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No. 125

GUIDANCE DOCUMENT ON HISTOPATHOLOGY FOR INHALATION TOXICITY STUDIES, SUPPORTING TG 412 (SUBACUTE INHALATION TOXICITY: 28-DAY STUDY) AND TG 413 (SUBCHRONIC INHALATION TOXICITY: 90-DAY STUDY)

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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No. 125 *Guidance Document on Histopathology for Inhalation Toxicity Studies, Supporting TG 412 (Subacute Inhalation Toxicity: 28-Day) and TG 413 (Subchronic Inhalation Toxicity: 90-Day) (2010)*

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This document presents the OECD Guidance Document on Histopathology for Inhalation Toxicity Studies, Supporting TG 412 (Subacute Inhalation Toxicity: 28-Day) and TG 413 (Subchronic Inhalation Toxicity: 90-Day).

Updated versions of TG 412 and TG 413 were published in 2009. The project for developing this Guidance Document was discussed at the meeting of the Working Group of National Coordinators of the Test Guidelines Programme (WNT) in 2008. An outline of a draft Guidance Document (GD) was developed in consultation with the expert group on TG 412 and TG 413, and a first version of the GD was developed by a consultant, in cooperation with a team of pathologists. Comments on successive drafts were requested from the WNT in June and October 2009.

The GD was approved by the WNT at its meeting held on 23-25 March 2010. The Joint Meeting of Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 5 May 2010.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

This document was prepared by a consultant contracted by the Secretariat. For the purpose of this document, the consultant chaired a team of pathologists. The document was revised several times to take into account the comments from the Working Group of National Coordinators of the Test Guidelines Programme. The final version was edited by the Secretariat.

Pathologists Team:

Roger A. Renne, DVM, DACVP (Chair)
Roger Renne ToxPath Consulting
Sumner, WA, USA

Jeffrey I. Everitt, DVM, DACVP
Director, Comparative Biology & Medicine
GlaxoSmithKline
Research Triangle Park, NC, USA

Jack R. Harkema, DVM, PhD, DACVP
University Distinguished Professor
Department of Pathobiology and Diagnostic Investigation
College of Veterinary Medicine
Michigan State University
East Lansing, MI, USA

Charles G. Plopper, PhD
Professor Emeritus
Anatomy Physiology and Cell Biology
University of California, Davis
Davis, CA, USA

Martin Rosenbruch, DVM, PhD
Toxicologic Pathology
Bayer Schering Pharma AG
Wuppertal, Germany

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INTRODUCTION

1. OECD Test Guidelines for 28 or 90 day inhalation studies (TG 412 and TG 413) were adopted in 1981 and updates of these two documents were published in 2009 (OECD, 2009a, b). The purpose of this Guidance Document (GD) is to provide pathologists, toxicologists, and associated scientists with recommended guidelines for performing the pathology tasks required by the specifications of TG 412 and 413, and for optimizing the value of pathology-related tasks in these studies. This document is structured to follow the specifications in TG 412 and TG 413 and to provide recommendations and supplementary information to these specifications in the temporal sequence one would encounter in planning, participating in, and reporting the results of pathology tasks in a 28 or 90 day inhalation toxicology study utilizing TG 412 and TG 413. It is also intended to conform to the recommendations provided in GD 39, Guidance Document on Acute Inhalation Testing, and to the OECD Principles of Good Laboratory Practice (GLP) (OECD, 1998).

2. General guidelines and recommended practices for histopathology tasks in toxicology studies have been published in the open literature (Hildebrandt, 1991; Crissman et al, 2004). Following these published practices will serve the pathologist well in avoiding the potential pitfalls associated with performing and reporting pathology tasks in inhalation studies. This Guidance Document provides pertinent information on each aspect of the pathology tasks for inhalation studies in rodents, and references to additional publications with details on methods and anticipated results.

Tasks Prior to Necropsy

Consideration of the potential effects of the physicochemical properties of the test article

3. As indicated in paragraph 3 of TG 412 and TG 413, it is important to utilize all available information on the physical and chemical characteristics of the test article in planning and making preliminary decisions regarding specific procedures in inhalation toxicity studies. This is especially true in the necropsy-related and histopathology tasks, where once animals are necropsied and tissues processed, choices may be very limited. Test articles with high water solubility and caustic properties are most likely to induce lesions in the uppermost areas of the respiratory tract (see more details below in the Tissue Trimming and Slide Preparation section). The mainstem bronchi and their immediate distal bifurcations are a primary location for goblet cell hypertrophy and hyperplasia induced by irritant aerosols such as acrolein or cigarette smoke (see also comment below under Special Stains). The amount of impact and/or deposition in airways or alveoli and clearance from the lung are dependent on particle or aerosol droplet size and respiratory rate and volume. Knowledge of these and other physical/chemical characteristics of the inhaled material are useful for the pathologist in determination of likely sites and types of effects in the airways and lung parenchyma. Critical decisions on the use of bronchoalveolar lavage, cell cycle studies, and other necropsy or histopathology-related tools may be made more accurately by the judicious use of information on the solubility, pH, aerosol characteristics, and other physicochemical data on the test material as it enters the respiratory tract of the animals being exposed.

Review of pertinent study data

4. It is recommended that all pertinent data generated on the study be available to the pathologist, toxicologist, and other appropriate staff prior to scheduled necropsies. This may include but is not limited to available data required by the study protocol on:

- Clinical signs, clinical pathology, pulmonary physiology, and any other tasks performed during the exposure or recovery phase of the study prior to the scheduled necropsy;

- Necropsy and histopathology data from unscheduled deaths or moribund sacrifices during the exposure or recovery phase of the study prior to the scheduled necropsy;

- Records indicating sufficient training in necropsy procedures by prosectors, technicians, and other staff involved in the pathology/clinical pathology tasks.

Clinical Pathology

5. Guidelines for clinical pathology tasks are provided in paragraphs 36 and 37 of TG 412 and paragraphs 37 and 38 of TG 413. Following is supplemental information for performing clinical pathology tasks:

6. Clinical pathology is a key tool in detection, quantitation, and interpretation of effects of exposure to toxicants in rodent toxicology studies. An extensive database of clinical pathology data on laboratory rodents generated from toxicology studies by pharmaceutical and chemical industries, regulatory agencies, contract research organizations, and academic institutions is available in the open literature (Loeb and Quimby, 1999; Moore, 2000; Car et al, 2006). These and similar literature sources may be utilized to optimize the value of clinical pathology data for specific study protocols and for other criteria such as gender, stock/strain of the animals and diet status (restricted or ad libitum).

7. Prior to collections of samples, it is recommended, and would be in line with OECD GLP Principles (OECD, 1998), to confirm that collection methods, fluids, containers, diluents, or vehicles to be used are correct and consistent for the clinical pathology tasks required in the protocol. Standard procedures may be utilized for randomization of samples and periodic insertion of quality control sera as described in the literature (Loeb and Quimby, 1999; Car et al, 2006) and in study-specific protocols. If blood samples are to be collected without anesthesia, the saphenous or submandibular veins are recommended as the collection site. Anesthesia is recommended if blood samples are taken from the retro-orbital sinus or by cardiac puncture.

8. As mentioned in Table 1 in TG 412 and 413, overnight fasting prior to blood sampling may be considered to insure uniformity of clinical chemistry and hematology data. The optimal time from last exposure to removal of food (fasting), blood and/or urine collection, euthanasia, bronchoalveolar lavage (BAL), and necropsy may vary with the physical or chemical characteristics of the test article and available information on the effect of the exposure on study animals. In typical inhalation studies animals are fasted for 16 hours (overnight) following the last exposure, although fasting prior to necropsy is not done universally. If data available from clinical signs, clinical pathology, or other information obtained during the study or from the literature indicate fasting may decrease rather than increase the value of clinical pathology or other data, fasting parameters may be changed or fasting deleted. Not fasting prior to necropsy may impact body and organ weight ratios, histology of liver (glycogen in hepatocytes), blood glucose, and other clinical pathology values when compared with data from the literature.

9. Methods of statistical analysis of clinical pathology data are provided in paragraph 98 of the Guidance Document on Acute Inhalation Toxicity Testing (GD 39). In assessing the results of clinical pathology data, comparisons may be made to concurrent cage, vehicle, excipient, or other control groups, and to a range (95% interval) of normal values based on samples from a large number (~50) of animals of similar age, stock/strain, and receiving similar feeding and husbandry practices. Interpretation of statistically significant differences among groups may be tempered by the realization that many clinical pathology parameters have wide ranges and are affected by a number of variables. Similarly, a lack of statistical

significance does not imply a lack of effect of exposure, and interpretation of the results may require careful evaluation and correlation of clinical pathology parameters with all other available toxicology data.

10. Urinalysis is an optional task in TG 412 or TG 413, based on observed or expected toxicity of the test material. Collection of urine samples free of external contamination and of volume adequate for testing may require special collection containers and caging and an overnight sampling period. Analysis of urine samples for cellular contents (red blood cells, inflammatory cells, casts, uroliths) is recommended to add to the list of items in Table 1 in TG 412 and TG 413. The use of metabonomics tools on urine is not part of TG 412 and TG 413, but it may be considered. Metabonomics has proven useful to detect outliers prior to initiation of the study (Car et al, 2006), and to detect nephrotoxicity earlier than standard histopathologic methods (Boudonck et al, 2009)

11. If collectable samples are available, clinical pathology measurements are recommended in moribund animals prior to unscheduled necropsies, in order to determine cause of moribund condition and possible relationship to exposure to the test article.

12. Hematology data collected using automated instrumentation may be routinely confirmed by manual counting and enumeration of cell types (Moore, 2000). Histologic examination of bone marrow, spleen, and liver is recommended to evaluate hematopoietic elements and detect fibrosis or necrosis. Flow cytometry may be used to differentiate hematopoietic lineages (Criswell et al., 1998), and may also be utilized to quantify micronuclei (Torous et al, 2003).

13. Evaluation of clinical pathology data in inhalation studies may need to consider the impact of stress related to exposure to the test material and to handling and change of environment during cage transfer and specimen collection. The most consistently affected parameters related to stress in rats are increased lymphocytes, glucose, adrenalin, and adrenocorticotrophic hormone (ACTH) and decreased eosinophils (Car et al, 2006). Published methods are recommended to acclimate animals to specialized housing and equipment (Damon et al., 1986).

Supplemental/Optional Tasks Prior to Necropsy

Ophthalmoscopic Examination

14. Ophthalmological examination is described in paragraph 40 of TG 413, but not in TG 412. Several additional details and tasks are recommended with regard to ophthalmoscopic findings.

15. Ophthalmoscopic examination should be conducted by a suitably trained and experienced ophthalmologist, preferably using indirect fundoscopic examination and slit-lamp evaluation. When ocular abnormalities are detected at ophthalmoscopy, collection of eyes at necropsy and histopathologic examination of the eyes is recommended to attempt to identify the morphologic correlate of the abnormality. If ocular abnormalities are focal, it is recommended to perform histologic examination of additional sections. Appropriate sections from all groups are recommended for adequate comparison with untreated controls and to establish a possible dose response. The study report should contain an integrated interpretation of all ocular findings (ophthalmoscopic, macroscopic, and microscopic examinations) along with pertinent individual animal data.

Respiratory Physiology

16. Pulmonary function measurements are recommended in paragraph 89 of GD 39 for acute inhalation studies, and may be considered for subacute (TG 412) and subchronic (TG 413) inhalation studies. Respiratory rate and tidal volume measurements provide useful information regarding inhaled dose and

may indicate the primary respiratory tract site of effect of the inhaled material (Boggs, 1992; Costa et al, 1992; Gad and Chengelis, 1998; Gad, 2006).

Bronchoalveolar Lavage

17. Methods and rationale for performance of bronchoalveolar lavage, provided in paragraph 90-94 of GD 39, are referenced in TG 412 and TG 413. Following is supplemental information for consideration in performing bronchoalveolar lavage.

18. General methods for BAL in rodents are available in the literature (Henderson 1984, 1988). Details of methods used for BAL will depend on animal species and parameters to be measured. Some potentially useful indicators of damage available in bronchoalveolar fluids are presented in Table 1 (modified from Henderson, 1984). Quantitation of types and numbers of granulocytes and macrophages in BAL fluid will provide an indication of severity and duration of inflammation (Henderson, 1984). Good technique is important to avoid leakage of blood into lavage fluid, which will confound assays for total protein, spectroscopic endpoints, or cell populations in the lavage fluid. Cell recovery from BAL fluid may be improved by performing and pooling several (2-3) lavages on the same lung prior to centrifugation. Wet/dry lung weight ratios may be obtained using an alternative method of tying off the right mainstem bronchus at the tracheal bifurcation, lavaging the left lobe, removing the lobus cranialis and using it for wet/dry weight ratio, and the remaining right lung lobes for histopathology. This alternative method obviously decreases the total amount of lung available for histopathology. Correlation of quantitative BAL data with qualitative histopathology data provides a compelling scientific argument for effects of inhaled test articles.

Table 1: Indicators of Acute Injury in Bronchoalveolar Fluids (from Henderson, 1984)

Parameter	*Location*	*Possible indication if elevated*
Lactate Dehydrogenase	Cytosol (glycolysis)	Cell damage (increased membrane permeability to frank cell lysis)
Glucose-6-phosphate-dehydrogenase	Cytosol (hexose monophosphate shunt)	Cell damage; leakage from cells undergoing repair
Lysosomal acid hydrolases	Lysosomes	Release during phagocytosis; granulocyte and/or macrophage damage
Alkaline phosphatase	Plasma membranes, Type II cell lamellar bodies, serum	Type II cell damage or increased secretions; transudation of serum proteins
Glutathione peroxidase Glutathione reductase	Cytosol	Protection mechanism activated against lipid peroxidation
Angiotensin converting enzyme	Endothelial cells	Endothelial cell damage
Total Protein	Extracellular	Transudation of proteins across alveolar-capillary barrier
Sialic acid	Mucus Glycoproteins	Increased mucus secretion Transudation of serum glycoproteins
Lymphocytes, mast cells, granulocytes Macrophages		Inflammation

Cytokinetic Studies

19. Changes in cell turnover rate are a useful tool which may be utilized to detect and quantify short or long term changes in tissues in response to toxicants, and may yield insight into their mechanism of action (Evans et al, 1991). Cell proliferation (Melnick et al, 1993) and cell death (Zhang et al, 1995; Gomez-Angelats et al, 2002; Kai et al, 2004; Elmore, 2007) have long been recognized as key factors in carcinogenesis, but are also useful tools to detect and quantify cellular changes in short term toxicology studies. Cell kinetics studies are based on the use of compounds that label cellular DNA and allow the microscopic detection and quantitation of cells undergoing DNA synthesis at the time of tissue fixation. Immunohistochemical detection using a monoclonal antibody to bromodeoxyuridine (BrdU) is the most widely accepted tool for this assay. Cells in the DNA synthesis (S) phase can be labeled over short (single pulse via intraperitoneal or intravascular injection) or long (subcutaneously implanted osmotic pump or oral dosing in water) periods of time (Goldsworthy et al, 1993). Endogenous markers of cell cycle status such as proliferating cell nuclear antigen (Eldridge et al, 1993) and Ki67 (Gerdes et al, 1983; Ignatadis and Sotiriou, 2008; Challen et al, 2009) may also be measured using immunohistochemical techniques. Cell proliferation studies have provided much useful information in studying toxicity of nasal (Monticello et al, 1993) and pulmonary (Haschek and Witschi, 1991) epithelium. However, analysis of these data requires caution and sample size is an important factor (Morris, 1993).

20. Programmed cell death (apoptosis) is a normal biological process which may be altered by exposure to toxicants or other pathologic events (Elmore, 2007). Apoptosis occurs via several pathways, and may be detected and quantified using light or electron microscopy, DNA fragmentation, detection of caspases using immunochemistry or DNA polymerase chain reaction (PCR) microarray technology (Elmore, 2007).

The Terminal dUTP Nick-End-Labeling (TUNEL) assay is a DNA fragmentation method frequently utilized to quantify apoptosis in rodent toxicology studies.

Necropsy

21. Methods for necropsy and weighing of tissues are provided in paragraphs 39-41 of TG 412 and paragraphs 41-43 of TG 413. The following paragraphs recommend additional details and procedures related to the necropsy task.

22. An exception to the stated need in TG 412 and 413 for a complete necropsy may be animals found dead or sacrificed due to moribund condition during the first few days of a study in which the study protocol specifically requires replacement of animals accidentally killed or injured early in the study by replacement animals. In this case the necropsy of the animal to be replaced may be a less complete necropsy to determine cause of death or moribundity but not intended to generate tissues for histopathology related to the goals of the study.

23. The importance of identifying and necropsying moribund or dead animals as soon as possible after death cannot be overemphasized. Loss or low quality of histopathology data resulting from autolysis of tissues can seriously hamper interpretation of the results and compromise the value of the study. Although TG 412 and 413 state that necropsies on early death or moribund animals should be performed within a day or two, it is strongly recommended to store the animals in a refrigerator at 4-8 degrees centigrade and proceed to necropsy no later than 24 hours following removal from the study.

Methods of euthanasia

24. Animals should be euthanized using a method appropriate for compliance with current local animal welfare licensing regulations. Methods of euthanasia for laboratory rats are described in a recent publication (Everitt and Gross, 2006). American Veterinary Medical Association (AVMA) Guidelines (AVMA, 2007) and European Commission Guidelines (European Commission, 1995) provide a general description of euthanasia methods and issues on a wide variety of animal species. Inhalation studies may require increased attention paid to the effect of the anesthetic and method of administration on the respiratory tract and associated tissues. The most frequently used methods of euthanasia of laboratory rodents are inhalation of halogenated ether anesthetics, inhalation of carbon dioxide/oxygen combinations in a closed chamber, and intravenous or intraperitoneal injection of a barbiturate anesthetic. Exsanguination of the animal via incision of the caudal vena cava or axillary vessels following complete cessation of heartbeat is recommended to improve the quality of fixed tissues for examination by removal of a portion of the circulating blood. Heartbeat should be completely stopped prior to start of the necropsy.

25. The choice of individual anesthetic/chemical agents for euthanasia may depend in part on the material being tested and the clinical pathology parameters being measured. Barbiturate anesthetics may have an effect on cytochrome P450 levels with particularly important effects in liver (Popp and Cattley, 1991) or nasal epithelium (Dahl and Hadley, 1991). Halogenated ether anesthetics are commonly used in rodent toxicology studies including inhalation studies, but have the potential for effects on the respiratory tract since they are administered via inhalation, and care must be taken to avoid exposure to humans.

26. A variety of halogenated ether anesthetics have been recommended for euthanasia of rodents including: halothane, enflurane, isoflurane, sevoflurane, methoxyflurane, and desflurane (AVMA, 2007). Carbon dioxide/oxygen combinations are useful for short term anesthesia to collect blood samples, and are used in combination with exsanguination for euthanasia prior to necropsy. However, failure to maintain a ratio of 70% carbon dioxide/30% oxygen may induce multiple small hemorrhages in lung parenchyma (Renne et al, 2007). Carbon dioxide/oxygen ratios higher than 70%/30% should be avoided. This Guidance

Document recommends the use of intravenous injection of a lethal dose of a barbiturate anesthetic followed by exsanguination following cessation of heartbeat.

Necropsy Technique

27. A document providing a description of the background, preparation and planning, equipment, and procedures for a general rodent necropsy as part of a toxicology study is available in the literature (Everitt and Gross, 2006), and is recommended to be used as the basic text in preparing for necropsy of rodents in toxicology studies.

28. Well trained, conscientious prosectors thoroughly familiar with the anatomy and dissection procedures of the animals to be necropsied and the specific study protocol would be required to comply with GLP principles, and are recommended as an essential tool for success in the necropsy task (Bucci, 1991). Training of these staff members should emphasize attention to detail and consistency combined with efficiency and appreciation of the importance of this critical task in which mistakes are almost never correctable. Training should be in the hands of an experienced supervisor and/or pathologist having sufficient bench/lab experience to appreciate the disastrous results of mistakes made in necropsy. The supervising pathologist and other staff supervising the necropsy should be active, informed participants in the generation and approval of the study protocol, and should be responsible to provide all protocol-specific necropsy, trimming, and histology information to the other staff involved in the necropsy.

29. Table 2 in TG 412 and TG 413 lists the organs and tissues to be weighed and/or preserved in a suitable fixative (see below) during necropsy for histopathological examination. Fewer tissues for fixation are listed in TG 412. The preservation of other organs and tissues depends on the test article being studied and is at the discretion of the study director. This Guidance Document recommends as an option the fixation of all the tissues listed for preservation in TG 413 to be included in the tissue list for TG 412, and also recommends the fixation of the following tissues in addition to those listed in TG 413 in both TG 412 and 413:

- Superficial and deep cervical lymph nodes (LNs)

- Popliteal and/or Lumbar LNs

- Mesenteric LNs

It is also recommended to weigh organs from animals at unscheduled necropsies when feasible.

30. This Guidance Document recommends weighing the lungs of rats after removal of the lung associated lymph nodes (LALN) (cervical, mediastinal, and tracheobronchial lymph nodes) and heart, and before inflation with fixative. The tracheobronchial LN of mice may be left on the lungs during the process of removal and weighing of the lungs. Other organs to be weighed may be specified in the individual study protocol. Tissues and organs should be fixed in 10% buffered formalin or another suitable fixative (see below) as soon as necropsy is performed, and fixation should continue for no less than 48 hours prior to trimming depending on the fixative to be used. Special attention should be paid to insure that tissues to be weighed are kept moist, and that time from removal from the carcass for weighing to placement in fixative is minimized to avoid autolysis.

31. Examination and recording of gross observations and dissection and placement in fixative of all protocol-required tissues is a critical part of every necropsy in toxicology studies. Standard protocols may require the prosectors to ensure by their signatures on the necropsy form that all protocol-required tissues were examined and placed in fixative. In cases of questionable gross findings the responsible pathologist should confirm and/or improve upon the description of findings by the prosector. If the pathologist is not

available, the lesion should be photographed and fixed *in situ*, and this information provided to the pathologist.

32. During scheduled necropsies, a responsible pathologist should be available or on call to supervise the prosectors. Unscheduled death or moribund animal necropsies may not be under the direct supervision of a pathologist. High quality animal husbandry and observation of animals by experienced animal care staff utilizing information available in GD 19 will minimize the number of unscheduled necropsies.

Figure 1

33. One item useful in assuring that all tissues were collected and examined is a tray divided into numerous compartments (Figure 1). The prosector can place tissues small enough to be contained in individual compartments in preset compartments within this tray, which contains fixative solution that allows the tissues to begin fixation within the tray. When the prosector has completed the necropsy he/she can call the pathologist to examine the tissues within the tray to confirm that they have been collected. Photographs of pertinent findings at necropsy may be taken if required by the study protocol or if necessary for the documentation of unusual findings. Photographs, in particular digital images, may not be considered as raw data from a GLP standpoint.

34. The recommended necropsy procedure document (Everitt and Gross, 2006) lists dissection of the thoracic viscera following dissection of the head and abdominal viscera. For inhalation studies the most crucial target organs are the upper and lower respiratory tract; therefore this Guidance Document recommends dissection of the thoracic viscera immediately following dissection of the head.

35. Special attention during dissection of the head and thoracic viscera should be paid to dissection, fixation, and identification of the mandibular LNs and LALN, since these are the terminal collection sites of lymphatic drainage for the respiratory tract (Tilney, 1971; Hebel and Stromberg, 1986). The mandibular LNs are usually easily visible craniolateral to the mandibular salivary glands. The cervical LNs are less obvious; the superficial cervical LN (lymphocenter cervicale superficiale, Hebel and Stromberg) is attached to the ventral muscles of the neck; the deep cervical LN (lymphocenter cervicale profundum, Hebel and Stromberg) is just lateral to the trachea. The mediastinal LNs are attached to the thymus and the precordial mediastinum. If the study protocol requires thymus weights, the mediastinal LNs should be removed from the thymus prior to weighing the thymus. The tracheobronchial LNs (lymphocenter bronchiale, Hebel and Sromberg) are located just cranial to the bifurcation of the trachea into mainstem

bronchi and between the mainstem bronchi. All the LNs described above can be placed in individually labeled cassettes to insure they are available for microscopic examination.

36. After the thoracic viscera are removed from the carcass as a single unit, the cranial surface of the larynx may be examined visually for the presence of foam, blood, or other material. The trachea may be transected midway between the larynx and the tracheal bifurcation, for example 5 rings below the larynx or 10 rings above the bifurcation, and its lumen examined for foam or other fluids, and the larynx and attached tongue placed in fixative. The nasal cavity should be fixed by retroflushing fixative through the nasopharyngeal opening and out the external nares, and then placed in fixative.

37. TG 412 and TG 413 require weighing of lungs, heart, thymus, and other selected tissues at necropsy. Thus, the heart should be dissected free from the other thoracic viscera prior to weighing. Special care should be taken when removing the heart from the thoracic viscera to avoid cutting through the wall of the trachea or mainstem bronchi. Perforation of the tracheal or bronchial wall will make it impossible to fully inflate the lungs with fixative or maintain the inflated state. Due to problems such as this and other inconsistencies in trimming tissues for weighing, only thoroughly trained and experienced necropsy staff is recommended to trim tissues for weighing (Bucci, 1991; Everitt and Gross, 2006). According to this recommendation, prosectors dissect out tissues to be weighed but do not finely dissect surrounding tissues. Tissues to be weighed are transferred to person(s) trimming the tissues to be weighed, weighing tissues and recording weights using a computerized balance system. This arrangement has decreased the problem of leakage due to accidentally punctured airways and concurrently improved consistency of organ weight data, as well as decreased the time between removal of lungs from the carcass and instillation with fixative.

38. As indicated in TG 412 and 413, it is recommended that lungs to be processed for histopathology be inflated with fixative at a pressure of 20-30 cm of water. This Guidance Document recommends inflation using a gravity flow apparatus (see for example Figure 2) (Renne et al, 2001; Everitt and Gross, 2006). This apparatus consists of a clear glass bottle containing fixative solution and sealed at the top with a rubber stopper containing a pipette. The tip of the pipette is set at a precise vertical distance (20-30 cm; see below) above the level of the lungs to be inflated with fixative. Flexible tubing connected to a spigot on the side of the bottle carries fixative to the tracheal opening. Proper operation of this apparatus requires an airtight seal of the stopper and a patent pipette lumen. This apparatus should be tested for accuracy and consistency of pressure prior to and at intervals during its use. The optimal pressure for instillation varies with the species and size of the animal and could also vary with the effects of exposure to the test material. The degree of inflation should be closely observed, starting with a pressure of 20 cm and not exceeding 30 cm of water, and continuing inflation until the lungs fill but do not protrude from the thoracic cavity, i.e., until complete expansion but not overexpansion of all lobes. Regardless of the set target pressure for inflation, close attention should be paid to consistent inflation of each set of lungs at the same pressure to assure that all lobes are inflated to full expansion. Macroscopic assessment of lung surface should be carried out prior to the instillation of the fixative as minor pulmonary lesions can disappear during fixation.

39. Once inflation to normal *in vivo* expansion is complete, the transected trachea may be ligated near its cut end. If the study protocol requires morphometry on lung sections for detection and quantitation of changes in airway or alveolar size, lungs should be held under constant inflation pressure for a period of at least two hours to allow fixation to occur in the presence of potential leaks caused by minute holes in the pleural surface. This can be accomplished using a series of manifolds holding groups of lungs under the correct pressure (Figure 2).

Figure 2

40. The standard fixative for light microscopy is 10% neutral buffered formalin (NBF). Davidson's fixative is optimal for fixation of rodent eyes and testes; glutaraldehyde and Karnovsky's fixatives are utilized for tissues to be examined with electron microscopy. Various fixatives are discussed in the referenced document (Everitt and Gross, 2006). A few drops of eosin dye added to the fixative solution turns it a faint pink color and identifies it as the fixative, avoiding the potentially disastrous mistake of accidentally placing tissues for fixation into saline solution, plain water, or other non-fixative solutions. An additional method for preserving lung tissues intended to be utilized for immunohistochemical analysis with immunofluorescence is slow intratracheal inflation to full expansion with Optimum Cutting Temperature (OCT) medium followed by ligation of the trachea, freezing and holding in liquid nitrogen at -70C.

Supplementary/Alternative Methods for Necropsy

Fixation of tissues by vascular perfusion

41. Vascular perfusion of fixative provides superior tissue fixation and may be used when electron microscopic (EM) examination is a primary goal. Whole body perfusion of glutaraldehyde or other EM fixative via the left ventricle or abdominal aorta is the standard technique. A flushing solution containing heparin is used to remove blood from the circulation prior to infusion with fixative (Nyska et al, 2004). The time and technical expertise required for vascular perfusion make it impractical for most routine toxicology studies.

Fixation of lung by immersion in fixative

42. An alternative method of lung fixation is immersing the lungs in fixative and covering the lungs with gauze to assure immersion in fixative. This method avoids movement or removal of exudates and inhaled test materials deposited in the respiratory airways and alveoli associated with intratracheal instillation of fixative, and may increase the ability to detect and quantify edema compared to the intratracheal instillation method. However, the majority opinion of a group of experienced pathologists (authors of the article cited in this paragraph) was that the intratracheal instillation method, by inflating the lungs to expand alveoli, provides a closer approximation of the lung *in vivo* than the immersion only method, and the immersion method makes evaluation of cellular inflammatory responses or interstitial responses much more difficult. The consensus of this group was that the advantages of intratracheal instillation of fixative outweigh the increased ability to detect subtle edema using the immersion method (Renne et al, 2001). This Guidance Document recommends intratracheal instillation of fixative for routine inhalation studies. The immersion only method may be considered to use in evaluating lungs in which pulmonary edema is the principal issue.

Tissue Trimming and Slide Preparation

43. The task of trimming tissues obtained at necropsy has the same requirements for close attention to detail, consistency, efficiency, and powers of observation that were described for the necropsy task (Bucci, 1991). Errors made during trimming are also seldom repairable and can also have an impact on the quality of the pathology data. Training of staff for tissue trimming should be done by supervisory personnel experienced in the procedure and very familiar with the protocols. Staff trimming tissues should pay close attention to the necropsy findings as well as the study protocol, and close communication among the necropsy and trimming technical staff, histology supervisor, and pathologist is very helpful in obtaining tissue sections that accurately reflect the necropsy findings and provide the pathologist tissue sections needed to interpret the results.

44. Publications providing specific techniques for tissue trimming are available in the literature (Young, 1981; Sminia et al, 1990; Uraih and Maronpot, 1990; Morgan,1991; Lewis, 1991; Renne et al, 1992; Sagartz et al, 1992; Bahnemann et al, 1995; Harkema and Morgan, 1996; Plopper, 1996; Germann et al, 1998; Hardisty et al, 1999; LeBlanc, 2000; Ruehl-Fehlert et al, 2003; Kittel et al, 2004; Morawietz et al, 2004; Kaufmann et al, 2009) and on the internet (http://reni.item.fraunhofer.de/reni/trimming/index.php) . Specific guides from the internet website listed above for trimming tissue sections from the nasal cavity, larynx, trachea, and lungs are presented below in Figures 3, 4, 5, and 6. The information provided in this internet site is the product of a large effort by a number of toxicologic pathologists and histologists with extensive experience in performing toxicology studies for industry and regulatory agencies. It provides a detailed introduction and descriptions and graphics of specific techniques for trimming rodent tissues, including the respiratory tract. The internet site document cited above should be used as a basic text for trimming rodent respiratory tract tissues, although there are other methods utilized, some of which are described below. The eight paragraphs below also provide amplifications, additions, and exceptions to the methods presented in the internet document.

45. The internet site trimming guide recommends four transverse nasal sections for rats and three for mice and provides text and graphics for obtaining sections at those sites. The number and location of nasal sections should be adequate to ensure adequate microscopic evaluation of all four types of mucosal epithelium, the nasopharyngeal duct, and nasal associated lymphoid tissue (NALT). The location of the most critical lesions may depend on the physical and chemical characteristics of the test article and previous knowledge of its effect on the respiratory tract. Highly water soluble or caustic test compounds may have a profound effect on the squamous epithelium of the nasal vestibule, requiring additional nasal sections through the nasal vestibule (Gross et al, 1994; Harkema et al, 2006). Test articles suspected or

known to have an effect on olfaction, olfactory epithelium or the central nervous system may require additional sections through the caudal portions of the nasal cavity containing olfactory epithelium and the olfactory bulb area of the brain. Publications providing detailed methods for preparation of multiple sections through the olfactory area of the nasal cavity and nomenclature for describing lesions in olfactory epithelium are present in the literature (Morgan, 1991; Brenneman et al, 2002; Mery et al, 1994).

46. Poor choices in methods and materials for decalcification of rodent nasal tissues may result in less than ideal quality of stained slides due to overexposure to relatively strong acid chemicals. Use of formic acid and a commercially available ion exchange resin to decalcify the skull prior to trimming nasal cavity sections may provide optimal cellular detail as well as faster decalcification (Sheehan and Hrapchak, 1980).

47. Consistency in trimming and embedding nasal and laryngeal tissues is critical for interpretation of mucosal changes related to exposure. The zones of transition from squamous to transitional to respiratory to olfactory epithelium are relatively narrow in rodents and errors in trimming or embedding can easily result in the pathologist being unable to examine these critical areas. Staff undertaking the trimming and embedding of these important tissues should have the training and experience necessary to ensure that the tissues are available to the pathologist, and communication between the pathologist and histology laboratory staff is an important factor in a successful inhalation study.

48. The goRENI internet site recommends three transverse laryngeal sections in rats and two in mice and provides graphics for obtaining these sections in both species. The internet site text indicates the most sensitive laryngeal site for an effect of inhaled toxicants is the ventral pouch and the medial surface of the arytenoid cartilages. However, according to several papers, the most sensitive site in rats or mice to inhaled toxicants is the transitional epithelium at the base of the epiglottis (Renne et al, 1992, 2007; Renne and Gideon, 2006; Kaufmann et al, 2009). It is somewhat challenging to consistently provide a precise section through the base of the epiglottis and it is possible that only one section of larynx (including the ventral pouch) will be available in some inhalation studies.

49. Most published reports utilize the multiple transverse sections method for larynx described in the internet site publication for routine inhalation studies. An alternative method utilizing longitudinal sections of larynx has been described (Germann et al, 1998). These authors of this description found the longitudinal sectioning method advantageous for detection of laryngeal or tracheal cartilage degeneration and granulomatous inflammation in the oropharyngeal cavity of Fischer 344 rats in a chronic gavage study. The longitudinal method provides a different perspective of the ventral pouch area at the expense of a full transverse view of the base of the epiglottis and the medial surface of the arytenoid processes.

50. The internet site publication provides a description and graphics for preparing longitudinal sections of tracheal bifurcation. In most studies in which the protocol also requires sections of thyroid parathyroid, a transverse section of trachea at the level of the thyroid gland will also be available. The internet site publication also describes the requirement for careful microtoming until the required section containing the carina is available for embedding. However, this procedure may result in loss of the tissue containing one or more of the tracheobronchial lymph nodes present adjacent to the tracheal bifurcation. For this reason, it is recommended (see in Necropsy section above) to remove all tracheobronchial lymph nodes and place them in labeled cassettes for histologic processing either at necropsy or trimming, prior to trimming and embedding the trachea.

51. The internet site publication provides a description and graphics for preparing multiple sections from all lobes of the lungs of rats and mice, resulting in five separate sections. An optional method for mice is also described in which all lobes are detached from the trachea and embedded together in one cassette. This method has been used successfully and is recommended for consideration as an optional

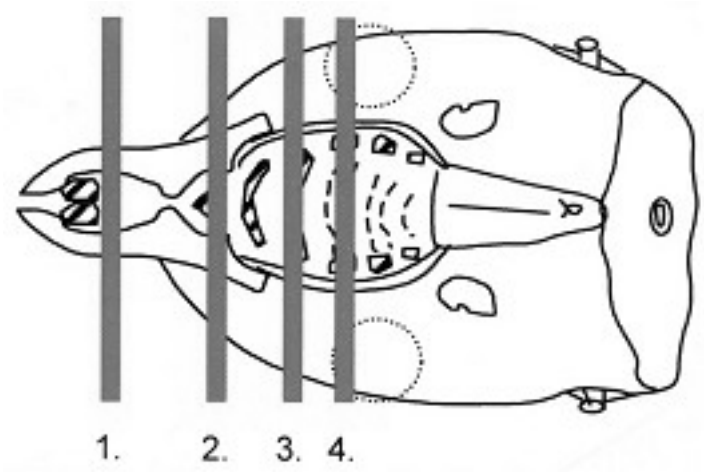
method, depending on the size of the lungs and the presumed degree of importance of the peripheral lung tissues.

52. An alternative method utilized successfully in preparing lung sections from smaller (Fischer 344) rats is separating the right and left lungs at the tracheal bifurcation and embedding each lung dorsal surface down, in two separate cassettes. The microtomer cuts through each coronal longitudinal section until the mainstem bronchus is clearly visible in the section, then takes a section for microscopy.

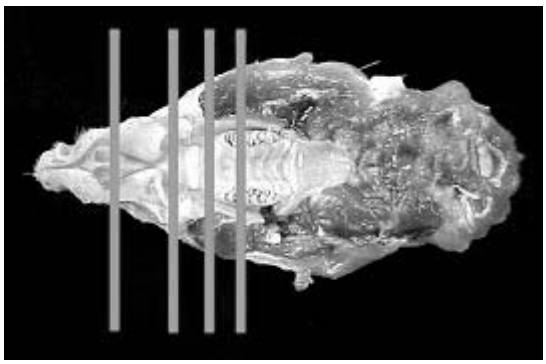
Figure 3: Trimming Guide, Nasal Cavity (from (http://reni.item.fraunhofer.de/reni/trimming/index.php)

NASAL CAVITY, NASOPHARYNX and PARANASAL SINUS

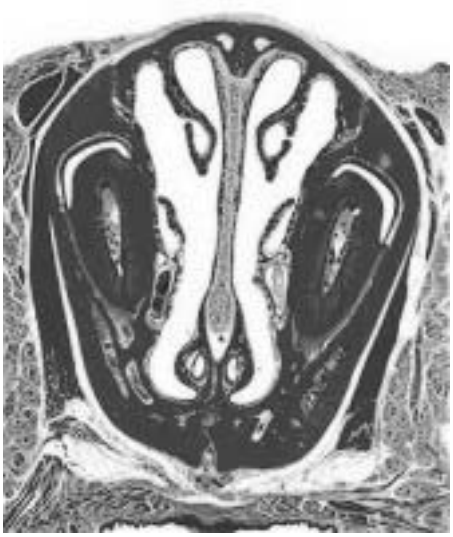
Species:	Rats and Mice
Organs:	Nasal cavity Nasopharynx Paranasal sinus
Localizations:	1) Posterior part of upper incisors 2) Incisive papilla 3) Second palatine crest 4) First molar teeth
Number of sections:	1 (oral toxicity study: third level) 4 (inhalation study)
Direction:	Transverse
Remarks:	Embedded with the rostral faces down Decalcified



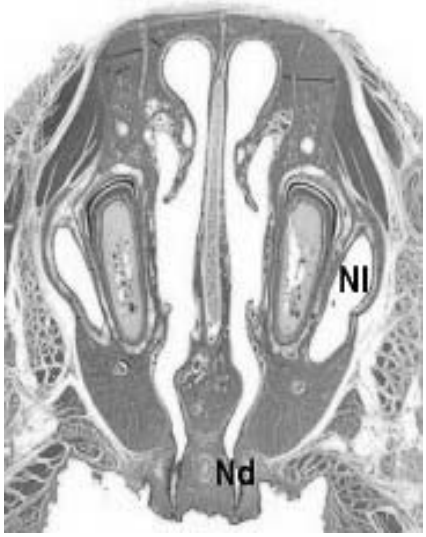
Nasal cavity, rat, 4 trimming locations.



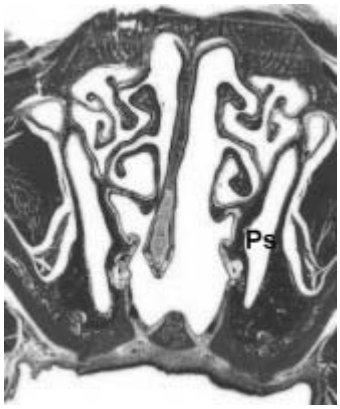
Nasal cavity, rat, 4 trimming locations.



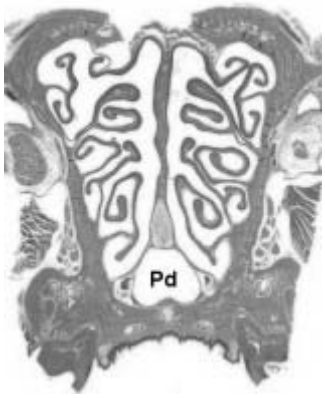
Nasal cavity, rat, location 1.



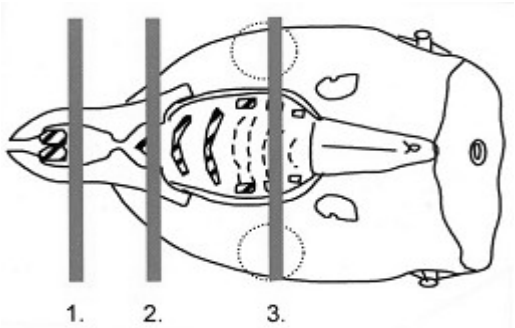
Nasal cavity, rat, location 2. Nd: nasopalatine (incisive) duct, Nl: nasolacrimal duct.



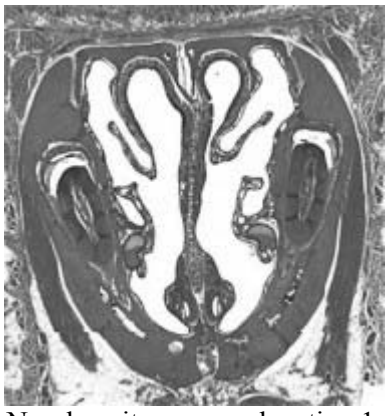
Nasal cavity, rat, location 3. Ps: paranasal sinus.



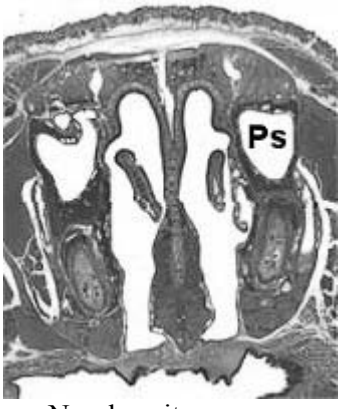
Nasal cavity, rat, location 4. Pd: pharyngeal duct (nasopharynx).



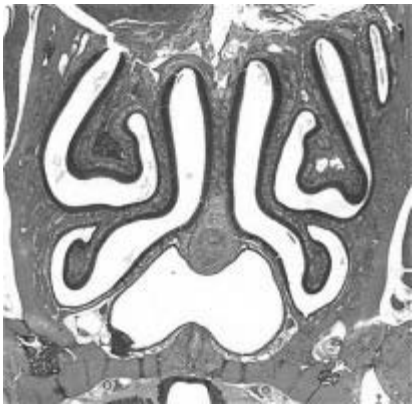
Nasal cavity, mouse, 3 trimming locations.



Nasal cavity, mouse, location 1.



Nasal cavity, mouse, location 2. Ps: paranasal sinus.



Nasal cavity, mouse, location 3.

The structures of the palate and the teeth are used for orientation to achieve transverse sections through the nasal cavity at certain levels.

In inhalation studies, four transverse tissue levels should be taken, because the examination of these sections at defined levels assures consistent recognition of degenerative and proliferative lesions of all different epithelial cell types of the nasal cavity and paranasal sinus. Neoplastic lesions occur more frequently in the anterior and middle portions of the nasal cavity, whereas some non-neoplastic and neoplastic lesions are observed exclusively in the olfactory epithelium. The third level includes respiratory and olfactory epithelial cells. The resulting slices of tissue are embedded with the rostral face down, because non-neoplastic lesions have been found to be most severe at the more rostral borders of the affected epithelium. Slight differences of the cut level may occur depending on anatomical variations in different strains. If more squamous epithelium is required for examination, a section rostral to level 1 should be performed. For examination of the olfactory bulb, a section caudal to level 4 is recommended.

Relevant differences between rats and mice

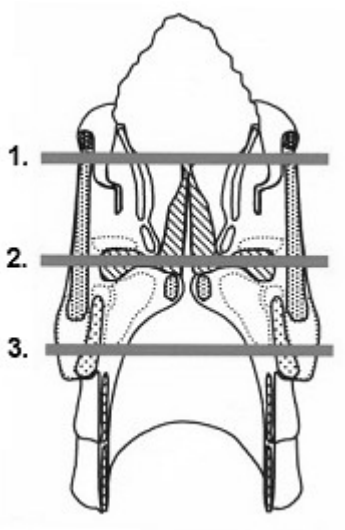
Mice should be trimmed in the same manner as rats. However, in inhalation studies with very young or very small mice (e.g., transgenic strains), it can be difficult to cut the nose in four levels. For those exceptions, the following three level-procedure is recommended:

1) Immediately posterior to the incisors,
2) At the level of the incisive papilla,
3) Through the middle of the second molar tooth.

Figure 4: Trimming Guide, Larynx (from (http://reni.item.fraunhofer.de/reni/trimming/index.php)

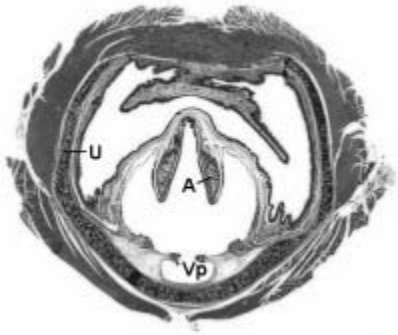
LARYNX

Species:	Rats and Mice
Organ:	Larynx
Localizations:	1) Base of epiglottis 2) Ventral pouch 3) Cricoid cartilage (rats only)
Number of sections:	Inhalation studies: rats 3, mouse 2 *Optional for rats and mice:* if necessary, the larynx can also be embedded in one block and step sections are taken at the predilection sites.
Direction:	Transverse
Remarks:	Since the larynx of mice is very small, only two pieces (level 1 and 2) are trimmed.

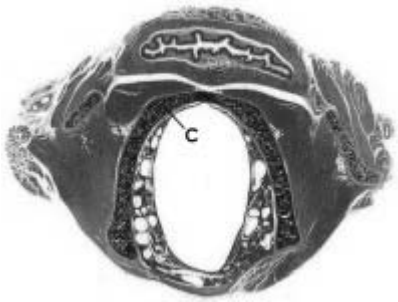


Larynx, inhalation studies.
Rats: levels 1-3, mice: levels 1 and 2.

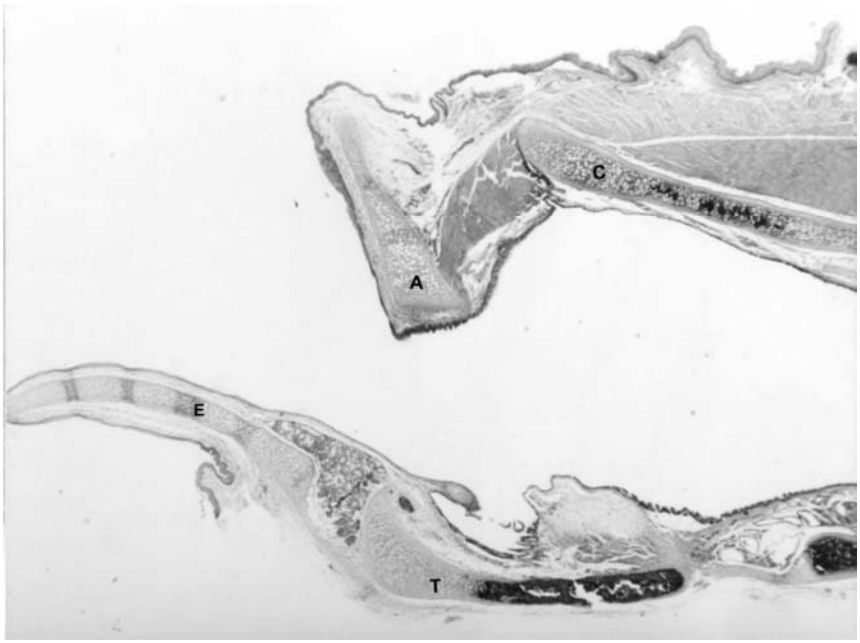
Larynx, level 1. Sg: seromucinous glands at the base of the epiglottis.



Larynx, level 2. Vp: ventral pouch, A: processes of the arytenoid cartilages.



Larynx, level 3. C: cricoid cartilage.



Longitudinal section, rat larynx.

A=arytenoid cartilage

E= epiglottal cartilage

T= thyroid cartilage

C= cricoid cartilage

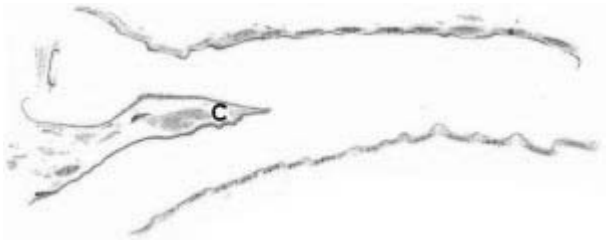
Only the cranial portion of the epiglottis is removed to ensure inclusion of the major predilection site for induced lesions. This site is primarily represented by the epithelial lining of the ventral and ventrolateral luminal surface of the larynx (cranial to the ventral laryngeal pouch). The remaining larynx is trimmed according to the proposed scheme at three levels including base of epiglottis, ventral diverticulum and cricoid cartilage. The three pieces are embedded with the cranial cut surface downwards. The three levels assure recognition of all different epithelial cell types of the larynx and underlying seromucinous glands.

Figure 5: Trimming Guide, Trachea (from (http://reni.item.fraunhofer.de/reni/trimming/index.php)

TRACHEA (inhalation Study)

Species:	Rats and Mice
Organ:	Trachea
Localization:	Including the bifurcation
Number of sections:	1 (2)
Direction:	Longitudinal horizontal *Optional*: transverse
Remarks:	Embedded *in toto*; careful microtome sectioning until recommended cutting level is obtained.

Trachea with bifurcation.



Trachea with bifurcation, C: carina.

3. In inhalation studies, tracheal epithelium including the epithelial lining of the bifurcation should be examined, because this is known as the most sensitive area to respond to inhaled particulate irritants. For this purpose, a longitudinal horizontal section should provide a long distance of the epithelial surface and the tip of the carina. For optimal estimation of e.g. mild hyperplasia, it can be helpful to have an optional transverse section.

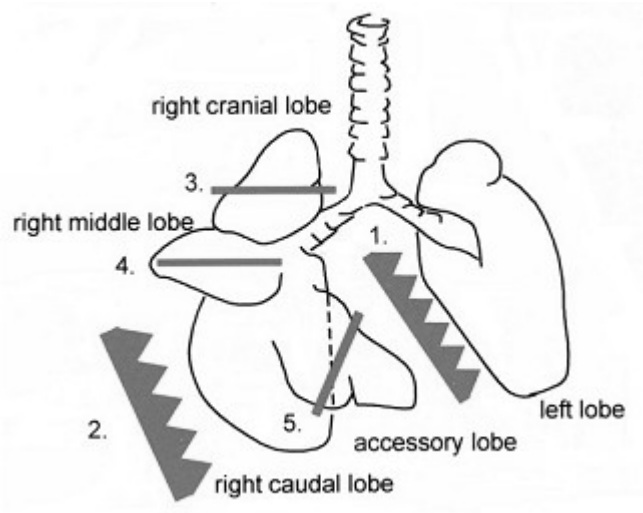
Figure 6: Trimming Guide, Lung (from (http://reni.item.fraunhofer.de/reni/trimming/index.php)

LUNG

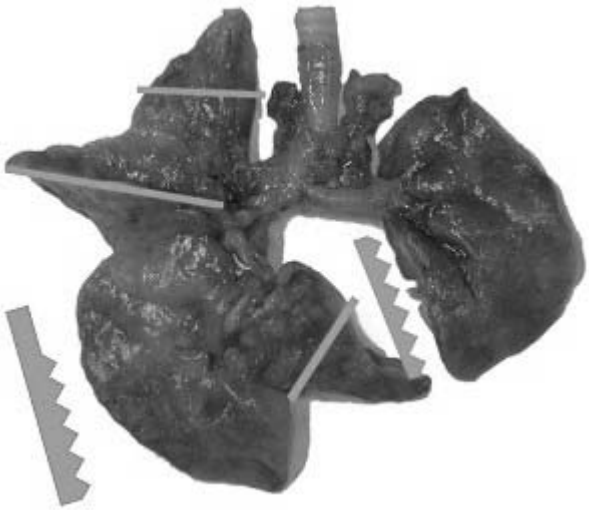
Inhalation study:

Species:	Rats
Localizations:	1) Left lobe 2) Right caudal lobe 3) Right cranial lobe 4) Right middle lobe 5) Accessory lobe
Number of sections:	5
Direction:	Sections 1, 2: longitudinal horizontal Sections 3, 5: transverse Section 4: longitudinal vertical
Remarks:	Instillation obligatory. Longitudinal horizontal section comprising the lobar bronchus and its main

branches.
Sample size(s) adapted to the size of the cassette(s); preferentially, the diaphragmatic margin is trimmed off.
Alternative procedure: right and left lobes (separate blocks) embedded ventral surface down.

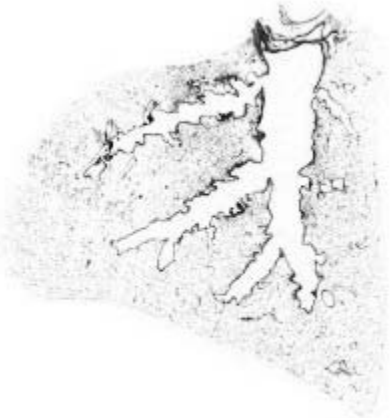


Lung, rat, ventral aspect, inhalation study.



Lung, rat, ventral aspect, inhalation study.

Lung, rat, location 1, left lobe.



Lung, rat, location 2, right caudal lobe.

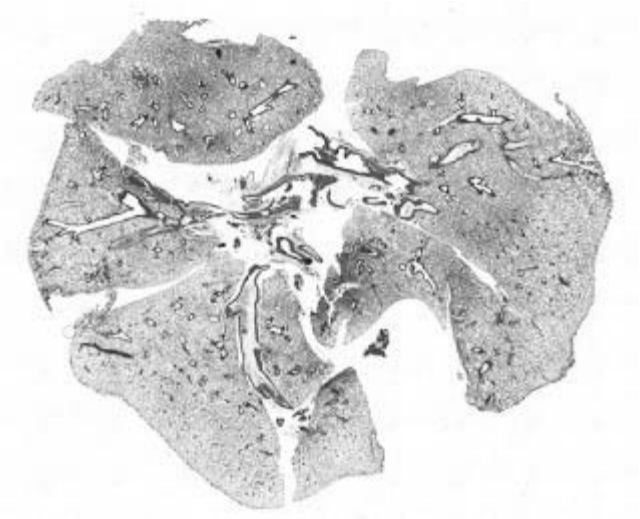
Lung, rat, location 3, right cranial lobe.

Lung, rat, location 4, right middle lobe.

Lung, rat, location 5, accessory lobe.

Inhalation study:

Species:	Mice
Localizations:	1) Left lobe 2) Right caudal lobe 3) Right cranial lobe 4) Right middle lobe 5) Accessory lobe
Number of sections:	5
Direction:	Sections 1, 2, 4, 5: longitudinal horizontal Section 3: transverse
Remarks:	Instillation obligatory. Similar procedure as in rats, but lobes are embedded *in toto*, ventral surface down and detached from the trachea. The five lobes normally fit into one cassette. *Option:* whole lung *in toto* (ventral surface down) without removal of the trachea. Microtome sectioning of left lobe and right caudal lobe until lobar bronchus and its main branches are visible (longitudinal-horizontal axis).



Lung, mouse, *in toto* (option).

Rats and mice:

Spontaneous neoplastic pulmonary lesions are rare in rats and arise mostly in the lung periphery whereas regenerative hyperplasia and squamous metaplasia occur mainly in the centroacinar region. Therefore tissue of the lung including parenchyma, bronchiolo-alveolar junctions and main bronchi should be investigated. In inhalation studies, sections of all five lobes should be examined according to the proposed scheme, which facilitates unambiguous identification of individual lung lobes. For histological identification of proliferative lesions in the lung, careful fixation by intratracheal instillation is recommended, even for oral studies.

Supplementary Tasks for Trimming and Slide Preparation

Airway Microdissection

53. A method for precise sampling of the bronchi and bronchioles of rodents by microdissection has been developed, which provides explants of intact airways to examine subpopulations of bronchial and bronchiolar epithelium (Plopper et al, 1991; Hyde et al, 1991). This method utilizes fixation of the lungs via intratracheal infusion of aldehydes or inflation of fresh lung with low temperature agarose, removing adjacent vessels and connective tissue, and longitudinally opening bronchi and bronchioles and numbering branches of airways as far as possible into the lung parenchyma. This research tool provides the opportunity to assess metabolic capabilities and do morphometry of airway cells from defined sites at the light microscopy or electron microscopy level. It is especially useful for assessments of airway toxicity when the aerodynamic properties of the inhaled material are such that its distribution within the lungs is highly dependent on the three-dimensional architecture of the airway tree.

Histopathology

54. Methods for histopathological examination of tissues are provided in TG 412 and TG 413. The following paragraphs recommend additional details and/or alternative methods related to the histopathology task.

55. Terminology used in histopathologic description, diagnosis, and reporting in rodent inhalation studies should follow standard pathology nomenclature, utilizing the wealth of information available in the published toxicologic pathology literature. The goRENI website (http://www.goreni.org) provides a standard reference for nomenclature and diagnostic criteria in toxicologic pathology, including respiratory tract tissues for rats and mice. This internet site is sponsored by the European, British, Japanese, and US Societies of Toxicology Pathology, and is the product of a large effort by a number of toxicologic pathologists and histologists with extensive experience in performing toxicology studies for industry and regulatory agencies. It provides a detailed introduction and descriptions and graphics of degenerative, inflammatory, and proliferative respiratory tract lesions in rats and mice, including criteria for diagnosis, differential diagnosis, comments on histogenesis and toxicologic significance, and an extensive bibliography. This internet site document is recommended as the basic text for describing, diagnosing, and reporting lesions observed in the respiratory tract and elsewhere, supplemented by journal articles and texts that provide information on subject matter relevant to specific study protocols. The bulk of the text and selected photomicrographs from the goRENI website are also available in published form (Renne et al, 2009). Selected journal articles and texts of special value for diagnostic methods and classification of lesions are listed below under specific areas of the respiratory tract.

Nasal Cavity/Nasopharynx

56. Adequate microscopic examination of respiratory tract tissues from rodent inhalation studies requires close comparison of carefully selected tissue sections from control and exposed animals. As noted above,

areas of normal transition from various epithelial types are relatively small but are important to evaluate, since these are often the site of subtle but meaningful lesions. An example is the nasal cavity of rodents, which is composed of four distinct epithelial subtypes, with well mapped transition zones (Harkema, 1991; Mery et al, 1994; Harkema et al, 2006). Recognition and documentation of metaplasia or hyperplasia of mucosal epithelium in these areas is easily missed without precisely located tissue sections and close comparison of control and exposed nasal sections by the pathologist. A useful stain in addition to the standard hematoxylin and eosin stain (H&E) to assist in the evaluation of changes in goblet cell populations in the nasal cavity and elsewhere is the Alcian blue/periodic acid Schiff stain, which stains the mucus in goblet cells a dark purple.

57. As mentioned above in the Trimming section, it is important to consider the physical and chemical characteristics of the test article in deciding the number and location of nasal sections for histopathologic examination. Test articles with high water solubility and caustic properties are most likely to induce lesions in the squamous epithelium of the nasal vestibule, in which case a section through this site would be necessary. Induced lesions in the stratified squamous epithelium lining the nasal vestibule may include hyperplasia, inflammation, and necrosis with ulceration. Examples of chemicals inducing lesions in this area include glutaraldehyde (Gross et al, 1994) and ammonia (Bolon et al, 1991).

58. The areas of transitional and respiratory nasal epithelium (distal third of the nasal and maxillary turbinates and adjacent lateral wall in the nasal section just caudal to the upper incisors, level I as described by Young, 1981) are frequently the most sensitive to inhaled toxicants (Harkema, 1990; Harkema, 1991; Harkema et al, 2006; Renne et al, 2007). The earliest change is an increase in thickness of the surface transitional epithelium and a minimal suppurative inflammatory infiltrate in the adjacent submucosa and glands. If this lesion progresses with increased dose or continued exposure, the affected epithelium will continue to thicken and may also undergo squamous metaplasia.

59. Lesions induced in olfactory epithelium most frequently arise in the rostral extension of olfactory epithelium lining the dorsal medial meatus in the section taken at the level of the incisive papilla (Young, 1981 section II). Induced lesions range from subtle degeneration of olfactory epithelium with regeneration to necrosis and ulceration of mucosa followed by atrophy or respiratory metaplasia (Harkema et al, 2006). Induced lesions may extend to similar areas of the dorsal meatus in more caudal nasal sections, depending on the concentration and inherent toxicity of the test material and the duration of exposure.

60. Although the rostral extension of olfactory epithelium is the most frequent site of olfactory epithelial lesions induced by inhaled toxicants, certain chemicals may initially induce olfactory lesions further caudally. The mode of action of these chemicals is frequently related to metabolism via cytochrome P-450 enzymes (Pino et al, 1999; Harkema et al, 2006). Inhalation of finely divided metal particles may result in deposition in the olfactory lobes of the brain via the olfactory neuroepithelium (Henrikkson and Tjalve, 2000; Dorman et al, 2002).

61. Olfactory lesions induced by a number of inhaled materials are described and illustrated in a paper by Hardisty et al (1999). This publication also provides information on nomenclature and criteria for diagnosis of induced olfactory lesions. Another source of information on methods of describing the location of induced nasal lesions in rodents is the publication by Mery et al (1994). These authors designed a system for mapping lesions in the rodent nose using a nomenclature that identified and numbered specific turbinate bones and cavities within the nose. Use of these two publications enables the pathologist to clearly locate and characterize lesions within the complex rodent nasal cavity.

62. Nasal-associated lymphoid tissue (NALT) is located in the ventral/lateral portion of the walls forming the rostral opening of the nasopharyngeal duct, level III (Young, 1981; Spit et al, 1989; Harkema, 1991; Asanuma et al, 1997; Elmore, 2006; Brandtzaeg et al, 2008). The efferent lymph drainage from this tissue

is to the posterior cervical lymph nodes (Koornstra et al, 1991; Kuper et al, 2003). Although less is known about the function of this lymphoid tissue compared to other mucosal-associated lymphocenters, it has been demonstrated to induce specific local immune responses and activate immune mucosal tissue elsewhere as well as the systemic immune system (Kuper et al, 2003). There is much current interest in the role of the immune system in toxic effects observed in laboratory animals (Kuper et al, 1990, 1992, 2003). Examination of the NALT should be included in standard inhalation toxicology studies, and special efforts made to provide adequate sections through this tissue.

Larynx

63. Many of the issues critical for successful microscopic examination of nasal tissues described above apply to histopathology of the rodent larynx. Areas requiring precisely located tissue sections and close comparison with controls for diagnosis of squamous metaplasia and/or hyperplasia of mucosal epithelium include the base of the epiglottis, ventral pouch, and medial surface of the arytenoid processes of the larynx (Lewis, 1991; Renne and Gideon, 2006; Kaufmann et al, 2009). The most frequent and earliest change often observed in the larynx is a loss of cilia and rounding or flattening of the surface transitional epithelium at the base of the epiglottis and the adjacent ventral pouch. Initial lesions which do not completely fulfil the attributes of metaplasia are diagnosed as "epithelial alteration". When the affected epithelium is clearly flattened and consists of more than one layer (stratified), the diagnosis of squamous metaplasia is applicable (Kaufmann et al, 2009). If this lesion progresses with increased dose or continued exposure, the affected epithelium will form additional layers (hyperplasia) and the surface layer (stratum corneum) will become thicker (hyperkeratosis). Inflammatory infiltrates in the adjacent submucosa may accompany these epithelial changes.

64. As mentioned above, an alternate method of examining larynx is via longitudinal/parasaggital sections, which provide a different perspective of the ventral pouch at the expense of a full transverse view of the base of the epiglottis and the medial surface of the arytenoid processes. The paper by Germann et al (1998) describes the technique and illustrates the advantages of longitudinal sections of larynx.

Trachea and mainstem bronchi

65. The trimming section above described methods that maximize the amount of longitudinal trachea and mainstem bronchi available for microscopic examination. The carina is of particular interest because it is a site of increased impact for inhaled test material and thus potentially a primary site for induced mucosal lesions. The mainstem bronchi and their immediate distal bifurcations are a primary location for goblet cell hypertrophy and hyperplasia induced by irritant aerosols such as acrolein or cigarette smoke (see also comment below under Special Stains). Microdissection of bronchi and bronchioles (see above under Trimming) is a useful research tool for morphometry and studying metabolism of test articles on subpopulations of airway epithelium.

Bronchioles, alveolar ducts, alveoli, pleura

66. Evaluation of the effects of inhaled test materials on the lower airway and alveolar parenchyma requires the same attention to detail and close comparison with concurrent control tissues as described for upper respiratory tract. A number of texts are available describing lesions induced in rodent lungs from inhaled xenobiotics (Boorman et al, 1990; Haschek et al, 2001; Greaves, 2007), as well as detailed information on the normal anatomy, physiology, and biology of rodent lungs (Parent, 1992). Furthermore, the goRENI website (http://www.goreni.org) provides a standard reference for nomenclature and diagnostic criteria for induced lesions in the respiratory tract tissues of rats and mice, with an extensive bibliography.

67. As noted above, the response in the deep lung is dependent not only on the irritant/toxic properties of the inhaled test material but on its physical characteristics. Amount of impact and/or deposition in airways or alveoli and clearance from the lung are dependent on particle or aerosol droplet size and respiratory rate and volume (Sweeney and Brain, 1991; Oberdörster, 1996; Pauluhn and Mohr, 2000; Pauluhn, 2009). Knowledge of these and other physical/chemical characteristics of the inhaled material are useful for the pathologist in determination of likely sites and types of effects in the airways and lung parenchyma. Inhaled particulates will often induce lesions in alveolar ducts and adjacent proximal alveoli; extension into more distal alveoli may depend on particle size and the toxic nature of the inhaled material. Exposure-related lesions often occur at the bronchiolar-alveolar junction, including inflammation, septal thickening and hyperplasia. Exposure-induced effects may be as subtle as minimal congestion, edema, acute inflammatory cell infiltrates, or slight changes in populations of alveolar macrophages, mucosal epithelium, or connective tissue in lung parenchyma. More severe inflammatory or degenerative pulmonary lesions may occur in 28 or 90 day studies, whereas proliferative findings are more likely to occur in chronic studies. In longer duration studies, differentiation of neoplastic from hyperplastic epithelial lesions is often of critical importance.

Lung-associated lymph nodes (LALN)

68. The necropsy and tissue trimming procedures described steps to assure availability of lung-associated lymph nodes (LALN) for microscopic examination. Examination of LALN provides useful information on numbers and types of cells, fluids, or foreign materials infiltrating the lung parenchyma of exposed animals (Moyer et al, 2002; Calderon-Garciduenas et al, 2002). Quantitative assay of LALN for inhaled material is a useful tool for confirming and quantifying retained dose of inhaled material.

Supplementary/Optional Tasks for Histopathology

Special Stains

69. Special stains of respiratory tract sections are often helpful in diagnosis and interpretation of induced lesions. Goblet cell hyperplasia or metaplasia, frequently induced in nasal and bronchial tissue in inhalation studies, are more easily detected and quantified using Acian Blue/Periodic Acid Schiff (AB/PAS) staining (Harkema et al, 1989). Trichrome, Sirius-red and Van Gieson's stains are helpful in evaluating alveolar fibrosis (Richards et al, 1991; Kamp et al, 1995). Congo Red and modified H&E stains are useful for quantifying eosinophils or differentiating eosinophils and neutrophils in formalin-fixed lung sections (Meyerholz et al, 2009)

Immunohistochemistry

70. Immunohistochemistry is a powerful tool utilized in rodent toxicology studies for identifying specific cells and/or tissues in respiratory tract tissues using antibodies linked to a chromogen (Burnett et al, 1997; Schlage et al, 1998; Ghio et al, 2000). However care should be taken to avoid nonspecific staining related to cross-reactive binding of the antibody to a non-target tissue, and to use appropriate controls (Johnson, 1999).

Morphometry

71. Morphometry provides the capability to quantify and thus more accurately evaluate and interpret a number of lesions induced in respiratory tract by inhaled toxicants (Hyde et al, 2006). Labeling nuclei of proliferating cells with a monoclonal antibody to 5-bromo-2'deoxyuridine (BrdU) enables the pathologist to not only detect but quantify hyperplasia of respiratory tract epithelium in the nose (Goldsworthy et al, 1993; Rios-Blanco et al, 2003) or lung (Yokohira et al, 2008). Proliferating cell nuclear antigen (PCNA) (Goldsworthy et al, 1993; Tuck et al, 2008) and Ki-67 (Tian et al, 2008) are two markers of cells in DNA

synthesis used to detect cell proliferation in rodent lungs. Measurement of total area of epithelium lining the base of the epiglottis using image analysis is useful for quantifying laryngeal squamous metaplasia and hyperplasia (Renne et al, 2007). Measurements of various parameters of pulmonary architecture (total lung volume, mean alveolar size) are keys to detect and quantify alveolar emphysema or fibrosis (Hyde et al, 1991). A recent document (Hsia, et al, 2008), available from the American Thoracic Society and the European Respiratory Society, provides detailed guidelines of the currently accepted standards and approaches for quantitative assessment of lung pathology.

72. Regardless of labeling or measurement method used, the pathologist should determine the appropriate procedures for assessing morphometry and cell proliferation and should apply appropriate statistical methods of evaluation (consulting a professional biostatistician if necessary).

Ultrastructural Examination

73. Although not routinely utilized in toxicology studies, transmission electron microscopy (TEM) is valuable for identification of induced lesions at the ultrastructural level. Scanning electron microscopy (SEM) provides clear, photogenic images of the internal surface of the nasal cavity, airways, and lung parenchyma, and may be valuable for confirmation and identification of particulate such as manufactured nanoparticles and agglomerates of particulates in the respiratory tract. SEM may be used with energy-dispersive X-ray spectroscopy to confirm the chemical composition of nanoparticle agglomerates.

Reporting

Report preparation and internal review

74. The contents of the pathology report text, tables, and figures are the primary responsibility of the study pathologist. Nonetheless, the format and content of the pathology report may vary with the goals of the study and the interests of the intended recipient. The study protocol may provide specific instructions on the content of the report and the relative depth and breadth of information presented. Study sponsors or regulatory agencies may have a preferred format for presentation of the tabular data and the text. Tables are the standard format for summarizing the pathology and related data, and individual animal pathology data are usually presented in tables in an appendix. All individual data (in an appendix or table), a summarizing table with all organs, and, optional, separate tables to detail a specific finding/findings should be included in the report. A narrative text provides a detailed description of pertinent histopathology findings, interpretation of the data, and conclusions reached, with references to the summary tables. The narrative portion of the report should also provide information about problems encountered during the pathology tasks that may have impacted data interpretation and conclusions reached.

75. The interpretation of the histopathology data are primarily the responsibility of the study pathologist. The depth of discussion of the findings may vary widely, depending on the needs of the study sponsor or regulatory agency and the format of the entire study report. Current guidance from regulatory agencies regarding format and content for the pathology report and the other portions of the overall study report varies with the organization. Some regulatory agencies require a separate pathology report within the overall study report and an overall conclusion/discussion section that integrates the pathology and other study data. Others prefer a completely separate pathology report. In any case the input of the pathologist is clearly a critical part of the study report, and the pathologist should be an active participant in the overall interpretation of the study results (Morton et al, 2006).

Statistics

76. Statistical analysis of histopathology data from 28 or 90 day studies should emphasize comparison of severity grade as well as incidence of lesions between exposed groups and controls. Choosing appropriate

statistical tests may require assistance from a professional biostatistician. The Fisher's Exact test, Mann-Whitney U-test, Wilcoxon Rank Sum test, or Kolmogorov-Smirnov tests may be appropriate for evaluating and interpreting the incidence and severity of experimentally induced lesions (Gad, 2001, Bolon et al, 2006). Because there are multiple exposed groups and there may be satellite and interim sacrifice groups, analyses will benefit from holistic or all-inclusive statistical consideration, with attention paid to the possible presence of a trend. In evaluating statistical data it is important to keep in mind the normal range of biological variation, utilize concurrent as well as historical control data, and consider findings that, although not statistically significant, may suggest an effect of exposure to the test material.

Quality Assessment

77. Laboratories performing toxicology studies under OECD TG 412 or TG 413 are in the usual case generating data for submission to regulatory agencies under Good Laboratory Practices (GLP) or similar regulatory guidelines. Quality Assurance (QA) is the basic task of a specific group of people responsible for assessing and assuring the quality of all aspects of the study. The many facets of the pathology tasks and the criticality of the data generated often present technical challenges to QA staff that require close cooperation between QA, pathologists, and histology and necropsy staff (Hildebrandt, 1991). The most effective way for pathology and histology staff to approach the issue of quality of pathology data is to always keep in mind that these critical data will be thoroughly audited, reviewed, and often challenged by knowledgeable and experienced pathologists and toxicologists within the regulatory agency receiving the data, as well as any industrial sponsor whose product's safety is being addressed by the study. Any deficiencies found in the pathology data will reflect directly on the pathology staff, not on the QA staff. Thus, it is clearly in the best interests of the pathologist and pathology staff to exercise vigorous and thorough quality control for all procedures and on all data, and assist QA staff in any way possible in their audit of the pathology data.

Data Review

78. Some form of at least informal peer review of pathology data should be a routine part of any histopathologic evaluation in which data are to be used for regulatory decisions or safety evaluation. This review of data (cross check, peer review) should be an established and reproducible process. Histopathology data in which there was never any room for questions or suggested alternative diagnoses is likely less valuable due to lack of effort. Formal peer review is an established process that is becoming routine in toxicology studies, resulting in greater consistency and higher quality of the data (Ward et al, 1995).

Pathology Working Groups

4. 79. In studies with controversy over the diagnostic classification or interpretation of important or critical microscopic lesions, a pathology working group may provide resolution of the issues. A pathology working group (PWG) is a assemblage of toxicologic pathologists with experience in a selected area or subject of toxicologic pathology meeting to examine selected specimens related to that subject, usually tissue sections, from one or more studies in which differences of opinion are present regarding the diagnoses, classification, and/or toxicologic significance of the findings on the sections (Hildebrandt, 1991; Crissman et al, 2004). Standard procedure is for each pathologist to examine the slides in question, usually without knowledge of the original diagnoses, and offer an opinion. Following tabulation of the results an attempt is made at a consensus opinion on individual lesions and an overall conclusion. The results are presented in a report to which all members of the PWG contribute. PWGs are useful tools which provide a valuable contribution to resolution of disagreements, enhance the quality of the pathology data, and in most cases speed up the process of acceptance of the study results by regulatory agencies.

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