered in all the proposed studies. The best approach to solving this problem would be to use a stable isotope of vanadium, either ^{50}Vn or ^{51}Vn , and inductively coupled plasma mass spectrometry (ICP-MS) for detection, otherwise the data would be confounded by background noise. It is important that speciation of vanadium be tracked within the animal and this will only be possible using stable isotopes. Metals behave similarly in tissue culture as they do *in vivo* and usually do not undergo complicated metabolism, except for vanadium that is reduced by ascorbic acid and oxidized by other cellular components.

Dr. Hooth said the oral route would be used throughout the studies with inclusion in either the feed or drinking water or given by gavage, depending on which compound is selected. The NTP will include *in vivo* micronucleus assays, as it is unlikely that the *in vitro* mutagenicity assays will prove whether vanadium is a mutagen or not. Data from the *in vivo* genotoxicity assays could possibly be used to partially explain any lesions that might be seen in a two-year bioassay. She thanked the reviewers for their suggestions of other studies that the NTP could perform with the collaboration of DIR investigators.

She asked Dr. Costa if he thought the isotopic studies could be used to investigate differences in uptake or the interconversion of the various vanadium species. Dr. Costa replied that one could track the stable isotope if it is given as the tetravalent form and it is then converted to the pentavalent form.

c. Public Comments

Mr. Manuppello, PETA said the weight of evidence suggests that vanadium is poorly absorbed in humans and generally exhibits low toxicity through oral exposure. An Expert Group on Vitamins and Minerals at the U.K. Food Standards Agency observed that there has been very few reported cases of vanadium toxicity when exposure is by routes other than inhalation. Typical exposures are well below levels that might cause concern. Adverse effects reported from short-term and subchronic toxicity studies with human volunteers included abdominal pain, nausea, and weight loss, which were reversed when consumption ended. PETA urged the NTP to assign this study a low priority and to rely on clinical and epidemiological studies.

BSC Discussion (continued)

Dr. Howard said that the FDA evaluated this nomination and is supportive of the proposed studies. Vanadium is used as a dietary supplement and the regulation of dietary supplements falls under the Dietary Supplement Health and Education Act, which limits the agency to ask for efficacy or safety data. Dietary supplements can be sold as long as there is no known toxicity of the material. Small subsets of the U.S. population consume dietary supplements in exceedingly high levels. Identification of a mechanism of action for vanadium and a potential population at risk will allow other governmental agencies to evaluate subsets of the population in epidemiological studies. The human studies cited by Mr. Manuppello are limited in number and scope, and probably lack the statistical power to draw conclusions about toxicity. He encouraged Dr. Hooth to design studies to

investigate a possible epigenetic mechanism.

Dr. Raymond Novak recommended that a metabolic profile of the lipids and glucose be included in the *in vivo* studies.

Dr. Bucher said the BSC would not be hearing about the activities of the Biomolecular Screening Branch, but one of its goals is to begin to identify mechanisms of action of chemicals *in vitro* before setting priorities for *in vivo* studies. The approaches discussed for vanadium are an excellent example of the kind of information the program will generate from *in vitro* studies that will help the NTP design better studies.

D. 4,7,10,-Trioxatridecane-1,13-diamine

a. Presentation

Dr. Masten said a research concept was not developed for 4,7,10,-trioxatridecane-1,13-diamine (TTD). It was nominated by NCI based on its high production volume, lack of adequate toxicity data, and potential exposure in the workplace. It is used as a curing agent in epoxy resins and as an intermediate in the production of other chemicals such as polymers, corrosion inhibitors, and textile and leather additives. Human exposure is expected to be primarily occupational, but there is no specific information regarding exposure levels in the workplace. The U.S. production was 500 million to 1 billion pounds in 1998 but less than one million pounds in 2002. There are no workplace regulations for TTD, but it is labeled as a corrosive. TTD is a liquid across a wide range of temperatures with a pH > 12. There are no carcinogenicity or mutagenicity data, but based on its structure and QSAR analysis, there is some suspicion of carcinogenicity.

Based on its LD50, TTD is classified as slightly toxic by the oral and dermal routes. It is a severe skin irritant in rabbits and a suspected eye and respiratory irritant. There is no information on its metabolism, but structurally it contains a diethylene glycol moiety in the center of the molecule and alkylamine groups at both ends. Metabolism is likely to occur at one of the amine groups forming a primary amine alcohol or carboxylic acid, but it is unlikely that diethylene glycol would be formed as a metabolite.

NTP's study recommendations are to include it in the high throughput screening (HTS) initiative that Dr. Bucher mentioned and to conduct *in vitro* genotoxicity studies. No *in vivo* studies are proposed due to its corrosivity, which would likely preclude its administration at doses where NTP studies would be informative.

b. BSC Discussion

Dr. Crump, a BSC reviewer, said the chemical is manufactured in a moderately high volume and there is a potential for occupational exposure. There is very little toxicity data and it seems a fairly modest proposal. Dr. Crump asked how this data might be used in the future.

Dr. Keith Soper, a BSC reviewer, said the summary was clear. He supports *in vitro* testing given TTD's properties and it seems unlikely *in vivo* testing is feasible. He was surprised at the change in production volume; from 1994 to 1998 a 1000-fold increase

was noted followed by a 500-fold decrease in production from 1998 to 2002. He wondered about the accuracy of the numbers. Since the QSAR models suggest TTD might be carcinogenic in male rats and female mice, he thought it worthy of investigation but the limited study proposed should suffice.

Dr. Masten responded that reporting of production volumes every four years to EPA is required and perhaps the volumes in a non-reporting year such as 1999 or 2001 might differ widely. In response to Dr. Crump's question, he said the main intent would be to use non-animal approaches to derive information that perhaps could be added to the MSDS, or used in a classification and labeling scheme for various hazards. With a pH of 12, the material will be difficult to study humanely.

Dr. Mirsalis was also struck by the drop in production volume and asked if commercial TTD sale and use is being phased out. If the compound will not be significantly used in five years it might not be worth studying. He suggested the NTP investigate this possibility before initiating further studies.

Mr. Janzen said it is difficult to comment on the validity and utility of the HTS data and hoped more information would be forthcoming at the next update on the HTS initiative in November.

E. Furan

a. Presentation

Dr. Daniel Doerge, NCTR, provided background information on furan, which was nominated by the FDA Center for Food Safety and Applied Nutrition for mechanistic testing and evaluating dose-response relationships in carcinogenicity assays. Furan is produced in a wide variety of foods during cooking and roasting, particularly in canned and jarred foods, baked and toasted cereal, and coffee. The level of furan in foods, ranges from 5-175 ppb, and depends on its thermal generation and dissipation by volatilization during heating. The estimated mean average daily intake across the whole adult diet is 0.25 µg/kg body weight/day, while babies on infant formula and jarred foods consume higher amounts of furan.

He described how scientists at the FDA used food concentration data in conjunction with food frequency data and substitution analyses to perform a Monte Carlo simulation of furan intake and develop a population distribution model. This simulation allowed for the investigation of substitution affects when various foods were removed from the diet or the exposure levels were modified in a particular food type. Coffee has the highest concentration of furan, but if eliminated from the diet, it had only a modest effect on total furan intake.

Furan is metabolized by CYP 2E1 to cis-2-butene-1,4-dial, an extremely reactive intermediate that is conjugated with glutathione and excreted. Furan can form DNA adducts and may form hemoglobin adducts.

He pointed out the similarities between furan and acrylamide: they are both formed during the cooking of common foods and are widely distributed in the food supply. Both compounds were negative in bacterial mutagenicity tests in the 1980s and 1990s using traditional *Salmonella* strains. Further investigation of their respective oxidative metabolites, glycidamide and cis-2-butene-1, 4-dial, showed that they are direct acting mutagens in strains that detect oxidized intermediates. Both compounds are carcinogenic in rodent bioassays with furan showing clear evidence of carcinogenicity in mice and rats based on increases of hepatocellular adenoma and carcinoma in mice and cholangiocarcinoma, hepatocellular adenoma and carcinoma, and mononuclear cell leukemia in rats. There is strong evidence for regenerative hyperplasia at the same doses that cause tumors in both species. Neonatal dosing with high concentrations during the first three weeks of life resulted in significant increases in liver carcinogenicity later in life.

The rationale for the proposed studies is to determine whether the dose-response curves for bile duct tumors in rats and the corresponding cytotoxicity in the liver differ from each other. Furan is regulated based on its mechanism of action, namely whether it is genotoxic or causes carcinogenicity via hepatotoxicity and subsequent cell proliferation. These studies will identify biomarkers of exposure in the rodent in relation to bile duct tumors and attempt to link rodent hepatocarcinogenicity with human exposure using these same biomarkers.

The first study would extend the dose-response range for the carcinogenicity of furan in the rat by testing lower doses than those used in the original bioassay. A second study would expose rats subchronically for less than a year to monitor for hepatotoxicity. If hepatotoxicity is noted, furan exposure will be stopped for some animals and they will be kept for up to a year to determine if the hepatotoxicity is reversible. The third study will measure the *in vivo* mutagenicity of furan in Big Blue transgenic rats at the *cII* locus as well as endogenous hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutations. Mutants can be sequenced to gain information about the mechanism of mutation. The fourth study will identify exposure biomarkers such as DNA and hemoglobin adducts and urinary metabolites in concert with toxicokinetic measurements following single and repeated dosing. Physiologically based pharmacokinetic modeling will then be developed to integrate the information that is already available on furan with data from these studies using low dose exposures.

b. BSC Discussion

Dr. Russell Cattley, a BSC reviewer, said this is a very ambitious proposal and represents a significant investment of effort. He questioned how a study using daily doses of furan lower than used in the NTP bioassays would improve the risk assessment. He believed that it would be more informative to address the dose responses for regenerative cell proliferation, and DNA damage from which hypotheses could be generated about the relative contribution of each of these processes. There was clear evidence that cytotoxicity and regenerative cell proliferation did occur under conditions of the bioassay, but there was no evidence that this is true for DNA damage. He said he was not familiar with using the Big Blue rat to detect DNA damage and did not know if this is an

appropriate and valid test system to use. He suggested that long-term labeling with bromodeoxyuridine would be more appropriate at the low end of the dose-response curve rather than Ki67 and proliferating cell nuclear antigen (PCNA) staining. Since furan induces both hepatocellular carcinoma and bile duct tumors, information about the cell of origin of these tumor types would be important. If the focus were on cholangiocarcinoma, it would be more appropriate to evaluate the mechanism in the bile duct epithelium as opposed to evaluating changes in the entire liver. He was skeptical that biomarkers of exposure in the human would be altered by small changes in dietary furan.

In conclusion, Dr. Cattley said the proposed concept does fit NTP's goals to strengthen the scientific basis whereby toxicology data can be applied to risk assessment. There is an increased risk of cholangiocarcinoma and proliferative effects in the bile duct epithelium in humans in countries where endemic bile duct fluke infestation occurs. This observation suggests that chronic injury to bile duct epithelium by a toxic chemical might be reasonably expected to increase risk of cholangiocarcinomas in humans, but this would be very difficult to assess for furan. Additionally, in terms of public health implications, any potential mitigation strategies would be interesting. He gave the program a moderate priority within the long-range plans for the NTP because he is unsure whether this information will contribute to public health.

Dr. Gregory Kedderis, an *ad hoc* reviewer, compared furan to acrylamide. Both are generated in food preparation, but chemically and toxicologically, they are very different compounds. Acrylamide is a direct acting neurotoxicant that is metabolized to a relatively stable epoxide that circulates in the blood. Furan is a stable compound and a potent hepatotoxicant once it is bioactivated, and the reactive intermediate binds predominantly to macromolecules in the liver. Thus, studying hemoglobin adducts of furan will be uninformative; rather one should measure glutathione-derived metabolites that are excreted from the liver.

He agreed with testing furan at lower doses than used in the bioassay. He cited a study by Dr. Robert Maronpot who found hepatocellular carcinomas in female mice administered 4 and 8 mg/kg furan in corn oil by gavage, but not at lower doses. He urged Dr. Doerge to consider this study in his experimental design as well as the observation that furan-derived DNA adducts are very unstable. The mutagenicity studies that suggested furan is mutagenic used impure solutions that contained hydrogen peroxide, which was detected by a *Salmonella* strain that responds to oxidative compounds. He believed that undue weight is being placed on evidence of a DNA adduct. Since the major public health concern is diet, a feeding study would be more appropriate than one using corn oil gavage. These questions need to be addressed at the beginning of the study. He was concerned that furan is measured by its volatility and headspace analysis. He was uncertain about the Big Blue assay as it does not detect deletions and if the mechanism of action of furan were via cytotoxicity, a deletion would be the type of genotoxic lesions that one would expect.

He believed that a developmental carcinogenicity study would be premature as it is

unknown how neonatal rats metabolize furan. This information should be known before embarking on such a study.

Dr. Doerge agreed that the program is ambitious and said the FDA has had extensive experience with acrylamide. The utility of the dataset for furan will reduce the uncertainty about risk particularly as it pertains to regulation of the generation of carcinogens in food during cooking. He agreed that even when the studies are completed and the uncertainties are reduced with modeling and biomarkers, it still might not be technologically feasible to remove furan from foods. The finding of acrylamide in foods fostered a huge effort worldwide to reduce the levels in various commercial food products but with mixed success. Since furan is more volatile, he is more optimistic about reducing its level in food.

The Big Blue rodent model has been used throughout world to study genotoxic mechanisms, but it is not a technique that has been embraced by the regulatory community *per se*. These mechanistic studies will investigate how ADME in a whole animal impacts the mutagenic process in specific target tissues. Ideally, the study would focus on the cells of origin of the tumor, but it is not clear how one would separate the bile duct cells; hence, a histopathological approach may be the best.

Both Dr. Cattley and Dr. Kedderis raised the issue of conducting developmental studies. The NTP's present default exposure paradigm begins while the animal is *in utero* and continues throughout life so that reproductive endpoints can be monitored.

Dr. Novak asked whether the incidence of hepatocellular carcinoma has increased in the population over the last decade, and Dr. McCarver responded that there are a number of other underlying factors affecting liver cancer in the U.S., primarily hepatitis C. Dr. Novak added that some people with hepatitis C seroconvert and others do not. Considering the classic initiation promotion scenario for hepatocellular carcinoma, he thought that a study incorporating cessation of treatment would be informative. He recommended that methylation patterns and comparative genomic hybridization using high throughput screening on chips be included in the experimental design. One approach for investigating the development of lesions in the bile duct might be to use whole animal imaging with an appropriate reporter for the bile duct cells.

Dr. Kedderis added that the Maronpot study did a stop-dosing group and no tumors were found. According to the literature, furan is not an initiator in that paradigm but a promoter, which is consistent with the hypothesis that it produces cell proliferation though a cytotoxic mechanism. Dr. Nigel Walker recalled that the rats in a different stop exposure study developed tumors whereas Dr. Maronpot's study was in the mouse.

Dr. Gail McCarver said the developmental studies are critical, because human infants are the group with the largest exposure due to their intake of infant food. She said CYP 2E1 is regulated differently in rodents and humans. It is expressed in the second trimester in human liver whereas in rodents it is expressed after birth.

Dr. Howard said the FDA mission is not only to regulate products, but also to provide the public with appropriate scientific data to make health risk decisions. Once information regarding acrylamide was made public, people became selective regarding their diet.

Dr. Mirsalis, who has extensive experience with the Big Blue assay, said there is a large database on the mouse but less on the Big Blue rat. The rat was chosen because of the cholangiocarcinomas, but there are no other studies on cholangiocarcinogens and he is not sure how the investigators will evaluate mutations only in bile duct cells. He suggested that the mouse might be a better species as hepatocellular carcinomas were observed. Alternatively, it is possible that furan is causing tumors in rats by a genotoxic mechanism and in mice by a cell proliferation non-genotoxic mechanism; hence, a study in both species might be informative. Although Dr. Kedderis rightly stated that Big Blue rodent does not detect large deletions outside the range of the transgene, it does detect small deletions. He recommended measuring cell proliferation in bile duct cells, hepatocytes, and Kupffer cells, as it would be important to determine whether mutations are identified at doses that induce proliferation. From a human health aspect, if furan were only mutagenic at doses that cause proliferation, monitoring of alanine aminotransferase (ALT) would provide some indication of risk. He recommended that a sample of the mutants be sequenced to ascertain whether an increase in mutations is due to clonal expansion.

Dr. Kerkvliet was not convinced about the public health significance of the animal studies. She believed human studies would provide a better sense of risk. Coffee has a four times higher level of intake than any other commodity and its concentration of furan is the highest of all foods tested. Dr. McCarver said the challenge with human studies is confounders such as hepatitis C and fatty liver.

Dr. Crump said there is extensive literature on furan and the objective of this study is to help a regulatory agency in its assessment of risk. The methods used by regulatory agencies to set action levels for humans are crude and not much of the animal data can be used. He believed the dose-response in animals has limited utility in humans and it only provides a crude measure of potency. He thought the FDA presently has enough information to develop action levels because there are already data from a gavage study. He thought repeating the same study at lower doses would be a huge expenditure of resources and animals. He believed that the best use of the animal data is for hazard identification.

F. Melamine and Cyanuric acid

a. Presentation

Dr. Gonçalo Gamboa da Costa, NCTR, introduced the concept by providing information on the death of a number of pets in 2007 that consumed pet food contaminated with melamine and cyanuric acid. Melamine is a high production volume triazine used in the manufacture of countertops, fabrics, glues, flame-retardants, and housewares. Significant amounts of melamine monomer (up to 2.5 mg/100 cm²) can leach from melamine-based kitchenware exposed to hot acidic food. Cyanuric acid is a hydroxylated triazine that

exists in equilibrium with its triketone tautomer. It too is a high production volume chemical used in the manufacture of herbicides, dyes, resins, antimicrobial agents, and as a stabilizer in swimming pools. Cyanuric acid is added as a swimming pool disinfectant up to 100 ppm. Children can ingest up to 154 mL of swimming pool water each time they swim which translates to 15.4 mg of cyanuric acid.

The reason for the toxicity in pets was the inclusion in the food of wheat gluten and rice protein that had been adulterated with scrap melamine. The melamine was added to meet the required nitrogen content of the pet food. Pathological examination of the affected animals revealed melamine cyanurate crystals in the distal and proximal tubules of the kidney. This acute renal toxicity was replicated in the laboratory.

Both melamine and cyanuric acid have low toxicities with LD50s in excess of 3 g/kg in rodents. The solubility of melamine and cyanuric acid are in the range of 2-3 g/L while melamine cyanurate has a solubility of 2.2 mg/L. Thus, it appears that the toxicity was triggered by the low solubility of the crystals that formed between melamine and cyanuric acid when both were administered simultaneously.

It became apparent that other than the pet industry, the contaminated gluten had been incorporated into feed for poultry, swine, and fish, which constituted a spillage into the human food chain. The FDA Center for Food Safety and Nutrition (CFSAN) conducted a risk assessment on melamine. It based the assessment on a level of melamine of 50 ppb in all food that was consumed and concluded that consumption of pork, chicken, and domestic fish and eggs from animals fed the tainted feed was unlikely to pose a human health risk. However, little was known at the time about the synergistic toxicity between the two triazines.

After CFSAN released the risk assessment, a review panel recommended (1) studies to better understand the pharmacokinetics of melamine, (2) a toxicological assessment of a combined exposure to melamine and cyanuric acid in different species, (3) the determination of biomarkers that might be predictive of renal failure to melamine and cyanuric acid, and (4) additional studies addressing the long term exposure of humans to melamine and cyanuric acid.

The proposed studies are based on the review panel's recommendations and include a sequence of studies with each study based on the outcome of the previous one. The first phase is to develop analytical methods to quantify cyanuric acid and melamine in the blood and urine of animals and use them to determine an appropriate non-toxic dose for toxicokinetic studies. The second phase is to conduct a pharmacokinetic study in the rat to determine the absorption, and distribution, metabolism and elimination (ADME) of melamine and cyanuric acid, administered individually, simultaneously, and in a time-staggered manner with exposure first to melamine and then to cyanuric acid. The third phase will determine the NOAEL for a combined gavage of melamine and cyanuric acid for 28 and 90 days, using the conditions determined in phase two, in which a nephrotoxic response is reported. In phase four, the FDA will identify metabolomic and proteomic biomarkers related to induced nephropathy. The urinary metabolomic profile will be

determined by nuclear magnetic imaging methodologies. The fifth phase will use the miniature pig model, which mimics human kidney anatomy and physiology, because it would be more appropriate in generating data for human risk assessment. Based on the subchronic studies, a sixth phase would be longer-term studies to evaluate chronic administration of melamine and cyanuric acid simultaneously.

b. BSC Discussion

Dr. Jim Riviere, a BSC reviewer, agreed that the rationale for studying the potential toxicity of a combination of melamine and cyanuric acid was clearly explained. There is a real concern for potential human exposure and the combination presents a novel toxicologic approach that has not been studied. The specific experimental design will address the research needs requested by the FDA review panel and specifically the kinetics of the formation of melamine cyanurate crystals in the rat. These studies are crucial since it is not known what the relationship is between exposure to the individual triazines and the ultimate renal effects. The 28 and 90-day toxicology studies should permit determination of the NOAEL. He approved of the planned metabolomic and proteomic studies. The demise of the dogs and cats was acute, and the question remains as to why certain dogs and cats were susceptible and others were not. With the potential for human exposure, it is important that the mechanism and toxicokinetics be determined. He agreed with conducting studies in the miniature pig, which is more comparable to the human on a body mass basis and has related physiology. It would be important to complete the *in vitro* studies on the stoichiometry of crystal formation as a function of melamine and cyanuric acid concentrations, rates of input, pH, and solution osmolarity using renal tissue slices from the rat and miniature pig. This study might also provide information on other potential markers to assess the *in vivo* studies that might link the two species.

Dr. Bunton agreed with Dr. Riviere's comments because of the potential for human nephrotoxicity. She suggested that a recovery phase be added to the 90-day study to assess any possible recovery. The NOAEL and possible biomarkers could differ in the rat and the miniature pig and this needs to be considered when extrapolating between species.

Dr. Gamboa da Costa agreed that the suggested *in vitro* studies should be undertaken first and he has begun to address the solubility of melamine and cyanuric acid as a function of pH while keeping the osmolarity fixed. He questioned why the precipitation is only found in the kidneys if both triazines are in circulation. It is possible that plasma proteins stabilize the triazines. He approved of the suggestion to monitor recovery in the 90-day study because most animals do not die from the acute exposure, which indicates a potential for clearing the crystals from the tubules if a certain percentage of renal function remains intact. Since neither blood urea nitrogen nor creatinine is sensitive enough as a biomarker, an effort to identify a more sensitive biomarker will be important.

Dr. Portier asked whether administration of the two compounds together would be considered as a mixtures experiment. Dr. Gamboa da Costa responded that it would not be possible to administer different ratios of the compounds, as this would expand the

study enormously.

V. Concept Review on the NTP and NIEHS Investigative Research Support Contract

a. Guidelines for Review

Ms. JoAnn Lewis, Office of Acquisitions, NIEHS, briefly outlined the guidelines for the BSC regarding the discussion of concept reviews. She asked the BSC to review the concept for its overall value and scientific relevance for fulfilling the program's goal of protecting public health. They should consider the scientific, technical, and programmatic significance of the proposed activities, availability and adequacy of the technology and other resources necessary to achieve the required goals, and the scientific or clinical uses of the anticipated data. The discussion should be limited to a review of the general purpose, scope, goal, and optional approaches to pursue the overall objectives. The meeting will be closed to the public should discussions turn to the development or selection of the details of the project's request for proposals such as specific technical approaches, protocol, statement of work, data format, or program specifications. A meeting is closed to protect free exchange of the advisory group members' opinions and to avoid premature release of the details of the proposed contract or request for proposal.

b. Presentation

Dr. David Malarkey, NIEHS, identified himself as the project officer and Ms. Colette Malone as the contract officer. He discussed the three areas relating to the contract: its history, purpose, and the highlights of the projects that have been completed through this contract over the >15 years of its existence. It is a consolidation of three older NTP contracts: the transgenic and National Center for Toxicogenomics contract, the genotyping contract, and a molecular oncology contract. In 2003, the Genetic Alterations in Cancer (GAC) database was added. GAC is a comprehensive database of genetic mutations in tumors from animals and humans exposed to environmental agents.

The purpose of the new contract is to provide scientific support by conducting *in vivo* and *in vitro* investigative research projects for NTP and NIEHS scientists. Some examples of the types of support are: conducting mechanistic studies for carcinogenesis bioassays, breeding or housing of pathogen-free animals, characterizing genetically altered models, and providing molecular biology. It will also support the development of biomarkers, and targeted pathology workshops.

During 2003-2007, about 40 different investigators at NIEHS used the contract to perform about 140 different projects. Studies included investigation of the toxicogenomics of the liver and heart, and genotyping of more than 20 genetically modified mouse models. Ongoing activities include the development of methods to measure troponin, magnetic resonance imaging in the liver, aryl hydrocarbon receptor (AhR)-mediated neurotoxicity, gene expression in liver slices exposed to perfluorinated chemicals, and the host susceptibility initiative.

Noteworthy findings over the past four years are: hepatic gene expression varies by type of toxicant, the time of day samples are taken, and the liver lobe analyzed; hair follicle stem cells give rise to skin tumors in TgAC mice; the constitutive androstane receptor (CAR) is required for liver carcinogenesis in the mouse; cyclooxygenase 2 receptors and non-steroidal anti-inflammatory drugs influence colon cancer in Min mice, and gene expression patterns in whole blood are influenced by liver injury.

c. BSC Discussion

Dr. Novak, a BSC reviewer, said the concept document summarizes the major goals. The provision of support and resources for the activities of NTP and NIEHS scientists appear reasonable. This activity facilitates translation of research in terms of biomarkers and cell-based assays, an activity in which additional high throughput technologies should be added. There are a number of imaging techniques that should be considered including whole body imaging with a reporter construct.

Mr. Janzen did not believe that the NTP would find a single vendor who would be able to perform all the activities ranging from translational medicine to high throughput screening to the molecular biology studies that Dr. Malarkey discussed.

Dr. Portier asked how this resource would be used. Dr. Malarkey replied that principal investigators at the NIEHS would contact him about the availability of contract funds. The scientists develop proposals that are peer reviewed for suitability of support before they are initiated.

Dr. Bradfield asked how the NTP determines the cost benefits of these projects. He did not understand why the NTP needs an outsourced resource for experiments that many scientists might consider as personal experiments. Dr. Malarkey replied that there might be a novel technique that DIR scientists cannot do in their laboratory that could be performed under this contract. Dr. Bucher added that the NTP is primarily a non-laboratory activity, but the program has laboratories that support pathology and clinical pathology for NIEHS. This contract provides an opportunity for the NTP to investigate specific questions that cannot be done through its large testing contracts. These studies fit between activities that intramural scientists tackle in their laboratories and those that can be done under contract.

Dr. Novak said the NTP has a repository of tissues from animals that have undergone various treatments and he asked if the NTP saves DNA samples for subsequent studies. Dr. Malarkey replied that the NTP determines for each study whether or not to archive tissue, and DNA is archived on a case-by-case basis. Tissues from NIEHS investigators are often archived in their own laboratories.

Dr. Novak motioned for approval and Dr. Mirsalis seconded the motion; 13 members voted yes, 0 voted no, and one abstained (Mr. Janzen). Mr. Janzen did not believe the NTP would find a single vendor to provide such a wide range of services.

VI. Technical Report Review Subcommittee Report

a. Presentation

Dr. Nancy Kerlvliet, Chair of the NTP BSC Technical Reports Review Subcommittee (“the Subcommittee”), summarized the actions on the Draft NTP Technical Reports from the peer review meeting on February 27-28, 2008. The Subcommittee reviewed the findings and conclusions from studies of dibromoacetonitrile, dibromoacetic acid, chromium picolinate monohydrate, 1,2 dibromo-2,4-dicyanobutane, isoeugenol and 5-hydroxymethyl)-2-furfural that used conventional F344 rat and B6C3F₁ mouse models, and the photocarcinogenicity study of different preparations of *Aloe vera* tested in SKH-1 mice exposed to simulated solar light. The Subcommittee also reviewed the findings and conclusions of a three-month study on estragole in conventional male rats.

- The Subcommittee voted 7 yes, 1 no, and 0 abstentions in favor of the conclusions that there was *clear evidence* of carcinogenic activity of dibromoacetonitrile in male F344/N rats based upon oral squamous cell papillomas or carcinomas, *some evidence* in female rats based on oral squamous cell papillomas and *clear evidence* in male and female B6C3F mice based upon squamous cell papillomas or carcinomas of the forestomach.
- The Subcommittee voted unanimously 8 yes, 0 no, and 0 abstentions in favor of the conclusions that there was *clear evidence* of carcinogenic activity of dibromoacetic acid in male and female F344/N rats due to adenomas of the large intestine in both genders and malignant mesothelioma in male rats, and *clear evidence* in male and female B6C3F1 mice based on hepatocellular neoplasms in both genders and hepatoblastomas in males.
- The Subcommittee reviewed a report on the photocarcinogenicity of different preparations of *Aloe vera*, applied topically for 46 weeks to SKH-1 mice exposed to simulated solar light. The Subcommittee voted unanimously 8 yes, 0 no, and 0 abstentions in favor of the conclusions that there was no effect in male mice and a weak enhancing effect in female mice exposed to aloe gel or aloe emodin, and a weak enhancing effect in both male and female mice for the whole leaf or decolorized whole leaf extract. For the whole leaf decolorized extract, there was an increased multiplicity of squamous cell neoplasms.
- The Subcommittee voted 7 yes, 0 no, and 1 abstention in favor of the conclusions that there was *equivocal evidence* for carcinogenic activity of chromium picolinate monohydrate in male F344/N rats due to preputial gland adenomas and *no evidence* of carcinogenic activity in female F344/N rats or male and female B6C3F1 mice.
- The Subcommittee voted unanimously 8 yes, 0 no, 0 abstentions in favor of the conclusions that there was *no evidence* of carcinogenic activity of 1,2 dibromo-2,4-dicyanobutane in either species or gender.
- The Subcommittee voted 8 yes, 0 no, and 1 abstention in favor of the conclusions that there was *equivocal evidence* of carcinogenic activity of isoeugenol in male F344/N rats based on thymomas and mammary gland carcinomas, *no evidence* in female rats, and *clear evidence* in male B6C3F1 mice based on hepatocellular adenoma and/or carcinoma and *equivocal evidence* in female mice based on histiocytic sarcoma.
- The Subcommittee voted unanimously 8 yes, 0 no, 0 abstentions in favor of the

- conclusions that there was *no evidence* of carcinogenic activity of 5-hydroxymethyl)-2-furfural in male or female F344/N rats or male B6C3F1 mice and *some evidence* in female mice based upon hepatocellular adenomas.
- The Subcommittee voted unanimously 8 yes, 0 no, 0 abstentions in favor of the conclusions that estragole showed carcinogenic activity based on the occurrence of two cholangiocarcinomas and one hepatocellular adenoma in the liver of three of ten male F344/N rats in the high dose group. Because rats and mice were exposed for only three months, these studies do not assess the full carcinogenic potential of estragole.

b. BSC Discussion

Dr. Mirsalis moved to accept the Subcommittee report as presented and Dr. Soper seconded the motion. The vote was unanimous in favor of the motion with 12 yes and 0 no votes and 0 abstentions.

VII. Criteria for Evaluating Outcomes for Reproductive, Developmental, and Immunotoxicology Studies.

a. Presentation

Dr. Paul Foster, NIEHS, discussed how the NTP is proposing to establish criteria to describe the results from immunotoxicology, reproductive, and developmental studies to indicate the strength of the evidence for NTP conclusions. These criteria would be similar to those used for its carcinogenicity studies. The carcinogenicity study criteria provide levels of evidence statements for clear evidence, some evidence, equivocal evidence, no evidence, and inadequate studies where there are issues with the conduct of the study.

Recently, the NTP completed large multigenerational studies on genistein and ethinyl estradiol and the draft reports were brought to the BSC Technical Reports Review Subcommittee for peer review. The NTP realized the need for criteria to express the studies' conclusions and is moving forward with this initiative.

The carcinogenic levels of evidence permit the NTP to compare a test article across studies with different strains and sexes and different test articles for the same strain and sex across studies. Having levels of evidence criteria for reproductive, developmental and immunotoxicology studies would enable the NTP to make similar types of comparisons. If possible, the NTP would like to pattern these new criteria to use the same level of evidence terminology as carcinogenic bioassays, i.e., clear, some, etc.

Dr. Foster and Dr. Dori Germolec are leading this initiative and are testing their draft criteria first through exercises in-house. The next step will be to form two BSC working groups, one to evaluate the immunotoxicology criteria and a second to evaluate the reproductive criteria and the developmental criteria. The working groups will be composed of both practitioners and users of these non-cancer data. Drs. Kerkvliet and Carney, two BSC members, will chair the immunology and reproduction/developmental working group meetings, respectively. Dr. Foster invited other BSC members to

participate in the working groups. At the working group meetings, the participants will work through case studies applying the draft criteria to data similar to those collected from these types of studies. The groups will determine the suitability and utility of the draft criteria and their application for distinguishing between different levels of evidence, e.g., “clear evidence” versus “equivocal evidence”. The recommendations from the working groups will be presented to the entire BSC at the meeting in November 2008.

b. BSC Discussion

Dr. Crump thought the idea of developing criteria to standardize outcomes from non-cancer studies would be meaningful and might be very useful because it would allow comparisons to be made across studies. He was pleased the NTP would take the same approach for these non-cancer end points as is being used for the carcinogenicity bioassays.

Dr. McCarver said it would be important to assess the utility before adopting the approach.

VIII Host Susceptibility Program

a. Presentation

Dr. John (Jef) French, NIEHS, provided an update on the development and implementation of the Host Susceptibility Program (HSP). Host susceptibility to disease is defined as the interaction between multiple host (genetics, behavior, nutritional status, life stage, etc.) and environmental factors (cold, heat, diet, infectious agents, environmental toxicants, etc.) that can modify an individual’s susceptibility. HSP will focus on the role of genetic variation in concert with toxicant exposure to determine the range of biological responses in genetically diverse and genetically defined animal models.

Structurally, the rodent and human genomes have a great deal of diversity in terms of single nucleotide polymorphisms, copy number variation including insertions and deletions, and linkage disequilibrium between genes. The laboratory mouse, which is based upon three subspecies, *domesticus*, *castaneus*, and *musculus*, were bred for specific coat colors, behavioral phenotypes, etc. that gave rise to the classical laboratory inbred mouse lines. From genotyping and sequencing studies, it is known that mouse lines share haplotype structures consistent with their ancestors of origin. Recently, the NTP under a research contract with Perlegen Sciences completed the resequencing of 15 strains of inbred lines for comparison to C57BL/6J mice. Significant genetic diversity was found in the mouse strains with approximately 2×10^7 single nucleotide polymorphisms (SNPs), which is approximately the same magnitude of SNPs as estimated in the human population.

The objective of the HSP research is to understand the role that both genetic and epigenetic variation plays in the development of complex diseases - not only single gene autosomal dominant or autosomal recessive diseases, but rather those that are polygenic in nature where high penetrant genes and low penetrant genes modulate the development

of the disease. The program plans to accomplish this objective by exposing the 15 defined mouse strains to specific environmental exposures under defined experimental conditions. It is believed that the data obtained will allow the prediction at the population level of a biological response to aid in a cross-species extrapolation and to provide information for risk characterization. Another aim is to identify and functionally validate candidate genes within quantitative trait loci, so that mouse genes can be linked with human orthologs in response to toxicant exposure. The final aim will be to identify the genetic basis and mode of action of a toxicant to cause disease in animal models and to correlate these findings with human disease genes. Because toxicity is complex and a single dose or multiple doses can result in many different phenotypes, the program will need to focus on a particular phenotype to obtain quantitative measures that can be associated with biomarkers of disease. Absorption, distribution, metabolism, and excretion (ADME) kinetics will play a critical role in the initial studies, as it is recognized that most inbred strains will metabolize chemicals at different rates and possibly produce a different spectrum of metabolites.

In the development of the HSP, the NTP has had extensive discussions with intramural scientists, the NTP BSC on two occasions, and extramural mouse geneticists. The NTP announced this initiative through presentations and interactions with a number of groups, and publication of information in the Federal Register and via a number of electronic email distribution list for genetics and toxicology. Through these announcements, the NTP received 27 unique responses from 21 national institutions that endorsed the initiative. The respondents considered genetic variation a critical parameter to the program's success. Secondly, they suggested that a systems biology or systems genetic approach would be necessary to understand not only the underlying genetic mechanisms, but also those signaling and structural pathways that might play a role in the modulation of disease incidence. A few individuals promoted the idea that development of NTP resource centers in bioinformatics, RNA and DNA microarrays, and high dimensional and high throughput data analysis that would be critical for this project.

When NTP was realigned within the NIEHS Division of Intramural Research in October 2007, the Host Susceptibility Branch was created. Expertise among staff affiliated with the branch is broad and includes ADME, carcinogenicity, toxicology, toxicogenomics, molecular pathology, and administration. A focus for future hires will be scientists with expertise in genetics and environmental genomics. Also, a number of adjunct staff from NIEHS with expertise in cardiotoxicity, biostatistics, and epidemiology will provide support and plans are underway to hire a bioinformaticist and computational biologist for NTP that will be located administratively in biostatistics.

The plan for testing is composed of two parts –intramural-initiated programmatic research to fulfill the needs of the NTP, and extramural research partnerships to capitalize on a new and novel gene environment research program. The HSP hopes to support small grants through RO3s and XO1s and to collaborate with RO1-supported researchers by providing samples from large-scale NTP animal studies. Peer review will be critical and the NIEHS Division of Extramural Research and Training (DERT) will provide oversight and management of this effort.

The HSP also consulted with Dr. Joe Tomaszewski, NCI, on the NIH Rapid Access and Intervention Development Program (RAID), which creates mechanisms by which individuals can gain access to NIH resource contracts. The HSP is considering this model as a possible means for supporting extramural research.

The first set of studies will be to evaluate the ADME of benzene and bis-chloroethoxymethane (BCEM) and short-term cancer bioassays using genetically modified mice. BCEM has a metabolite in common with a number of cardiotoxins of interest to the NTP. Benzene was chosen from a nomination by two investigators at University of California at Berkeley who are interested in corroborating their human benzene genotoxicity data that was collected at very low exposures with results from the 16 resequenced strains of mice. FDA Center for Drug Research and Evaluation has suggested that the NTP study various cardiotoxins and hepatotoxins that have been problematic for pharma in recent years.

The next steps are to (1) increase the expertise in the branch, (2) work on the development of programmatic research to complement the needs of participating NTP agencies, (3) identify and develop intramural collaborative research partnerships, (4) encourage agent-specific nominations from federal agencies as well as intramural and extramural scientists, (5) evaluate the pilot projects and the development of the requisite tools required for this type of research effort, (6) work-out a mechanism to undertake peer review of the extramural research, and (7) develop partnerships using available mechanisms.

b. BSC Discussion

Dr. Mirsalis was skeptical whether a program analogous to the NCI/NIH RAID program is appropriate. He said RAID is a very specific program where an investigator provides a drug or vaccine with a particular end point in mind and the RAID program allows the investigator to bridge a gap using various resource contracts that are in place. The HSP is different and scientists may want to use resources supplied by the NTP to develop data to write another grant or get a paper published.

Dr. French agreed about the comments relating to the RAID model, but he investigated its possible usefulness for the HSP because it is an existing successful program. The purpose of the HSP is to carry out the mandated NTP mission, but also to include extramural investigators so that the program might develop into a multi-disciplinary collaborative research effort. The collaboration may be through R03 or X01 grants, the latter of which could be used to develop preliminary data for an R01 grant. The NTP does not believe that it can develop this area alone and it would be advantageous if the NTP could collaborate with experts who are studying genetic variation.

Dr. Bradfield said he was confused about the mechanism going forward. He asked if Dr. French was talking about investigators writing R03 and R01 grants and how the NTP would identify them. Dr. French answered that the grant process would be through an RFP or RFA and the NTP would be divorced from the process. It would be exclusively

an extramural DERT activity, but the NTP could provide guidance about the area of focus for the research.

Dr. Bradfield then asked what the advantage would be for the investigator and whether the NTP would provide reagents such as the outbred strains. Dr. French replied that the NTP might provide resources beyond the R03s and R01s, such as samples from studies in genetically defined rodents exposed to defined experimental conditions.

Dr. Bradfield encouraged the NTP to figure out a way to mine the existing database of R01s and R03s to ascertain who is working in this area and Dr. French replied that the NTP has reviewed the CRISP database and has a list. The HSP was advertised through the NIH Guide and Federal Register to identify individuals with the required expertise. The NTP received a good response to its request for information as mentioned earlier. Dr. Bradfield said he was thinking about others like himself who are uninformed as this is the first time that he has been made aware that the NTP might be interested in interacting with extramural scientists. Dr. French said the NTP must do a better job of communicating with the bench researcher.

Dr. Bucher said there are two levels for interaction in HSP. One is through NTP's solicitation of projects that will be peer reviewed for their validity and their scientific merit. The second is through NTP making resources from its studies available competitively to anybody who wants to access them for their own particular projects. Obviously, those scientists who nominate a project will have a competitive advantage in applying for grant funding or obtaining a supplement to an existing R01. This is the reason it has taken some time to sort through these alternatives. The NTP wants to be fair but also do the best science possible. It is still not defined exactly how this program will work.

Dr. Novak hoped that the HSP would also be an opportunity to evaluate epigenetic end points.

Dr. French agreed that epigenetic events are important. He said there are a number of fixed structural alterations in the mouse and human genomes, that most likely will have an effect on susceptibility, but the epigenome is probably dynamic over a lifetime and differs at the various life stages. As an example, he quoted the twin studies where methylation patterns are very similar in young siblings, but by the time the twins reach adulthood, they diverge.

IX. Adjournment

Dr. Bucher thanked the BSC for its advice throughout the meeting and especially thanked Dr. McCarver for her leadership and service as chair