NATIONAL TOXICOLOGY PROGRAM
BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

Peer Review of Draft Technical Reports of
Toxicology and Carcinogenesis Studies
by the Technical Reports Review Subcommittee

May 3, 2001

Summary Minutes

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NTP Board of Scientific Counselors Technical Reports Review Subcommittee
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The meeting began at 8:30 a.m. on May 3, 2001 in the Rodbell Conference Center of the David P. Rall Building, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Members of the subcommittee attending were Drs. Steven Hecht (chairperson), Linda Chatman, Harold Davis, Yvonne Dragan, Norman Drinkwater, James Klaunig, David Malarkey, Michele Medinsky, Walter Piegorsch, and Mary Anna Thrall. Dr. Medinsky was not present. For further information, contact Dr. Mary S. Wolfe, Executive Secretary, at 919-541-3971 or wolfe@niehs.nih.gov.

NTP Technical Report Reviews

***o*-Nitrotoluene**. Dr. June Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of *o*-nitrotoluene by discussing the uses of the chemical and the rationale for the study, describing the experimental design, reporting on survival and body weight effects, and commenting on chemical-related neoplasms in male and female rats and mice. The proposed conclusions for the report were:

> Under the conditions of these studies, there **was clear evidence of carcinogenic activity** of *o*-nitrotoluene in male rats based on increased incidences of malignant mesothelioma, subcutaneous skin neoplasms, mammary gland fibroadenoma, and liver neoplasms. The increased incidences of lung neoplasms in male rats were also considered to be exposure related. There was **clear evidence of carcinogenic activity** of *o*-nitrotoluene in female rats based on increased incidences of subcutaneous skin neoplasms and mammary gland fibroadenomas. An increased incidence of hepatocellular adenoma in female rats was also considered to be exposure related. There **was clear evidence of carcinogenic activity** of *o*-nitrotoluene in male and female mice based on increased incidences of hemangiosarcoma, carcinoma of the large intestine (cecum), and hepatocellular neoplasms (females only).
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> Exposure to *o*-nitrotoluene caused increased incidences of nonneoplastic lesions of the mammary gland (females only), liver, bone marrow, spleen, lung, and mandibular lymph node (females only) in male and female rats and of the liver, kidney, and nose in male and female mice.
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> Decreased incidences of mononuclear cell leukemia occurred in exposed groups of rats; the incidence of testicular interstitial cell adenoma was decreased in male rats.

Dr. R. Sills, NIEHS, presented results of analyses of mutations in *ras* oncogenes and p53 tumor suppressor genes extracted from hemangiosarcomas found in *o*-nitrotoluene-treated mice. While the mutation rate in *ras* oncogenes was similar in hemangiosarcomas from chemical treated animals and controls, mutations occurred in almost all the p53 tumor suppressor genes from *o*-nitrotoluene-induced hemangiosarcomas but rarely in spontaneously occurring tumors. Ms. T.

Devereux, NIEHS, showed results of analyses for mutations that disrupt the regulation of β-catenin from the same set of hemangiosarcoma tissue samples. About half of the *o*-nitrotoluene-induced hemangiosarcomas had such mutations, while none were seen in spontaneously occurring hemangiosarcomas.

Dr. Medinsky, a principal reviewer, was unable to attend the meeting and her comments were read into the record by Dr. M. Wolfe, NIEHS. Dr. Medinsky agreed with the proposed conclusions. She asked for clarification of whether instability of the test chemical in feed affected the actual doses administered and what were the consequences of irradiating the feed. Dr. Dunnick replied that the chemical was stored in refrigerators and mixed with feed every two or three days to minimize loss due to instability. Dr. G.N. Rao, NIEHS, explained that to prevent pathogen contamination the NTP has begun irradiating the animal feed because the prepared diets were not formulated to be autoclaved. The nutrient composition of the diets was unaffected by the irradiation.

Dr. Medinsky also questioned the appropriateness of analyzing metabolite data normalized to creatinine levels rather than total metabolite excreted. Dr. L.T. Burka, NIEHS, explained that creatinine production, which is related to muscle mass, is a good surrogate measure of body weight and also provides a convenient method of factoring differences in age and sex for animals of different size. In addition, bulk measures of urine volume are less precise than metabolite concentrations and thus less useful for mathematical modeling.

Dr. Malarkey, the second principal reviewer, agreed with the conclusions. He praised the inclusion of a stop study and molecular biology studies and asked for clarification of how specific were the mutations observed and what were the sites of β-catenin and p53 protein accumulation. Dr. Sills replied that the p53 protein was located primarily in the nucleus and β-catenin predominantly in the cell membrane, with nuclear accumulation seen in some tumor types such as hepatoblastomas. Dr. Malarkey suggested the lesions in the reproductive tract and endocrine glands might indicate an endocrine disrupting mechanism of toxicity by the chemical. As a general question for all NTP studies, he asked for details of monitoring for possible *Helicobacter hepaticus* contamination of the animal rooms. Dr. Rao described the monitoring procedures used to detect *Helicobacter* infection following an outbreak in the late 1980s and noted that the NTP studies have been free of known viral and bacterial pathogens since 1991.

Dr. Klaunig, the third principal reviewer, also agreed with the conclusions. He asked for clarification of the cause of death of the animals and for more information on the site of origin of the various mesotheliomas. Dr. Dunnick replied that mesotheliomas were indeed a significant cause of mortality and the majority of the mesotheliomas originated in the tunica vaginalis.

Dr. Chatman questioned the phrasing of the proposed conclusions and suggested that listing certain neoplasms as evidence of carcinogenic activity and others as "also exposure related" or "may have been related" was potentially confusing. Dr. Davis also questioned whether the additional statements implied the lesions were treatment related. Dr. C. Portier, NIEHS, explained that for each sex/species group there is one overall conclusion category for the

chemical, rather than different categories for different tissue sites. Thus to convey fully the study results, the NTP mentions not only the neoplasms giving the strongest indication of carcinogenic activity but also other chemically related effects. Dr. Chatman proposed that the lung neoplasms in male rats and liver neoplasms in female rats be included in the **clear evidence** statements. Dr. J. Bucher, NIEHS, said the proposed phrasing was chosen to distinguish that the strength of response for the lung and liver neoplasms was less than for the other tumor types. Dr. Dunnick noted that, were the other neoplasms not present, the lung neoplasms would be classified only as **some evidence**. Dr. Drinkwater agreed that the lung and liver effects were weaker than clear evidence and moved that the conclusions be accepted as written. Dr. Klaunig seconded the motion, which was approved by six yes votes to two no votes (Drs. Chatman and Davis).

***p*-Nitrotoluene** Dr. June Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of *p*-nitrotoluene by discussing the uses of the chemical and the rationale for the study, describing the experimental design, reporting on survival and body weight effects, and commenting on chemical-related neoplasms in male and female rats and mice. The proposed conclusions for the report were:

> Under the conditions of these 2-year feed studies there **was equivocal evidence of carcinogenic activity** of *p*-nitrotoluene in male F344/N rats based on increased incidences of subcutaneous skin neoplasms. There was **some evidence of carcinogenic activity** of *p*-nitrotoluene in female F344/N rats based on increased incidences of clitoral gland neoplasms. There was **equivocal evidence of carcinogenic activity** of *p*-nitrotoluene in male B6C3F_1 mice based on increased incidences of alveolar/bronchiolar neoplasms. There **was no evidence of carcinogenic activity** of *p*-nitrotoluene in female B6C3F_1 mice exposed to 1,2500, 2,500, or 5,000 ppm.
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> Exposure to *p*-nitrotoluene caused increased incidences of nonneoplastic lesions of the kidney, spleen, and liver in male and female rats, testis in male rats, and lung in male and female mice.
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> Decreased incidences of mononuclear cell leukemia in male and female rats and testicular interstitial cell adenomas in male rats were attributed to exposure to *p*-nitrotoluene.

Dr. Davis, the first principal reviewer, questioned the use of the term "uncertain findings" to describe conclusions of equivocal evidence. He disagreed with the statement in the report that hematopoietic cell proliferation increased in the female 5,000-ppm rats. Dr. Dunnick concurred. Dr. Davis also questioned whether there could be a relation between testicular interstitial cell adenomas and atrophy when the incidences of the former decreased while the latter increased. Dr. J. Mahler, NIEHS, explained that while atrophy can occur as a secondary effect of an

adenoma, the absence of a neoplasm may increase the possibility of detecting a primary atrophic change. Dr. Davis also encouraged the inclusion of human exposure data whenever available.

Dr. Medinsky, the second primary reviewer, was unable to attend the meeting and her comments were read into the record by Dr. M. Wolfe, NIEHS. Dr. Medinsky agreed with the conclusions and focused on details of the discussion of metabolism and urinary biomarker data. Dr. Dunnick indicated that communications between NTP staff and the reviewer had resolved these questions.

Dr. Klaunig, the third principal reviewer, inquired about the cause of apparent lower survival in control male rats compared with the high dose males. Dr. Dunnick noted that the survival in control male rats was normal for NTP studies, with mononuclear cell leukemia being one of the main causes of early deaths. However, in the treated animals, splenic toxicity caused by the chemical inhibited the occurrence of mononuclear cell leukemia.

Dr. Davis moved that the conclusions be accepted as written and Dr. Klaunig seconded the motion, which was approved unanimously with eight votes.

Acrylonitrile Dr. Burhan Ghanayem, NIEHS, noted that acrylonitrile is a recognized carcinogen in several strains of rats and described the experimental design and results of the toxicology and carcinogenesis studies in male and female mice. The proposed conclusions were:

> Under the conditions of this 2-year gavage study, there **was clear evidence of carcinogenic activity** of acrylonitrile in male and female B6C3F$_1$ male based on increased incidences of forestomach and harderian gland neoplasms. Neoplasms of the ovary and lung in female mice may have been related to administration of acrylonitrile.
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> Nonneoplastic lesions of the forestomach and harderian gland in males and of the forestomach and ovary in females were associated with administration of acrylonitrile by gavage for 2 years.

Dr. Drinkwater, the first principal reviewer, inquired about details of urinary metabolite collection and the apparent increase in excretion of two metabolites with age. Dr. Ghanayem replied that while there was increased variation with age, examination of 15 metabolites and creatinine did not reveal a consistent pattern of change with age.

Dr. Chatman, the second principal reviewer, inquired about the classification of the ovary and lung tumors as "uncertain" findings in the summary table. Dr. J. Bucher, NIEHS, said that henceforth that subheading would be changed to "equivocal." Dr. Chatman asked about the appropriateness of grouping various types of ovarian cysts for analysis. Dr. A. Nyska, NIEHS, explained that particularly in older animals one cannot determine the cell of origin of cysts and the program policy is simply to combine them under one category.

Dr. Dragan, the third principal reviewer, suggested that pharmacokinetic data could be helpful in setting doses for long-term studies. Dr. Ghanayem replied that for this particular chemical, the acute toxicity due to formation of cyanide as a metabolite was the determining factor in dose selection.

Dr. Thrall inquired if a decrease in hematocrit in female mice could be attributed to Heinz body formation and also commented on the lack of hematology measures in the long-term studies. Dr. G. Travlos, NIEHS, said that Heinz bodies were indeed looked for, but were not detected. He explained that hematology measurements were limited in 2-year studies to avoid bleeding core study animals and also because disease processes in older animals made such measures more variable and less amenable to interpretation.

Dr. Dragan moved, and Dr. Drinkwater seconded, that the conclusion be modified to indicate that the neoplasms of the ovary and lung in female mice were not dose dependent. After discussion among the panel the motion was withdrawn. Dr. Drinkwater then moved that the conclusions be accepted as written. Dr. Dragan seconded the motion, which was approved with seven votes and one abstention (Dr. Klaunig).

Methacrylonitrile Dr. Burhan Ghanayem, NIEHS, introduced the toxicology and carcinogenesis studies of methacrylonitrile by comparing the toxicity and metabolism of methacrylonitrile and acrylonitrile, describing the experimental protocols, and summarizing the body weight, survival, and histopathologic lesions in rats and mice. The proposed conclusions were:

> Under the conditions of these 2-year gavage studies, there **was no evidence of carcinogenic activity** in male or female F344/N rats administered 3, 10, or 30 mg/kg. There was **no evidence of carcinogenic activity** of methacrylonitrile in male or female B6C3F_1 mice administered 1.5, 3, or 6 mg/kg.
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> In male and female mice, methacrylonitrile administration caused significant increases in the incidences of nonneoplastic lesions of the nose and liver.

Dr. Chatman, the first principal reviewer, questioned whether a maximum tolerated dose was achieved for the mouse study. Dr. Drinkwater, the second principal reviewer, also wondered if the mice could have tolerated a higher dose and if the loss of some animals due to accidental deaths significantly reduced the sensitivity of the study. Dr. Dragan, the third principal reviewer, suggested that inhalation might have been a more appropriate route of exposure.

Dr. Ghanayem justified the selection of 6 mg/kg as the top dose for mice by noting that in the precursor 90-day study, two mice receiving 12 mg/kg died from acute toxicity. He also noted that, on a molar basis, the top dose in this study was double the lowest dose at which neoplasms were seen in mice in the companion acrylonitrile study. Dr. J. Haseman, NIEHS, presented calculations indicating that the loss of part of the high dose group of male mice reduced the

theoretical sensitivity of the study to discern a chemical-related increase in neoplasms only by about 10%.

Dr. Chatman moved that a statement be added to the conclusion to indicate that mice may have been able to tolerate higher doses. Dr. Piegorsch seconded the motion. Dr. Drinkwater inquired if any data on cyanide levels were available to indicate that doses of 12 mg/kg would not have been tolerated. Dr. Ghanayem said that mortality, tremors, and convulsions were the indicators of acute toxicity that were used.

Dr. C Portier, NIEHS, asked the panel for a more precise statement and asked if the implication was a conclusion of **inadequate study**. Dr. Hecht did not feel the study was inadequate because the goal of providing a comparison between methacrylonitrile and acrylonitrile was achieved. Dr. J. Bucher, NIEHS, said the dose selection criteria used in this study followed the standard practice: because 12 mg/kg caused lethality and acute toxicity in a 90-day study, half that dose was chosen for the 2-year study. The motion to amend the concluding statement failed by three yes votes to four no votes with one abstention (Dr. Klaunig). Dr. Davis then moved that the conclusions be accepted as written. Dr. Malarkey seconded the motion, which was approved by five yes votes to one no vote and two abstentions (Drs. Klaunig and Dragan).

Citral Dr. Nancy Ress, NIEHS, introduced the toxicology and carcinogenesis studies of citral by describing the properties and uses of the chemical, the study rationale, the protocol including the inclusion of the chemical in microcapsules in the animals' feed, and the lesions observed in rats and mice. The proposed conclusions were:

> Under the conditions of this 2-year feed study, there **was no evidence of carcinogenic activity** of citral in male or female F344/N rats exposed to 1,000, 2,000, or 4,000 ppm. There was **no evidence of carcinogenic activity** in male B6C3F1 mice exposed to 500, 1,000, or 2,000 ppm. There was **some evidence of carcinogenic activity** in female B6C3F1 mice based on increased incidences of malignant lymphoma.

Dr. Davis, the first principal reviewer, said that because lymphomas are a fairly common neoplasm he thought a conclusion of **equivocal evidence** was more appropriate for the female mouse study. Dr. Piegorsch, the second principal reviewer, noted the frequent comparisons of concurrent study results with historical control data and wondered if formal statistical comparisons with historical data might be considered. Dr. J. Haseman, NIEHS, replied that, because of inevitable differences in diagnostic criteria and laboratory conditions between different studies, such formal comparisons might not be warranted and the historical data would be used informally for comparative purposes. Dr. Piegorsch felt the response in mice was greater than equivocal based on the significant increase in lymphomas and the unusually high incidence of lymphomas in the EMF study in the historical control database. Drs. Davis and Thrall questioned whether hematologic changes ought to be attributed to reduced feed

consumption. Dr. Thrall also noted that the reduced urea nitrogen concentration might have been due to dehydration or nephropathy.

Public Comment: Ms. Jennifer Cocchiara, representing the Research Institute for Fragrance Materials, inquired if the ability of citral to inhibit the conversion of retinol to retinoic acid (a vitamin A precursor) could have added nutritional stress to the study animals and contributed to their body weight decreases. Dr. Ress felt the toxicity of the chemical rather than nutritional deprivation was the main cause of lowered body weights.

Dr. Davis moved that the conclusion be accepted as written, with the exception that the conclusion for female mice be changed to **equivocal evidence of carcinogenic activity**. Dr. Thrall seconded the motion. The vote on the motion was four yes votes and four no votes, whereupon Dr. Hecht, as chair, broke the tie and the motion was approved.