3-16-92

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Additional Issues Requested by the Peer Review Committee in Connection with the Carcinogenicity Classification of SAN582H

TO: Esther Rinde, Ph.D.
Manager, Peer Review for Carcinogenicity

FROM: Deborah L. McCall DMcCall 3-16-92
Toxicology Branch II / Section III / (H7509C)

THROUGH: James Rowe, Ph.D., Section Head James Rowe 3/16/92
Toxicology Branch II / Section III / (H7509C)

and

Marcia Van Gemert, Ph.D., Branch Chief K. Clark Swentzel for 3/16/92
Toxicology Branch II / HED (H7509C)

Background:
On March 4, 1992, a Peer Review for Carcinogenicity was held for the chemical - SAN582H, a herbicide. The registrant is Sandoz Crop Protection Corporation. The general consensus was that SAN582H would be classified as a "C" carcinogen without quantification due to the ovarian and benign liver tumors. Also, some questions were raised by the committee due to its structural similarity to Acetochlor and Propachlor. The questions were: 1) since nasal papillary tumors were found in high incidences in Acetochlor, do we know how the nasal cavity was examined in this study (were 3 sections examined)?; 2) what type of ovarian tumors were found in Propachlor?

1) Acetochlor was fed to Sprague-Dawley rats in doses of 500, 1500, and 5000 ppm (Accession # 40484801). After the nasal cavities were reexamined there was evidence of dose-related increases in nasal papillary adenomas in male rats with statistically significant differences at the 1500 and 5000 ppm dose levels. Papillary adenocarcinoma were also present in two 5000 ppm males. No dose-related finding for nasal tumors were noted in the female rats (see Attachment 1).

The registrant has supplied information on the processing technique used in the SAN582H rat study. The pathologist examined two cross-sections of the nasal cavity, one from region II and one at region III (see Attachment 2, figure 1).

2) The Propachlor study was conducted with the same strain of rats (Sprague Dawley), at dose levels of 0, 10, 50, 500 ppm, but it was conducted at a different laboratory - Hazleton Laboratories America in 1987. The study was classified as Core-Supplementary by the reviewer (L. Taylor) due to: the lack of historical control data on the ovarian tumors, and the MTD might not have been attained. The reviewer noted that the study authors had not discussed the increased ovarian tumors and tabulated the following information in the DER:

OVARY (ppm)	0	10	50	500
Benign granulosa/theca cell tumor	0	0	1	4
Malignant granulosa/theca cell tumor	0	0	0	1
Sarcoma, undifferentiated	0	0	1	0

There was an increased incidence of ovarian tumors ($p<0.05$), reported) in the high-dose group compared with the controls (Fishers' Exact Test). No historical control incidence data was provided.

Selected Histopathology findings (from pp. 1 and 2 of pathology section)

No. tissues examined: Dose group:	(69) 0ppm	(70) 500ppm	(69) 1500ppm	(69) 5000ppm
(MALES)				
NOSE/TURBINATES				
-autolysis	2	0	0	0
-inflammation, nasolacrimal duct	1	8	5	6
-inflammation, nasal mucosa	3	9	7	16**
-inflammatory epithelial hyperpl.	1	0	3	2
-papillary adenomas	0	1	6*	18**
-Squamous cell carcinoma	0	1	0	1
-Squamous papilloma	0	0	1	0
-osteoma, maxillary (benign)				
-papillary adenocarcinoma	0	0	0	2
-Esthesioneuroma (benign tumor)	0	0	0	1
No. tissues examined:	(69)	(68)	(70)	(69)
(FEMALES)				
NOSE/TURBINATES				
-inflammation, nasolacrimal duct	5	1	2	2
-inflammation, nasal mucosa	2	8	6	8
-inflammatory epithelial hyperpl.	1	0	2	0
-Papillary adenomas	0	0	2	1
-Squamous cell carcinoma	1	2	1	0
-carcinoma in-situ	0	0	1	0
-epithel. inflamm. squamous metaplasia	0	0	1	0
-submucosal glandular hyperplasia	0	0	0	2

* significantly different ($p \leq 0.05$) from control using Fisher's Exact Test with Bonferroni Inequality
** significantly different ($p \leq 0.01$) from control using Fisher's Exact Test with Bonferroni Inequality

Peto test* for trend:	"p"
nasal papillary adenoma, males	0.000
nasal papillary adenoma, females	0.055
nasal papillary adenoma, both sexes	0.000
papillary adenocarcinoma, males	0.027
esthesioneuroma, males	0.062
all nasal malignancies, males	0.031

* taken from p. 5 of EHL 86027 report

*** END-OF DOCUMENT *** — Attachment II

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ATTENTION OF: Dennis Arnold

FROM: S. A. Ruckman

COMPANY: 1 Sandoz

DATE: 10 March 1992

FAX NUMBER: 708 390 3941

MESSAGE:

Re: SAN 582 H: Rat Tumorigenicity Study (SDZ 335/891445)

Please find below the information you requested last Thursday. I am sorry it has taken longer than I anticipated to obtain replies to your questions:

1) Sections of nasal turbinates were taken according to "Histopathological examination of the rat nasal cavity" (John T. Young; "Fundamental and Applied Toxicology", 1: 309-312 (1981)).

2) Two cross-sections were taken, one at level II and one at level III, for each rat from groups 1 and 4. Any macroscopic abnormality in this region from any animal from any group was also included.

3) Both sections included the nasal turbinates of both sides of the nasal cavity.

I hope I have answered your questions satisfactorily. If you need to know any more, please contact me.

With kind regards,

S A Ruckman

Stephen A. Ruckman,
Study Director
Division of Toxicology

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FUNDAMENTAL AND APPLIED TOXICOLOGY 1:309-312 (1981)

Histopathologic Examination of the Rat Nasal Cavity

JOHN T. YOUNG
Pathology Section, Toxicology Research Laboratory, Health and Environmental Sciences, Dow Chemical, U.S.A., Midland, MI 48640

ABSTRACT

Histopathologic Examination of the Rat Nasal Cavity. Young, J.T. (1981). *Fundam. Appl. Toxicol.* 1:309-312. A method for preparation of the rat nasal cavity for histopathologic examination is presented, and the anatomic features of the sections obtained are described. The method results in four tissue sections which are selected because of their reproducibility based on landmarks on the dorsal aspect of the mouth and because of the anatomic features of each section which contribute to a thorough examination of the nasal cavity. The four areas examined include transverse sections taken at the following points: (1) immediately posterior to the upper incisor teeth, (2) at the incisive papilla, (3) at the second palatal ridge, and (4) at the level of the first upper molar teeth. The value of each section to the overall evaluation of the nasal cavity and adjacent structures is also discussed. The method described is applicable to both short-term and chronic toxicity studies, and allows for a thorough examination of the nasal cavity without destructive invasion at the time of necropsy.

INTRODUCTION

Because the laboratory rat is an obligate nasal breather, the nasal cavity serves not only as an organ of olfaction but also as the only functional air passage between the external environment and the larynx. As such, the mucous membranes of the nasal cavity are the first structures exposed to inhaled toxicants. In spite of its anatomic location, implying a propensity for insult via the inhalation route, the nasal cavity of rodents has generally been ignored in animal testing protocols in favor of examining lower parts of the respiratory tract. Numerous inhalation studies in our laboratory of various materials have identified inflammatory, degenerative, hyperplastic, metaplastic, and/or neoplastic lesions of the nasal mucosa and emphasized the sensitivity of this tissue to toxic insult. Comparative histopathologic assessment of the nasal cavity, however, has been hampered in some of these studies by incomplete examination and nonstandard sectioning procedures.

Tumors of the nasal cavity in rats associated with various chemical agents have been reported by others, not only by the inhalation route of exposure (Kuschner, *et al.*, 1975; Kociba, *et al.*, 1975; Laskin, *et al.*, 1980; Zapp, 1975) but also when administered in the feed (Isaka, *et al.*, 1979), drinking water (Pelfrene and Garcia, 1976; Singer and Taylor, 1976), and even by subcutaneous injection (Taylor and Nettesheim, 1975). In most instances, however, tumors of the nasal cavity are detected only when they are large enough to be physically deforming rather than by a systematic examination of nasal cavity, and rarely are the antecedent changes to tumor development and concurrent lesions described.

The purpose of this report is to detail a standard method of preparing the rat nasal cavity for histopathologic examination. The following procedure should result in four histologic sections of the nasal cavity from standard comparable regions which will be of sufficient quality to permit assessment and interpretation of subtle changes and to engender confidence that such interpretations have been made with a minimum of processing variables.

METHODS

Preparation at necropsy

Following decapitation and opthalmic examination, the eyes are removed and the integument is removed from the

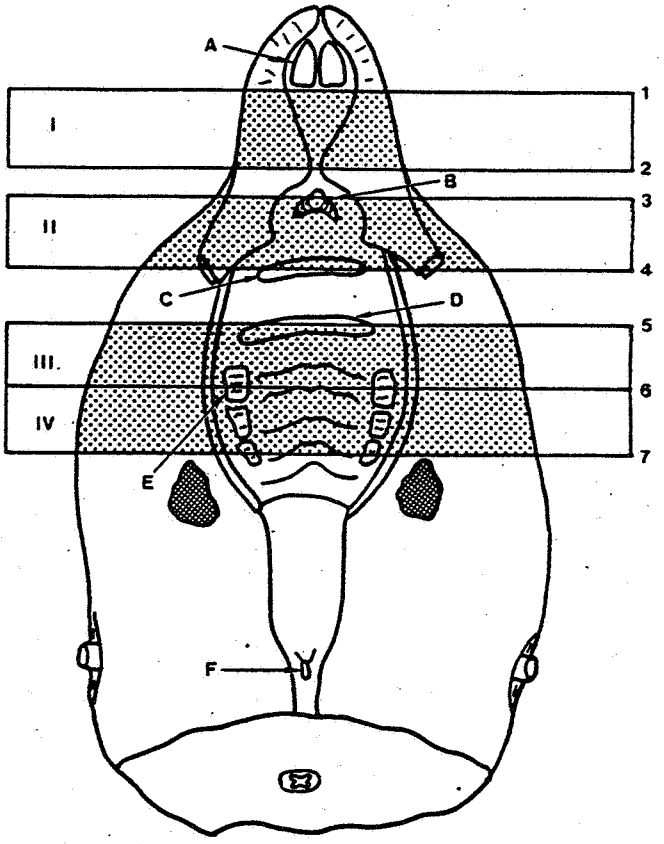

FIG. 1. Ventral view of the rat hard palate region, with the lower jaw removed, indicating the four tissue slices, I—IV (stippled areas), which will be embedded anterior face down. The numbers on the right-hand side indicate the levels of the seven cuts necessary to produce the four slices. A, upper incisor teeth; B, incisive papilla; C, first palatal ridge; D, second palatal ridge; E, first upper molar tooth; F, posterior opening of the pharyngeal duct (nasopharynx).

head. The lower jaw, brain, and pituitary are also removed, and the nasal cavity is gently flushed with 10-15 mL of neutral phosphate-buffered 10% formalin by means of a 20 or 30 mL syringe fitted with a blunt 15 ga needle which is inserted approximately 0.5 cm into the posterior opening of the pharyngeal duct (nasopharynx). If inserted too deeply, only half of the nasal cavity will be flushed. The head is immediately placed in 10% formalin after flushing and fixed for at least five days.

Decalcification

If the ears and/or Zymbal glands are not required for examination, the floor of the cranial vault and associated structures may be removed prior to decalcification. The formic acid-sodium citrate method of decalcification (Luna, 1968) is considered optimal, and 7-10 days with frequent changes of solution is usually sufficient. Following decalcification the tissues should be washed for 8-24 hours in running tap water and then returned to formalin until trimmed for processing.

Trimming

The object of trimming the heads is to produce four tissue slices which, after embedding and sectioning, will permit histopathologic evaluation of transverse sections of the nasal cavity at the following levels: 1) immediately posterior to the upper incisor teeth, 2) at the incisive papilla, 3) at the second palatal ridge, and 4) at the middle of the first upper molar teeth. The decalcified head should be removed from formalin and placed on a cutting board such that the roof of the mouth (hard palate) is facing up. With a *sharp* razor blade or scalpel, cuts are made perpendicular to the plane of the hard palate and perpendicular to the plane of the nasal septum at the following levels (see Figure 1):

cut 1: immediately posterior to the upper incisor teeth.
The tip of the nose is examined and returned to the formalin fixative with the other tissues.

cut 2: 2-3 mm posterior to cut 1.
This will produce the first tissue slice for embedding (I). The tissue slice is examined and placed anterior face down in a cassette.

cut 3: through the incisive papilla.
This will produce a thin slice of tissue which is examined and returned to the formalin fixative.

cut 4: through the first palatal ridge.
This will produce the second tissue slice for embedding (II). Examine this slice and place it anterior face down in a cassette.

cut 5: through the second palatal ridge.
The free slice produced by this cut should be examined and returned to the formalin fixative.

cut 6: through the middle of the first upper molar teeth. This cut should pass through the anterior (medial) portion of both eye orbits.
This cut will produce the third slice for embedding (III). This is examined and placed anterior face down in a cassette.

cut 7: through the third upper molar teeth.
This cut will produce the fourth slice (IV) for embedding. This is also examined and placed anterior face down in the cassette.
Examine the remainder of the head and return it to the formalin fixative if no lesions are noted.

The examination of each tissue slice at the time of trimming is an important step in the examination of the nasal cavity. If discrete lesions are noted, sections will generally be taken through them at the expense of the four standard sections, or the blocks will be identified in such a way that they will be sectioned by microtomy deep enough to include the lesions on the slides to be examined histologically.

The four tissue slices should always be placed *anterior face down* in the cassettes for processing, and ultimately embedded in the same manner. This ensures that all recuts of a block will be deeper (more posteriorly) into the nasal cavity.

Processing

Processing is done in a conventional manner used for the remainder of the tissues.

Embedding

All four sections are embedded with the anterior face down (sectioned first) with no more than two tissues per paraffin block. The two most anterior slices should be embedded in the same block and oriented in the same direction, preferably with the nasal septum perpendicular to the long axis of the block.

Sectioning

The same general principles which apply to sectioning any decalcified tissue are applicable to the nasal cavity blocks. If the tissue is slightly under-decalcified, prolonged soaking of the blocks and the use of well-sharpened steel knives, in lieu of the disposable variety, may be helpful. The emphasis, histologically, will be placed on the delicate nasal turbinates, and a small extra amount of albumin in the water bath may be useful in preventing folds and wash-offs in these areas.

Staining

Staining with hematoxylin and eosin should be accomplished in the conventional manner used for the remainder of the tissues.

RESULTS

The described method will result in four sections for histopathologic examination. An adequate anatomic description of the nasal cavity and associated structures at various levels is available in Hebel and Stromberg (1976). A general description of anatomic features in the four sections produced by the method presented here is as follows:

Level 1 (Fig. 2). This section, which is the most anterior of the four, is taken immediately posterior to the incisor teeth. The nasal cavity is completely separated by the cartilaginous septum into two chambers each with a dorsal, middle, and ventral meatus. The paired vomeronasal organ is visible near the ventral aspect of the septum. The two turbinates present at this level are the nasoturbinate extending ventrally from the nasal bone and the maxilloturbinate projecting dorsally from the maxilla medial to the roots of the upper incisor tooth on either side. The paired nasolacrimal ducts at this level are near their anterior terminus in the vestibule and are present just ventral to the maxilloturbinate and ventromedial to the roots of the incisor teeth. The entire nasal cavity at this level, with the exception of the ventral meatus at or below the vomeronasal organ, is lined by ciliated respiratory epithelium. The ventral meatus is lined by stratified squamous epithelium.

Level 2 (Fig. 3). At the level of the papilla the paired incisive ducts on either side communicate with the oral cavity The

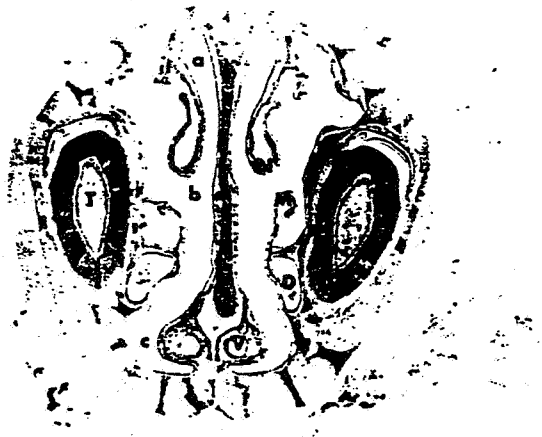

FIG. 2. Level 1 of the rat nasal cavity. This section is just posterior to the upper incisor teeth and consists primarily of respiratory epithelium except for the ventral meatus. Dorsal meatus (a), middle meatus (b), ventral meatus (c), nasoturbinate (N), maxilloturbinate (M), nasolacrimal duct (D), vomeronasal organ (V), nasal septum (S), root of upper incisor tooth (T). H and E stain (×2.5).

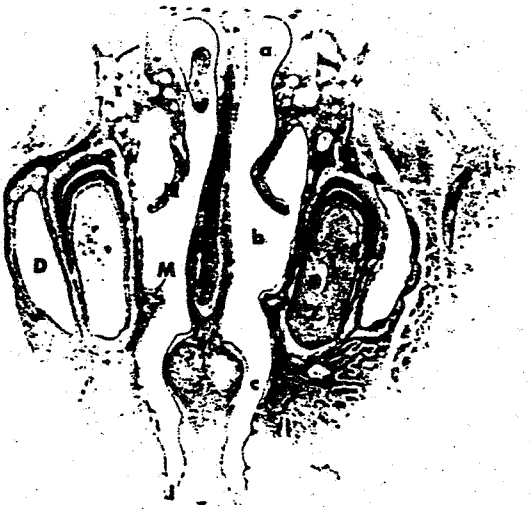

FIG. 3. Level 2 of the rat nasal cavity. This section is at the incisive papilla and contains all three epithelial types, respiratory, olfactory, and stratified squamous. Dorsal meatus (a), middle meatus (b), ventral meatus (c), nasoturbinate (N), maxilloturbinate (M), incisive duct (I), nasolacrimal duct (D), root of upper incisor tooth (T), nasal septum (S). H and E stain (×2.5).

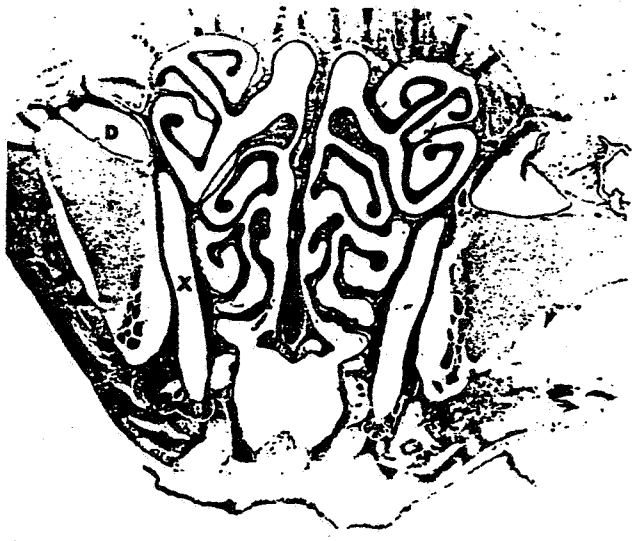

FIG. 4. Level 3 of the rat nasal cavity. This section of the ethmoid recess, taken at the level of the second palatal ridge, is lined primarily by olfactory epithelium. Ectoturbinates (1', 2'), endoturbinates (1, 2, 3), nasolacrimal duct (D), maxillary sinus (X), lateral nasal glands (G), nasal septum (S). H and E stain (×2.5).

FIG. 5. Level 4, which occurs at the level of the first upper molar teeth, is deep in the ethmoid recess and is lined primarily by olfactory epithelium. Ectoturbinate (2'), endoturbinates (2, 3, 4), pharyngeal duct (P), olfactory lobe of brain (O), harderian gland (H), nasal septum (S). H and E stain (×2.5).

nasoturbinates are still prominent in this section, but the maxilloturbinate is reduced to a small projection or is completely absent. The nasolacrimal ducts are located lateral to the roots of the incisor teeth. The vomeronasal organ is sectioned near its posterior end, and the lumen may or may not be visible. Occasionally, portions of the endoturbinates may be cut near their most anterior excursion and appear to be free in the dorsal meatus. All three epithelial types are present in this section: olfactory epithelium lines the dorsal meatus, stratified squamous epithelium lines the incisive ducts and papilla, and the remainder of the nasal cavity is lined by respiratory epithelium.

Level 3 (Fig. 4). The third level of examination is through the ethmoid recess at or near the anterior end of the pharyngeal duct. The two halves of the nasal cavity may or may not communicate at this level depending on the location of the section relative to the fully formed pharyngeal duct. Each side of the cavity has two ectoturbinates and three endoturbinates, some of which have several lamellae. These turbinates are lined primarily by olfactory epithelium except for the inner aspects of some of the scrolls which are lined by respiratory epithelium. The nasolacrimal ducts are located dorsally at this level nearing their origin at the medial canthus of the eyes. The roots of the incisor teeth are no longer present. The maxillary

sinus is present lateral to the origins of endoturbinates, and it is surrounded by the lateral nasal glands of Steno and the maxillary glands. Respiratory epithelium lines the maxillary sinus.

Level 4 (Fig. 5): The section produced at the level of the first molar teeth is deep into the ethmoid recess, but identifiable areas of the ecto- and endoturbinates are still present. The pharyngeal duct is fully formed ventrally and does not again communicate with the nasal cavity in its course to the respiratory pharynx. In the dorsal part of the section the olfactory lobes of the brain are visible, and lateral to the ethmoid recess a full section of the harderian glands is present (if they have not been intentionally removed previously). Virtually the entire ethmoid recess is lined by olfactory epithelium, and respiratory epithelium lines the pharyngeal duct.

DISCUSSION

Years of experience with both inhalation and ingestion toxicity studies by pathologists in our laboratory and a realization that a thorough examination of an animal used in hazard testing systems, particularly when exposed via the inhalation route, would have to include an examination of the nasal cavity and associated structures dictated that a standard method be developed to facilitate an adequate examination of the nose. It has been our experience that certain compounds selectively affect respiratory epithelium, some affect only olfactory, and many are nonspecific as to the epithelial type affected. Regardless of specificity, however, most agents produce their effects in a gradient fashion not only based on dose level but also relative to depth within the nasal cavity. In general, nonneoplastic, compound-induced effects have been found to be most severe at the more anterior limits of the affected epithelial-type, whether respiratory or olfactory, and decrease in severity in the posterior direction.

It was this principle, plus the need for easily identifiable landmarks that would make the sections easily reproducible, which dictated selection of the four sections in the present method. Each has characteristics which are unique and increase its usefulness in the total examination.

The most anterior section, or level 1, is lined primarily by respiratory epithelium with the exception of the ventral meatus. By sectioning posterior to the incisor teeth the junction between respiratory epithelium and the stratified squamous epithelium of the vestibule is avoided, and subtle changes in the respiratory epithelium can be interpreted with confidence that normal junctional variability is not a factor. Level 2 has two important advantages: it provides for a more posterior evaluation of respiratory epithelium, and it provides the most anterior examination of olfactory epithelium possible. For compounds which selectively affect olfactory epithelium, the dorsal meatus of level 2 is the area where the effect will generally be most readily apparent at the lowest concentrations.

Level 3 provides an extensive area of olfactory epithelium for examination covering most of the ecto- and endoturbinates and the nasal septum. A semiquantitative evaluation of the severity of induced changes can generally be made on this section by estimating the percent of surface area affected. Level 4 allows an examination of the deeper reaches of the ethmoid recess where there is generally little air circulation. Extensive changes this far posteriorly are rare since the required degree of insult would probably produce life-threatening changes in the anterior air passages. In addition, the olfactory lobes of the brain are present at this level and central neuronal changes secondary to neuroepithelial changes (or vice versa) can be evaluated.

Other structures which can be evaluated with the sections of nasal cavity include harderian gland, nasolacrimal ducts, maxillary sinuses, masseter muscle, nerve (trigeminal), bone, skin, and hard palate. The evaluation of harderian glands is especially useful since it precludes the necessity of removing them from the orbit, trimming and embedding them separately.

The examination of the nasal cavity in the manner described is useful both for short-term inhalation studies and all chronic studies (the utility for short-term oral studies is probably limited unless nasal effects are anticipated). Destructive invasion of the nasal cavity at necropsy for gross examination severely limits useful histopathologic examination for nonneoplastic effects, which are very important for toxicologic evaluation. It also is a less than optimal technique for detecting small proliferative lesions, including tumors. Examination of the fixed and decalcified tissue at the time of trimming, however, permits a thorough gross examination of the nasal cavity without destroying anatomic relationships and allows detection of even the smallest space-occupying lesions.

The author wishes to thank Drs. Kociba, Quast, Jersey, Burek, and Johnson for their contributions to the development of the method; Jane Schuetz and Ralph Albee for their advice on histology; and Fran Stafford for typing the manuscript.

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