EXTERNAL LETTER PEER REVIEW OF A REPORT BY BIO-RESEARCH LABORATORIES, LTD.:

"SUPPORT: FINAL REPORT FOR A 90-DAY INHALATION NEUROTOXICITY AND TOXICITY STUDY BY EXPOSURE TO A DRY POWDER AEROSOL OF CERIC OXIDE IN THE ALBINO RAT WITH COVER LETTER DATED 013095."

FINAL REPORT

Prepared for
Integrated Risk Information System (IRIS) Program
Office of Research and Development
National Center for Environmental Assessment
U.S. Environmental Protection Agency

Prepared by
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Oak Ridge Institute for Science and Education
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August 2006

This document was prepared for the EPA by ORISE under Interagency Agreement No. DW-89939822-01-0 between EPA and the U.S. Department of Energy. ORISE is managed by Oak Ridge Associated Universities under a contract with DOE.

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Ceric Oxide Exposure Study Review

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REVIEWER COMMENTS BY QUESTION

External Letter Peer Review of a report by Bio-Research Laboratories, Ltd.: "Support: Final Report for a 90-day Inhalation Neurotoxicity and Toxicity Study by Exposure to a Dry Powder Aerosol of Ceric Oxide in the Albino Rat with cover letter dated 013095."

The U.S. Environmental Protection Agency's (EPA) National Center for Environmental Assessment (NCEA) is developing a "Toxicological Review of Cerium and Cerium Compounds." The Bio-Research Laboratories, Ltd (BRL, 1994) Final Report is a proposed principal study, but has not undergone a formal peer review. It is important that selected outside experts evaluate the accuracy of the experimental procedures, results, and interpretation and discussion of the findings presented in this investigational report.

The BRL (1994) study exposed four groups of Sprague-Dawley rats, 15 rats/ sex/ group, via nose-only inhalation to a dry powder aerosol of ceric oxide for 6 hours a day, 5 days a week, for 13 weeks at dose levels of 0, 5, 50, and 500 mg/m^3. Behavorial effects of exposure were not observed, nor were effects on the liver or kidney function or reproductive organs. Increased lung weights were evident and histological examination of the lung revealed pigment accumulation at all dose levels and alveolar hyperplasia at the two highest doses. Pigment accumulation was also observed in the nasal cavity, bronchi and trachea, and liver and spleen. The bronchial lymph nodes were macroscopically enlarged and microscopic findings included pigment accumulation and hyperplasia. Segmented neutrophil counts were elevated in females at all dose levels and the two highest dose levels for males.

1. Based on your knowledge of toxicological protocols, please comment on the experimental design of this investigation. Do you see any significant issues with the test system or article, inhalation exposure equipment and monitoring of atmosphere, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?

R. R. Dietert, Ph.D.

The study design incorporated 15 Sprague-Dawley rats of each sex for each of four groups: a control and three cerix oxide dose groups (exposure via inhalation). Sexes were started into the protocol in a slightly staggered time course to permit complete animal assessment to be accomplished within labor/facility constraints. The exposure protocol as well as the atmosphere monitoring were appropriate and with the stated exception of a single animal death due to an equipment sizing problem during exposure, all seems to have gone as designed. Atmospheric levels of the ceric oxide powder were in the ranges anticipated and the fluctuations across the exposure period were not problematic. The data collection, quality assurance oversight, and statistical analyses were conducted appropriately. Some neurotoxicologic endpoints had to be evaluated as qualitative or semi-quantitative assessments, but this was to be expected given protocols at the time the study was conducted. Overall, the study was well-conceived and appears to have been executed with considerable care.

Jie Liu, Ph.D.

The experimental design fits well with the title (goal) of this report "A 13-week inhalation toxicity and neurotoxicity study by nose-only exposure of a dry powder aerosol of ceric oxide in the albino rat." Inhalation to ceric oxide aerosols occurs at workplace where pollucite is mined or cerium compounds are manufactured. Inhalation exposure to airborne ceric oxide is also a possible route for the general population. Thus, to study the health effects from inhalation of ceric

oxide is of toxicological significance. Adult SD rats were selected because rat is a commonly used rodent species for inhalation toxicity and for neurotoxicity studies. A total of 120 qualified adult rats (15 male and 15 female rats/group x 4 groups) are sufficient and typical in such a 90-day subchronic study. The dose levels (0, 5, 50, and 500 mg/m3) were selected according to the potential human exposure (TLV of 5 mg/m3). Thus, the three dose level selections were appropriate and the highest dose is 100 times the TLV value for ceric oxide. All rats were examined before, during, and after exposure for any overt signs of reaction to treatment, including clinical observation, food consumption, body weight, Functional Observation Battery, laboratory investigations, and ophthalmologic examinations. At the end of the experiments, a complete necropsy and histopathology were performed. This was a well-designed and well-conducted study.

Overall, no specific issues are raised with the entire test system.

a. Animals. The 60 qualified animals/sex were selected from 85 rats and randomly assigned to different test groups and acclimated for two weeks prior to treatment as detailed on Page 13. This is a very good approach. Additional 10 rats were used to obtain the normal values for laboratory investigations and gross pathology. All animals were clearly labeled to trace their reactions to treatment as reported in the entire experiment (a minor comment: air control males were actually numbered 1001-1017, not from 1001 to 1015, similar to other groups). The animal housing, diet and water system were certified (page 1293-1310), and met the criteria of NIH Guidelines for Humane Use and Care of Animals. No significant issues in animals were found.

b. Test chemical. The identity and chemical analysis of test chemical were detailed on page 14, with certification on page 1311-1312. The stability of the test compounds during the experiment was also analyzed (page 1344 to 1352). No questions about the purity of ceric oxide used in the study.

c. Inhalation exposure equipment. The "nose-only" exposure equipment as depicted in Fig. 2 (page 48) was described in detail on page 14-16. No questions on the equipment system.

d. Monitoring of the experimental atmosphere. The exposure atmospheres were adequately described (page 16-17). For the three ceric oxide groups, daily camber concentrations (page 51-66), daily nominal concentrations (page 67-68), weekly particulate size (page 69-76), all were documented in detail. No questions.

e. Observations. The observations were adequately described in general on page 17 to 22, and documented in details in later sections:

i. Clinical examinations and mortality were summarized on page 26-27. A accidental death was described in detail and the conclusion that it is unrelated to treatment was valid. Table 6 (page 77-90) summarized the reddish staining among groups (Minor: please check page 79, on Day 37, group 3, pre-staining). The frequencies were low and also seen in controls, and the conclusion that these reactions were associated with exposure, not treatment, is valid. Other clinical signs were recorded in detail on page 278-282, and these occasionally occurred clinical symptoms were not consistent and are unlikely related to cerix oxide treatment.

ii. The animal body weight, body weight gain, food consumption were summarized in table 7-9 (page 91-96), with detailed individual animals on page 283-307. The individual numbers were correctly summarized.

iii. Ophthalmology summary reports (page 27, page 33) and the individual documentations (page 308-310) supported the conclusion that ceric oxide inhalation did not produce any ocular changes.

iv. Functional Observation Battery for neurotoxicty was a major goal of this study, and the comprehensive observations included home cage observation, removal observation, arena observation, handling, grip strength, and body temperature from pre-study, on Day 1, week 4, 8, and 13, were documented in detail. These observations were complete and summarized in table 10 (page 97-187), with individual animal report from page 311-670. These huge individual data were appropriately summarized, and supported the conclusion that no clear behavioral changes are associated with ceric oxide treatment (page 27). Minor: The page 438 (female week 8, group 4) should be placed after page 462 (female, week8, group 3).

v. Motor Activities are a major endpoint for neurotoxicity. They were depicted in Fig. 1 (page 40-47), summarized in Table 11 (page 188-195), with individual records from page 671-801. All the data supported the conclusion that no toxicologically significant differences in total motor activity between control and ceric oxide treatments were evident. (Minor: page 716, animal 3011 could be "T." as the number was too low and the same animal was normal on the 13 week, page 732).

vi. Laboratory Investigations. Detailed descriptions of Methods, Equipments, Symbols, and Criteria are included as Appendix 7 (page 802-812). Complete batteries of laboratory investigations were clearly documented. These included hematology summary in Table 12 (page 196-219) with individuals on appendix 8 (page 813-903), clinical biochemistry in Table 13 (page 220-237) with individuals on appendix 9 (page 904-958), and urinalysis with individuals in appendix 10 (page 959-995). These investigations were complete and supported the conclusion that ceric oxide treatment did not cause alterations on these 65 parameters. Minor: Clotted blood occurred on week 8 sampling (about 40% on page 835 and page 854), but clotted blood was avoided at week 13 sampling, which is the most important time point. Animal 4503 at 13 weeks had slightly higher ALT (page 956), but histology did not reveal significant change (page 1252), and other parameters were in normal range. Thus, this slight increase in ALT does not matter and does not affect the conclusion.

b. Terminal

i. Organ weights. At animal termination, the weights of 11 major organs were recorded in detail (appendix 11, page 998-1021) and summarized in Table 14. Ceric oxide exposure increased lung organ weight in both male and females in a clear dose-dependent fashion. The same conclusions were reached when the absolute organ weights were normalized to body weight (appendix 12, page 1022-1045, and Table 15, page 244-249), or to brain weight (appendix 13, page 1046 to 1069, Table 16, page 250-255). All these data clearly indicate the increases in lung weight are related to ceric oxide exposure. However, slightly, but significantly increases in spleen weights (only in male high dose group 4) may be related to ceric oxide exposure. The spleen weights (may be related) would be better separated from lung weights (clearly related to ceric oxide

exposure) on page 29 description. Increases in thymus weights in group 3 of male and female rats are also significant when normalized with the body weight, and thus they may also be related to ceric oxide exposure, rather than "treatment-unrelated" (page 29), which could be grouped together with spleen, as both organs are likely involved in clearance of transferred ceric oxide, as pigment accumulation was evident in spleen, and lymphonodes (page 29-32, although thymus was not examined, a few hemorrhage was seen in males at the high dose (page 267).

ii. Histopathology. Gross histopathology was summarized in Table 17 (page 256-260). In addition to the lung, the gross alterations in lymphonodes were evident (page 29-32, and Table 18, page 261-275).

iii. Histology procedures were described in detail on page 23-25. A detailed individual histopathology was reported on appendix 14, page 1070-1291, and partially summarized on page 29-32. In general, Histopathology was well conducted and the pathology findings supported laboratory analysis and gross pathological observations. Minor comments: some pages were misplaced: page 1210 and 1211 should be exchanged; page 1254-1262 should be followed by page 1216; page 1218 should be placed before page 1168. These pathological reports were appropriately summarized as notable pathological findings on page 34-36, and the conclusions reached are valid.

c. Statistics. The procedures of statistics were described adequately on page 25. Data were expressed as mean and SD, and analyzed ANOVA, followed by Dunnett's, Kruskal-Wallis, or Dunn's test. The frequency data and pathology were analyzed by Fisher's exact test. All these analysis are correct.

d. Quality Assurance. Quality assurance statement was described on page 25, and the statements were presented on Page 38-39. No questions on quality assurance issues.

Mark Noble, Ph.D.

The behavioral tests employed are not subtle and would be expected to only reveal dramatic alterations in function. Observational tests (locomotor activity level, arousal, grooming, defecation, urination, olfactory response, handling, salivation, etc.) all also represent crude tests, and there are few toxic insults in an environmentally relevant range that would affect any of these parameters. For example, it is not even clear whether any of these tests would detect toxicity of lead (Pb) when applied at environmentally relevant levels that now are believed to be associated with clear evidence of adverse effects on development of the CNS.

It is also noted that the standard deviation on some of these tests may be so large as to make it impossible to observe differences that are less than catastrophic. For example, consider on document pg. 188 the motor activity scores. Mean scores in the range of 255-290 are associated with standard deviations ranging from 89-135. From the small number of observations made, it would be statistically impossible to discern differences between groups.

David B. Warheit, Ph.D.

This is, in general, a well conducted study with a couple of limitations. There are, however, a few weaknesses which, while they do not compromise the integrity of the study, would have strengthened the quality of the study. These include the following:

1) There appears to be a significant lack of physicochemical characterization of the test article/test sample. This Reviewer makes a distinction between what would have been

required today (2006) vs. what should have been required in 1993, when the study was undertaken. In 2006, more extensive physicochemical characterization would have included the following: mean particle size and size distribution of the bulk material, surface area determinations, aggregation potential, surface coatings (if any), crystal structure, AS WELL AS the characterization that should have been included in 1993 – i.e., data regarding the composition, purity, solubility, and contaminants of the test article. This is rather important because the inhaled ceric oxide particles appeared to translocate from alveolar regions to extrapulmonary organs such as the liver, spleen and other organs – therefore it would be important to know whether the ceric oxide particles were soluble or insoluble.

2) The absence of an additional postexposure (sacrifice) period of evaluation is unfortunate. The experimental design described a 13 week inhalation exposure period, followed by an immediate postexposure sacrifice period. In order to gauge the reversibility of the effects observed or measured, it would have been necessary to add an additional 1 or 3-month postexposure period of evaluation.

3) It is very interesting that the authors have concluded that inhalation of ceric oxide particles produced antigenic stimulation. This would seem to be an unusual response following inhalation of dust particles. It seems likely to this Reviewer that inhaled particles could translocate from sites of particle deposition (bronchoalveolar junctions) to the corresponding interstitial compartment and connecting lymph nodes, wherein the particles may lie freely or be phagocytized by macrophages residing in the lymph nodes. However, the finding of lymphoid hyperplasia seems to be an unusual response for an inhaled dust. Therefore, it would have been very helpful to have information on the solubility of the ceric oxide dust and to have scheduled an additional postexposure time period to gauge the possible reversibility of the response.

4) It seems very interesting, given that ceric oxide particles are a white powder, that the pathologist reported that the pigment present both intra- and extracellularly within the respiratory system presented histologically as distinctly, round, blackish green material. Again, it would have been interesting to have more information on the physicochemical characteristics of the ceric oxide test article – as well as an additional postexposure time period.

Do you see any significant issues with the test system or article, inhalation exposure equipment and monitoring of atmosphere, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?

1) As stated above, this appears to be a well conducted study. This Reviewer has already discussed some significant issues related to the lack of physicochemical characterization of the test article and the experimental design of the study. The inhalation exposure equipment, monitoring of atmospheres, endpoints recorded, terminal procedures, statistical analyses, and quality assurance appear to be acceptable. It is unclear whether the authors considered the pigmentation/discoloration to be an adverse effect.

Some of the authors' conclusions were a bit confusing. Moreover, some aspects of the study results were interesting. These are detailed below:

1) In the summary and conclusion sections (page 32) it is written "An overall no-effect level cannot be established based on marginally reduced body weight gains recorded

in Group 4 animals, as well as other changes recorded in treated groups including increased segmented neutrophil counts, higher lung and spleen weights ….. "

The reference to the body weight gains recorded in Group 4 animals is confusing. According to the assessment of this Reviewer – the reason that a no-effect level could not be established was because – there were clearly significant biological effects vs. controls in Groups 3 and 4 animals; and more importantly, in Group 2 females – there was a significant increase in segmented neutrophil counts (hematology), concomitant with bronchial lymph node hyperplastic effects in both male and female group 2 animals as well as mediastinal lymph node hyperplasia in group 2 female animals.

2) The authors concluded that systemic dissemination of the test article was evident in Group 4 animals (without defining the term "systemic dissemination"). Was there not translocation (i.e., systemic dissemination) of ceric oxide particles from sites of particle deposition to systemic circulation (i.e., spleen, liver, etc.) at all exposure levels?

3) This Reviewer was intrigued by the relative lack of dose response effects when comparing the low (5 mg/m3) and higher exposure concentrations (50 and 500 mg/m3). The differences in exposure concentrations and corresponding doses (lung burdens were not measured) between 5 and 500 mg/m3 are substantial (100-fold!) Yet, although there was a (minor) dose response trend, with respect to the measured endpoints, it is remarkable that there were not greater histopathological differences between the responses to the low and high exposure concentrations.

4) This Reviewer was also surprised that exposures to 500 mg/m3 ceric oxide did not produce more substantial pulmonary effects such as (lung fibrosis). The major pulmonary effects were alveolar hyperplasia, lung weight increases and pigment accumulation. However, metaplasia in the larynx was noted for all treatment groups; and the lymph node effects have also been noted.

5) One must conclude, however, that since an overall no-effect level could not be established from this 13-week inhalation study, the currently proposed "nuisance dust" exposure level of 5 mg/m3 for ceric oxide particulates should be reduced.

2. Are there endpoints that should have been monitored that were not part of the investigation?

R. R. Dietert, Ph.D.

Given the time of the study (1993-1994), the endpoints monitored were appropriate for general toxicity and neurological toxicity evaluation. Most of the neurotoxicologic endpoints pertained to motor function rather than behavioral assessment. If the study were redone today, additional behavioral measures such as learning and memory-related endpoints might have been added. Some of the endpoints evaluated might have been quantitative instead of semi-quantitative as well. The same is true for lung, blood and lymphoid organ assessment. The measures obtained in 1993 were appropriate with the toxicological assessment norm for that time. However, if the study were repeated today, additional parameters would have been assessed to adequately analyze the hematopoietic, inflammatory and immune considerations. Lung cytokines could be evaluated and immune cell populations examined in lymph nodes, spleen and thymus. Again, these

evaluations were not all routine in rats in 1993 and, hence, the study parameters were appropriate given the study is now over a decade old.

Jie Liu, Ph.D.
For the goal and scope of this 90-day inhalation study, all the endpoints used are sufficient and no additional endpoints are necessary.

Mark Noble, Ph.D.
The information provided in this report clearly indicates a number of endpoints that should have been part of this investigation. These are considered in greater detail under heading 5, but are equally applicable to this question.

- **a.** How long does the inflammation caused by ceric oxide particle inhalation last? It is clear that industrial and environmental exposure will last for far longer than the two week end point utilized in this study. The truncated time point used provides no information on delayed effects, or changes over time.
- **b.** Are the hyperplastic and neoplastic changes seen indications of carcinogenic potential of exposure? When metaplastic changes are seen in the context of an examination in the clinic, one's immediate concern is to determine whether such changes are associated with pre-neoplastic states. Experiments that could be used to provide such information are considered under point 5.
- **c.** How do effects change with repeated exposure? It is suggested by the submitters of this trial that that the inflammatory changes seen are associated with stimulation of an immune response. Yet, there were no attempts to define whether this is so, nor what the consequences would be in the context of repeated challenge (let alone on an asthmatic background, for example).
- **d.** How do results differ in different strains? The results of analysis within a single strain are impossible to interpret in a meaningful way. Recommendations on other strains to use are provided under point 5.
- **e.** Combined insults: Single insult paradigms are highly artificial, and provide little resemblance to the effects of exposure in the real word. Co-exposures that would be likely to occur with ceric oxide exposure (e.g, other industrial contaminants likely to be found in the same workplace, exposure via diesel emissions.

David B. Warheit, Ph.D.
The endpoints monitored for this study were adequate.

3. Please comment on the strength, credibility, and relevance of the toxicological results. Were the results of the individual rodents correctly summarized and interpreted?

R. R. Dietert, Ph.D.
The toxicological results of the study do reflect the profiles from the individual rodents as far as the parameters evaluated. The observations appear to have been recorded with care and with appropriate oversight. As previously-mentioned some parameters were only semi-quantitative and if performed today, might have been done using quantitative imaging programs that would permit more extensive comparisons. Having said that, the histological results are clear-cut and the results did not suffer from the methodologies employed. In fact, there are very few gray areas in the study such as might have occurred where serious individual problems were noted but were not reflected as statistical analyses were performed. Such examples were minimal to non-existent. In summary, the results do reflect underlying individual rodent observations and the

profile of results can be viewed as credible.

Jie Liu, Ph.D.
This is a well-designed and well-performed study. The results obtained are solid, the data interpretations are appropriate. The conclusions reached are sound. All the individual data are correctly presented and summarized. These observations should be of toxicological significance.

Mark Noble, Ph.D.
Within the tests that do show changes, there also some peculiar observations that raise concern about the outcomes reported. For example in gross pathological observations of the lung, 15/15 animals in the moderate exposure group showed pale areas in the lung, but 0/15 animals in the low or high dose exposure groups (page 256). How is it that such effects are seen in all animals in an intermediate exposure group, but in none of the animals receiving either a lower exposure or a higher exposure? The most likely interpretation is of a flaw in the investigation, an issue that is un-addressed.

Bronchial and mediastinal lymph nodes in moderate and high exposure groups had a high frequency of enlargement and discoloration, and the potential negative implications of such finding were un-explored.

David B. Warheit, Ph.D.
As discussed above, the strength, credibility, and relevance of the toxicological results are adequate – under the conditions of this study. The study would have been strengthened if the characterization of the test material had been more robust and an additional postexposure period of evaluation (i.e., sacrifice) had been added to the experimental design of the study.

The results of the individual rodent data appear to be correctly summarized in this report.

4. Were the conclusions supported by the data? Are there any observations that were excluded from the conclusion that should have been included? More specifically, were there any observations that were excluded from the conclusions that are contradictory?

R. R. Dietert, Ph.D.
The most significant questions with the present study concern the interpretations of the findings, the fact that additional tests would have been conducted had the study been performed in this decade, and the projected ramifications of the reported findings.

In the overall conclusions the report states there were no behavioral effects observed associated with the test chemical treatment (page 1). Yet, the decrease in forelimb grip in the high dose females was significant and rather marked (page 27, data on page 177). It was, however, restricted to the one sex. It remains an open question in terms of the significance of this one dose group one sex effect.

Other issues surround the immune-inflammatory responses. The report states that the inflammation in the lungs (and also reflected by swelling in the spleen and lymph nodes) reflected little more than normal clearance of the particles. This led to a dramatic increase in circulating neutrophils as well as lung infiltration. Alveolar macrophages were largely responsible for particle uptake and this was particularly seen at lower exposure doses (page 35). The report speculates on the likelihood that the observed inflammatory findings would have been reversed following an appropriate recovery period (page 2). That is certainly one possibility and

might be the case. However, it is only one of several possibilities given the full range of observations in this report. The report indicates that lymphoid hyperplasia occurred at even low doses in both sexes in several lymph nodes and the lung (page 35). The reason for this is speculated to be immune stimulation by the test chemical. If indeed the report's authors are correct, then the test compound is antigenic and has induced a significant lymphoid immune response, the profile of it is uncharacterized. In effect, it is not known if this response would prove to be clinically uneventful and subside as such or if it might become problematic elevating the risk of hypersensitivity/autoimmune reactions. None of the parameters measured address the nature of the host immune stimulation beyond the clear lymphoid hyperplasia response. Therefore, it remains unanswered whether the host response is not problematic or potentially similar to what might be seen with exposure to certain nickel compounds. Clearly, the longer-term disposition of the lung inflammatory response is highly dependent upon the underlying nature of the lymphoid response via antigenic stimulation. For this reason, long term immune effects resulting from these exposures are simply unknown and were not addressed within this specific protocol.

Jie Liu, Ph.D.

All the data are carefully analyzed and appropriated interpreted. The major conclusions are supported by the data obtained, and no contradictory is evident. Thus, inhalation exposure of rats to ceric oxide at doses up to 100-fold the TLV value (500 mg/m^3) for 6 hours/day, 5 days a week for 13 weeks did not produce neurotoxicity, did not affect liver and kidney function or reproductive organs. The notable changes were the increased lung weights with pigment accumulation in a dose-dependent fashion following subchronic ceric oxide inhalation exposure. Pigment accumulation was also evident throughout the respiratory tract, and the immune organs (spleen, liver, lymphonode, and likely in thymus) with slight organ enlargement at the higher doses. All these changes could be envisioned as the adaptive mechanism to ceric oxide exposure, and the "threshold" to produce overt pathology changes is perhaps not reached. In addition, segmented neutropil counts were elevated in females at all doses, and in males at the higher doses. The significance of such an increase is not clear, but may also be related to immune reaction to ceric oxide exposure.

Mark Noble, Ph.D.

There is clear evidence of inhalation toxicity of ceric oxide. While the authors contend this is not evidence of direct toxicity, that is solely because experimental design was not such as to distinguish between direct and indirect effects. Moreover, it is of concern that changes of both hyperplasia and metaplasia were observed. While it is stated that "it is probable that the observed findings would be reversible following an appopriate recovery period," no evidence is provided to support such a claim.

While it is stated (page 39, document page 31) that "the presence of lymphoid hyperplasia in the lymph nodes and lungs is consistent with antigenic stimulation by the compound," it is also consistent with more adverse effects. Of particular concern in this regard was the metaplasia seen in the larynx.

While it was suggested that the lymphoid hyperplasia could be interpreted as antigenic stimulation by the compound, no tests on whether a T- or B-cell response is initiated were offered. This is of particular importance in the context of repeated exposure. If this is a substance that is allergenic, this would be of great concern in raising the possibility that reactions would become more serious over time. This critical question was not addressed.

While it was reported that "the pulmonary change was remarkable for the somewhat contained

inflammatory response despite the copious amount of pigmented material present" but was a finding "compatible with the minimal clinical signs noted" it is striking how discussion of worrisome data is consistently skewed as being of no significance when a more negative interpretation of the data is in fact more reasonable.

In he larynx, at the low exposure levels already 3/15 animals showed evidence of metaplasia, increasing to 9/15 and 13/15 in moderate and high dose respectively (pg 263). Hyperplasia was seen in bronchial and mediastinal lymph nodes in almost all animals, even in the lwo exposure group (pg. 267, 275). The laryngeal metaplasia was interpreted as being adaptive to the insult and "can be reversible." Once again, however, no observations are supplied to support this view and the data is automatically interpreted in favor of the there being no cause for concern.

David B. Warheit, Ph.D.
In general, the conclusions were supported by the data. Comments on the experimental design and characterization of the test material are detailed above.

5. In your opinion, was this investigation properly planned, conducted, and reported? Are there any procedures, observations or analyses that would have added to the quality of this investigation?

R. R. Dietert, Ph.D.
The study was well-conceived and conducted with care. The overall group summaries do reflect the underlying individual observations. In general, the conclusions are fully supported by the data with the possible exception of some speculation regarding outcomes that would extend beyond the timeframe of the direct observations. The study employed appropriate assessment tools available at the time of the investigation. This reviewer would consider the study protocol and subsequent results to be highly reliable to the extent parameters were examined.

If the study were conducted today, assessment measures would certainly be different. For example, with compounds such as cerix oxide, neurobehavioral assessment might well include parameters associated with learning and memory. Even some histological assessment would probably employ more quantitative measures using imaging equipment and software programs vs. qualitative subjective scoring. Additionally, it is likely bronchial-alveolar lavage would be collected for animals and analyzed for inflammatory cell profiles as well as cytokine profiles. This would provide a much better indication of the nature and potential changes in lung inflammation during the course of exposure. More information concerning the extensive lymphoid hyperplasia would be important as well. At present, little can be concluded relative to potential immunotoxicity based on the parameters examined.

In summary, the study appears to have been well conceived and the conclusions should be reliable. The utility of the findings is somewhat limited because of the historical nature of the study. It was performed more than a decade ago and, in some cases, used less sensitive assessment tools for neurobehavioral toxicity and immunotoxicity than are available today.

Jie Liu, Ph.D.
In summary, this 90-day subchronic study was well planed, professionally performed, detailed documented, and appropriately reported.

Mark Noble, Ph.D.

These studies raise serious concerns about potential toxicity of ceric oxide that need to be addressed with more precisely targeted investigations. Information that would seem of particular interest in establishing a better foundation for appropriate regulation of ceric oxide is as follows:

- **a.** How long does the inflammation last? Considering the known data (discussed in Section B) on clearance of ceric oxide, it is possible that the changes in induced are not readily resolved. Proper evaluation of ceric oxide toxicity requires information on longer exposure times (as individuals in the industrial environment, in particular, are likely to be exposed for periods far longer for 15 days) and also on effects that mayh occur at delayed time points after exposure ceases.
- **b.** Are the hyperplastic and neoplastic changes seen indications of carcinogenic potential of exposure? When one observes such morphological changes in tissue structure, the issue of transition to a neoplastic phenotype becomes of primary concern. The question of carcinogenic potential could be addressed in several ways. For example, inhalation exposure in one of the multiple mice strains that harbor a cancer-predisposing gene (generically referred to as "Onco-mice") could be used to provide a test of carcinogenic potential. In the present era, one could also conduct microrarray studies if a sufficient proportion of cells in the tissue are affected (or one could use laser-capture to cut out affected regions and conduct microarray analysis on mRNA amplified from these regions) to look for changes that would be indicative of neoplastic initiation and/or progression.
- **c.** How do effects change with repeated exposure? It is suggested that the inflammatory changes seen are associated with stimulation of an immune response. If this is the case, then exposing animals for 15 days, resting them for a month, and then re-exposing them would be associated with a more dramatic response in the second exposure trial. Due to the large number of individuals in the population with asthma, the question of whether ceric oxide can stimulate an immune response that might trigger asthmatic attack is considered of great concern. (In this respect, one is also concerned with the question of what effects ceric oxide exposure, in general, might have when combined with another inflammatory insult.)
- **d.** How do results differ in different strains? It has become absolutely clear that different strains of experimental animals provide very different outcomes in situations of toxicant exposure. One example of such differences is in the effects of methylmercury and thimerosal on different mouse strains. In the SJL strain it is very easy to detect adverse neurological effects of exposure to these substances at environmentally relevant levels, while in other strains of mice effects may be more limited. It is therefore recommended that studies need to consider this possibility, as choosing a particularly healthy strain of laboratory animals may provide a false sense of security (particularly in the context of the controlled diet of such animals, as discussed below).

  As breeding of SJL mice is problematic, a compromise strain that appears to be readily susceptible to chemical insults would be C57Bl/6. As most rat strains are not truly syngeneic (as determined by the need for immunosuppression when carrying out transplantation experimentation), this introduces another problem in interpretation of toxicological studies. The one strain of rats that is sufficiently inbred to allow cell transplantation without use of immunosuppressive drugs (the most rigorous test of whether animals are truly syngeneic) is the Fisher 344 strain.
- **e.** Combined insults: All toxicological insults in the real world represent combined insults. Some combinations that are relevant are combinations of environmental toxicants, which are well-established to cause different outcomes when applied in combination than when they are applied alone. For example, recent studies have shown that the combination of paraquat and maneb exposure in the rat produces CNS damage like that seen in

Parkinson's disease, while exposure to either agent alone does not have such effects. Other co-exposures need to be thought of in the context of the exposure itself. For example, exposure via diesel emissions might have different interacting factors than exposure in the context of glass polishing.

f. Dietary effects: In the context of industrial or ambient exposure to ceric oxide, dietary contributions may be of particular concern. The diets used for care of animals are often far superior to diets in human populations, in containing large amounts of fibre and vitamins, no refined sugars, and little or no trans-saturated fatty acids of the sort available in "junk food." As vitamin deficiencies, hyperglycemia and trans-saturated fatty acids all are known to cause oxidative stress to cells, any or all of these dietary problems could exacerbate the effects of exposure to a physiological stressor. For example, it has been shown that exposure to omega-3 fatty acids improve the healing response to spinal cord injury, while omega-6 fatty acid exposure actually worsens outcome (King et al., (2006) J. Neurosci., 26:4672-4680). For a more general review of the role of diet in neuroprotection, see, e.g., Mattson et al. (2002), Physiol. Rev. 82:637-642).

g. Particle size: It has become very clear that nano-particles can exhibit physicochemical properties very different from larger particles made out of the same substance, and may in fact be more reactive. As fine particulates will contain a contribution from nano-particles, and the use of nano-scale ceric oxide is already increasing (and can be predicted to increase still further), it is felt to be important to implement studies that allow the comparative properties of particulates of ceric oxide and nano-scale particulates of ceric oxide to be directly compared as the nano-scale particulates may be more toxic than those examined in the submitted studies. In these studies, concerns regarding strain differences and dietary contributions also pertain.

David B. Warheit, Ph.D.

In general, the conclusions were supported by the data. Comments on the experimental design and characterization of the test material are detailed above.

Other comments

Jie Liu, Ph.D.

This 90-day subchronic inhalation toxicity study of ceric oxide aerosol was well-designed, nicely performed, and all the procedures and results were very detailed reported. The data obtained were sound and appropriately analyzed. The conclusions reached are solid. There is no similar cerium toxicity and neurotoxicity study following inhalation exposure available in the literature, and this work would add to our understanding of toxicological profile of cesium.

Mark Noble, Ph.D.

A. Summary

This is a toxicity study on a hydrolytically stable high molecular weight cationic polymer of ceric oxide. Testing was via inhalation for 13 weeks in groups of 15 male and female rats. Animals were examined monthly for signs of toxicity, neurological testing (functional observational battery and motor activity testing), standard laboratory tests (hematology, clinical biochemistry, urinalysis), ophthalmoscopy and gross pathology.

This study was completed in 1994.

This study provides sufficient evidence of ceric oxide toxicity in the lungs as to provide cause for concern. There are also multiple examples of flaws in the study design. These include the use of

tests that would not readily detect subtle effects and/or have standard deviations so large that recognition of anything other than catastrophic results might not be possible. Moreover, evidence of adverse effects is consistently interpreted with a bias towards concern not being necessary, despite the fact that no data is presented to obviate the concerns raised.

Even with the flaws in this study, the evidence indicating a need for concern is readily apparent, and needs to be addressed by further experimentation. Specific recommendations for experimentation to resolve critical issues are offered, along with discussion of the flaws in study design.

B. Background

There is an increasing recognition that substances believed to inert, or otherwise toxicologically benign, actually may have significant adverse effects on the organism. One of the most recent examples of such a change in our understanding of the toxicological potential of a widely used compound is the 4-carbon molecule diacetyl, which is used to provide the aroma of butter in microwave popcorn and other baking uses. For many years of usage, no toxicological effects were reported. More recently, however, it has become clear that inhalation exposure to diacetyl, at least in industrial settings, causes extensive and very severe lung damage.

B.1. Ceric oxide:

The following information is summarized from the Chemical Information Profile for Ceric Oxide [CAS No. 1306-38-3] prepared in February 2006 for the National Toxicology Program by Integrated Laboratory Systems, Inc. (Research Triangle Park, NC) under Contract No. N01-ES-35515. Information provided in this previous report is considered highly pertinent to analysis of the present report. Detailed references for all of the following information is provided in this report, which is available at http://ntp.niehs.nih.gov/files/Ceric_oxide2.pdf.

Ceric oxide is used in an increasing variety of industrial applications, including petroleum refining (catalytic cracking catalysts), glass products, polishing powder, ceramics, crystals (e.g., for lasers and garnets), phosphors, automotive catalytic converters, as an additive to promote combustion of diesel fuels, as a pigment in dermatological preparations, and in nanoparticulate form as a carrier for otic and ophthalmic compositions. Projected uses include usages in cosmetics and lipsticks, as a matrix for anti-bacterial or bacteriostatic compositions. There has also been developing interest in nanoscale ceric oxide, which is being used as a fuel additive for diesel powered vehicles to increase fuel efficiency. Nanoparticles of ceric oxide are also used in the semi-conductor industry in chemical-mechanical polishing/planarization. Proposed nanoparticle usage includes as catalysts for chemical scrubers, nanoparticle coatings, as components of fuel cells, in electro-optical devices and even as an additive to cigarettes that emit low sidestream smoke.

Current exposure is not limited, by any regulations other than as might relate to exposure to particulates that are not otherwise regulated. These values are 10 mg/m^3 time weighted average (TWA) inhalable particulate containing no asbestos and <1% crystalline silica; 3 mg/m^3 TWA (respirable particulate); OSHA PEL: 15 mg/m^3 TWA (total dust) and 5 mg/m^3 TWA (respirable fraction)

B.2. Occupational Exposure to ceric oxide

NIOSH considered ceric oxide exposure in a report from 1981-1983.. At this time it was suggested that 25,130 workers (13,436 females) were potentially exposed to CeO2 in three industrial categories and 11 job categories including electrical and electronic technicians (17,417

[8,599 females]); grinding, abrading, buffing, and polishing machine operators (2,946 [2,448 females]); and optical goods workers (2,188 [2,448 females]). Exposure was confirmed by autopsy of workers with and without pneumoconiosis and workers who used carbon arc lamps or lens grinders.

B.3. Exposure to ambient particles

Data from the Health Effects Institute (HEI, 2001) suggests that there may be an increase in ambient ceric oxide particles in air (currently approximately 1 ng/m3) in areas of high traffic to levels of >1μg/m3. Diesel engine emissions are a particular source of interest due to the use of ceric oxide as a fuel additive, and emission factors for fuel containing 100 ppm CeO2 have been calculated to be 0.3 to 3.3 mg/km, depending on vehicle type. In tests with a Pt/Ce catalyst (0.5 ppm Pt and 7.5 ppm Ce in the diesel fuel), filtered emissions had 4.7 μg Ce/bhp-hr and 1.1 μg Pt/bhp-hr (brake-horsepower hour). It is estimated that by 2010 cerium emissions from use of diesel fuel in the European Union could total 1.3 million pounds annually (worst-case scenario: 22 million pounds).

Another source of ceric oxide emissions is municipal and hospital waste incineration. The mean cerium concentration found in bottom ash from incineration of various wastes was: food scrap – 8.57 ppm, animal waste – 23.5 ppm, horticultural wastes – 27.3 ppm, sewage sludge – 35.4 ppm, and municipal waste – 24.6 ppm.

In a subchronic inhalation toxicity study of microscale ceric oxide, there were significant increases in lung weights, concentration-related metaplasia of the larynx, and alveolar epithelial hyperplasia for mid- and high-dose male and female rats. The human equivalent Lowest Observable Effect Level (LOEL) derived from this study was 1 mg Ce/m3. Using data from this study and an uncertainty factor of 3000, a human equivalent Reference Concentration (RfC) of 0.3 μg Ce/m3 was developed (TERA, 1999). Intratracheal instillation of fine particles of CeO2 (size not given) induce primary lung lesions (i.e., pulmonary fibrosis and alveolar proteinosis and granulomas) but coarse particles did not.

No toxicological studies of nanoscale ceric oxide are available, and there are no adequate studies in mice for evaluating the potential inhalation hazard of either nanoscale or microscale ceric oxide. Given the concern that nanoscale metal oxides may be more toxic per unit mass than microscale metal oxides (due to the larger surface area per unit mass), the toxicological potency of nanoscale ceric oxide may be greater per unit mass. Consequently a human equivalent RfC for nanoscale ceric oxide may be considerably lower than 0.3 μg Ce/m3 and far lower than that predicted to occur from the use of nanoscale ceric oxide as a diesel additive.

B.4. Animal Studies:

Several interesting pieces of information pertinent to analysis of ceric oxide are available from previous animals studies, including the ones that are the subject of the present analysis. This information is contained in the February 2006 Chemical Information Profile for Ceric Oxide (Supporting Nomination for Toxicological Evaluation by the National Toxicology Program). The Chemical Information Profile included the statement that, for both sexes, “statistically significant lung weight increases, concentration-related metaplasia of the larynx, and alveolar epithelial hyperplasia for mid- and high- dose; pigment accumulated in lungs at all doses; and significant increase in lymphoid hyperplasia in bronchial lymph nodes that correlated with pigment volume.”

Based on previous studies, a No Observed Adverse Effect Level (NOAEL) of 0.41-0.43 mg Ce/m3 was determined for alveolar epithelial hyperplasia and of 0.55 mg Ce/m3 for increased

lung weight. Critically, the LOAEL (i.e., the lowest amount or concentration of an agent, found by experiment or observation, that causes an adverse alteration of morphology, functional capacity, growth, development or life span in an organism, system or (sub)population) was only marginally higher, and was suggested as 0.85 mg/m^3 in males and 0.82 mg Ce/m^3 in females (equivalent to ~1.0 mg CeO2/m^3).

What is important about the above information is that it indicates defining 5 mg/m^3 as low exposure and 50 mg/m^3 as moderate exposure, as in the studies under review, appears to be excessive in respect to suggested calculated LOAEL levels.

Other potentially pertinent information that has emerged from animal studies is that:
-Repeated doses increased retention half-times.
- Intratracheal administration of coarse particles did not induce lesions in rat lung, but finer particles did (size specifics not provided). Typical lesions were pulmonary fibrosis, alveolar proteinosis and granulomas.

B.5. Human Toxicity:

Studies on human toxicity are relatively rare. RE pneumoconiosis has been reported in several case studies of workers exposed to ceric and other RE oxides via inhalation, but these workers were also exposed to other particulates. Other rare observations have also been made, of which the most pertinent seems to be a report from Russia on children near a phosphate fertilizer plant in Russia, who were exposed to cerium concentrations decreasing from 10.2 ng/m3 at 200 m to 3.6 ng/m3 at 2500 m were 1.5 times more likely to have respiratory diseases, chronic inflammation of the tonsils, etc. (Volokh et al., 1990; PMID:2169646).

What has emerged as of particular interest from previous studies is that the less-soluble forms of inhaled cerium (e.g., ceric oxide) may remain in the lung and lymph nodes for years. Cerium deposits were found in the alveoli and interstitial tissue of an optical lens grinder 20 years after exposure to CeO2 powder particulates (<1 - 10 μm); some cerium deposits were also found inside the cells.

B.6. Other information of potential interest:

- When inhaled as CeO2, cerium precipitates in the lysosomes of alveolar macrophages as insoluble phosphates in fine needles or granules.
- Lung clearance rate is measured in years.
- Approximately 10% of absorbed cerium is excreted in the feces and urine with retention of 45% in the liver, 35% in the skeleton, and 10% in other organs (primarily, the spleen and kidneys).
− Ln3+ ions (which have the same valency shell as cerium) are well known inhibitors of Ca2+-dependent physiological processes such as those involved in blood clotting (e.g., prothrombin activation) and neuronal and muscular functions. It has been shown that trivalent cerium compounds inhibit active transport of Ca2+ through mitochondrial membranes, calcium and potassium channels, calcium-dependent hemolysis in burn patients, calcium- dependent enzymes, and contractility in cardiac, skeletal, and smooth muscle (e.g., intestinal) (Jakupec et al., 2005; PMID:15674649).

− Possible connection between cerium toxicity/magnesium deficiency and endomyocardial fibrosis has been reported (Brown et al., 2004; PMID:15275858; Eapen et al., 1996; PMID:8720088). The combination has promoted fibrogenesis in rat heart (Kumar et al., 1996; PMID:8694866). Cerium had an inhibitory effect on protein synthesis in cultured cardiac

myocytes and lung fibroblasts exposed to normal- and low-levels of Mg2+ (Shivakumar and Nair, 1991; PMID:2051999).

C. Evaluation of the current submission:

C.1. Summary of effects noted in the submission:

No effects were seen at a gross level, but higher lung weights were seen in the two higher dose levels of 50 and 500 mg/m(3). Lungs in all dose groups had pale areas macroscopically. There was pigment accumulation at all doses levels with alveolar hyperplasia. All treatment groups showed pigment accumulation in the nasal cavity, and the two higher doses levels showed pigment accumulation and hyperplasia. The highest dose showed pigment accumulation in liver and spleen.

Notably, bronchial lymph nodes were enlarged macroscopically at all dose levels in males and the two higher doses in females, and bronchial and other lymph nodes showed pigmenta accumulation and hyperplasia at all dose levels. The larynx of animals in all treatment groups showed pigment accumulation and metaplasia.

In addition, there were higher neutrophil counts in all dose levels in females and the two higher dose levels in males.

Pigment was thought to be evidence of failed clearance of the ceric oxide. That pigment was also found in the spleen and liver, it is apparent that respiratory exposure was associated with distribution in non-respiratory sites.

David B. Warheit, Ph.D.
Reviewer's notes-

A 13-week inhalation toxicity and neurotoxicity study by nose-only exposures of a dry powder aerosol of ceric oxide in the albino rat.

Fours groups each – 15 males and 15 females
0, 5, 50 and 500 mg/m3 6 hr/d 5 d/wk for 13 weeks nose-only
Groups 1 (0) ; 2 (5 mg/m3); 3 (50 mg/m3) and 4 (500 mg/m3)

MMAD = 1.8 – 2.2 μm

Body weights
Food consumption – weekly
Behavioral testing
Functional observation battery (FOB)
Activity levels?

- hematology
- clinical biochemistry
- urinalysis
- ophthalmological exams
- gross pathology
- histopathology

Results

- No clinical signs

- Overall body weight gain and food consumption of Group 4 was marginally inferior to controls – treatment related
- Treatment related effects in hematology were observed as higher segmented neutrophils counts in Group 2 females and Groups 3 and 4 of both sexes at 6 and 13 weeks
- No behavioral changes
- A trend for higher lung weights of males and females from Groups 2,3, and 4, and higher spleen weights of males from Group 4, were considered to be related to ceric oxide treatment

- Macroscopically, pertinent changes in the lungs – such as discoloration or pale areas (30 each in Groups 3 and 4), pale foci (4 animals in Group 2), and uncollapsed parenchyma (30 in Group 4 and 2 in Group 3). In addition, enlargement and/or pale discoloration of the bronchial lymph nodes (30 in Groups 3 and 4, and 28 in Group 2) and mediastinal (20 in Group 4, 18 in Group 3, 12 in Group 2 and 1 in Group 1) and pancreatic lymph nodes (3 in Group 1, 1 each in Groups 3 and 4) were primarily evident in animals treated with ceric oxide and considered to be induced by the test material.

- Histopathologically, pigment accumulation and/or alveolar epithelial/lymphoid hyperplasia in the lungs (30 each in Groups 2,3, and 4), lymphoid hyperplasia of the bronchial (30 each in Groups 3 and 4, 24 in Group 2), mediastinal (18 each in Groups 3 and 4, 12 in Group 2) and pancreatic (1 in Group 3) lymph nodes, metaplasia and/or pigment accumulation ion the larynx (22 in Group 4, 16 in Group 3, and 9 in Group 2), and pigment accumulation in the bronchial lymph node (30 each in Groups 3 and 4, and 27 in Group 2), nasal cavity (30 in Group 4, 26 in Group 3 and 15 in Group 2), bronchi (30 in Group 4, 9 in Group 3 and 1 in Group 2), trachea (28 in Group 4, 2 in Group 3), mediastinal lymph node (18 in Group 4, 17 in Group 3 and 12 in Group 2), liver (11 in Group 4), mandibular lymph node (12 in Group 4); spleen (9 in Group 4) and pancreatic lymph node (1 each in Groups 3 and 4) were considered to be induced by the test material.

- Conclusions ---
- An overall no-effect level cannot be established based on marginally reduced body weight gains recorded in Group 4 animals?, as well as other changes recorded in treated groups and including increased segmented neutrophil counts, higher lung and spleen weights, discoloration of the lungs and discoloration/enlargement of lymph nodes.

- Histopathologically, pigmented material accumulation in the lungs, bronchial, mandibular and mediastinal or pancreatic lymph nodes, trachea, bronchi, larynx, nasal cavity, liver and spleen, as well as alveolar epithelial hyperplasia (lungs), metaplasia (larynx) and lymphoid hyperplasia (bronchial and mediastinal or pancreatic lymph nodes, lungs) were seen in all treated groups. Systemic dissemination of the test article was evident in Group 4 animals.

  Results

  Clinical signs – not significant

  Body weights
    - Statistically significant lower mean body weight gains were recorded in high dose males vs. controls in weeks 2 and 8.

- The overall body weight gain of Group 4 was marginally inferior vs. controls – not statistically significant
- Statistically significant lower mean body weight gains were recorded in Group 2 females at week 7 and Group 2,3 and 4 females at week 10 vs. controls

Food consumption – NS

Ophthamalogy – NS

FOBs – No clear behavior changes were observed between the control and treated groups for qualitative assessment at any of the time points measured. However, a significantly reduced forelimb grip strength was recorded in Group 4 females at week 13.

Motor Activity – NS

Hematology

- Treatment-related effects were observed in segmented neutrophil counts in Group 2 females and Groups 3 and 4 of both sexes.
- Statistically significant elevated segmented neutrophils counts in Group 2 and 3 females and Group 4 animals – recorded at weeks 6 and/or 13 vs. controls.

Clinical Biochemistry – NS

Urinalysis – NS

Terminal Studies
Organ weights

- Treatment-related effects were seen in lung and spleen weights when expressed as absolute or relative to organ and brain weights.
- Higher lung weights were statistically significant in Groups 3 and 4 vs. controls
- Higher spleen weights (relative to body wt) were stat significant in Group 4 males vs. controls
- Spleen and lung weight changes correlated with gross and/or microscopic findings and were considered to be treatment-related.
- Significantly higher thymus wt recorded in Group 3 males vs. controls – not considered to be treatment-related.

Histopathology

- Histopathologically, pigment accumulation and/or alveolar epithelial/lymphoid hyperplasia in the lungs (30 each in Groups 2,3, and 4), lymphoid hyperplasia of the bronchial (30 each in Groups 3 and 4, 24 in Group 2), mediastinal (18 each in Groups 3 and 4, 12 in Group 2) and pancreatic (1 in Group 3) lymph nodes, metaplasia and/or pigment accumulation ion the larynx (22 in Group 4, 16 in Group 3, and 9 in Group 2), and pigment accumulation in the bronchial lymph node (30 each in Groups 3 and 4, and 27 in Group 2), nasal cavity (30 in Group 4, 26 in Group 3 and 15 in Group 2), bronchi (30 in Group 4, 9 in Group 3 and 1 in Group 2), trachea (28 in Group 4, 2 in Group 3), mediastinal lymph node (18 in Group 4, 17 in Group 3 and 12 in Group 2), liver (11 in Group 4), mandibular lymph node (12 in Group 4); spleen (9 in Group 4) and pancreatic

lymph node (1 each in Groups 3 and 4) were considered to be induced by the test material.

- A no-effect level was not present in Groups 2, 3 and 4, and systemic dissemination of the test article was evident in Group 4 animals. A dose-related effect of the compound was clearly evident.