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Respiratory Toxicologic Pathology of Inhaled Diacetyl in Sprague-Dawley Rats

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Abstract

Inhalation of butter flavoring vapors by food manufacturing workers causes an emerging lung disease clinically resembling bronchiolitis obliterans. Diacetyl, an α-diketone, is a major component of these vapors. In rats, we investigated the toxicity of inhaled diacetyl at concentrations of up to 365 ppm (time weighted average), either as six-hour continuous exposures or as four brief, intense exposures over six hours. A separate group inhaled a single pulse of ~1800 ppm diacetyl (92.9 ppm six-hour average). Rats were necropsied 18 to 20 hours after exposure. Diacetyl inhalation caused epithelial necrosis and suppurative to fibrinosuppurative inflammation in the nose, larynx, trachea, and bronchi. Bronchi were affected at diacetyl concentrations of 294.6 ppm or greater; the trachea and larynx were affected at diacetyl concentrations of 224 ppm or greater. Both pulsed and continuous exposure patterns caused epithelial injury. The nose had the greatest sensitivity to diacetyl. Ultrastructural changes in the tracheal epithelium included whorling and dilation of the rough endoplasmic reticulum, chromatin clumping beneath the nuclear membrane, vacuolation, increased intercellular space and foci of denuded basement membrane. Edema and hemorrhage extended into the lamina propria. These findings are consistent with the conclusion that inhaled diacetyl is a respiratory hazard.

Keywords: diacetyl; bronchiolitis obliterans; flavorings; airways obstruction; food processing workers; ketones; 2,3-butanedione.

Introduction

In May 2000, an unusual cluster of fixed airways obstruction in workers brought national attention to a microwave popcorn plant in Missouri (Akpinar-Elci et al. 2004; Kreiss et al. 2002). The overall rate for airway obstruction in workers in the plant was 3.3 times the expected rate, and nonsmokers had a 10.8-fold increase in the rate of airway obstruction (Kreiss et al. 2002). Moderate to severe impairment of pulmonary function was seen in nine former workers, with the forced expiratory volume in one second (FEV1) ranging from 14.0% to 66.8% of predicted values and the ratio of FEV1 to forced vital capacity ranging from 24% to 84% of the predicted value (Akpinar-Elci et al. 2004). The disease in these workers clinically resembled bronchiolitis obliterans. Occupational exposure to noxious gases is a potential cause of constrictive bronchiolitis obliterans, a fibrosing process in the small airways that results from damage to the bronchiolar epithelium (King 1989). However, known causes of bronchiolitis obliterans were not present in the small Missouri plant (Akpinar-Elci et al. 2004; Kreiss et al. 2002). Instead, more than 100 volatile compounds were detected, with diacetyl (2,3-butanedione), an α-diketone that imparts the odor and flavor of butter to food, being the predominant vapor. Estimated cumulative diacetyl exposure correlated with lung disease in the plant (Kreiss et al. 2002).

The morphologic changes in the lung that caused the altered lung function in popcorn workers are being elucidated. Eight former workers at the index plant had expiratory high-resolution computer tomography (HRCT) scans of their chests, which demonstrated air trapping and marked thickening of the bronchial walls in these patients. Five of these eight patients had cylindrical bronchiectasis, and three cases had subpleural nodularity with volume loss in the upper lobe, suggesting fibrosis. Three of the eight former workers had lung biopsies, and biopsies in two of these patients demonstrated excess fibrous connective tissue beneath the airway epithelium, which constricted the bronchiolar lumen, changes consistent with constrictive bronchiolitis obliterans (Akpinar-Elci et al. 2004). Thus, constrictive bronchiolitis obliterans is present in at least some of the patients, and the expiratory HRCT scans also demonstrate large airways disease as a

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Abbreviations: FEV1, forced expiratory volume in one second; HEPA filtered, high-efficiency particulate air filtered; HRCT, high-resolution computer tomography; ppm, parts per million; T1, first nasal section; T2, second nasal section; T3, third nasal section; T4, fourth nasal section; TWA, time-weight average; VOC, volatile organic compounds meter.

TABLE 1.—Experimental design.

Exposure Level	Experiment 1: Target TWA	Experiment 2: Target TWA	Experiment 1: Target Pattern	Experiment 2: Target Pattern
Control	0	0	N/A	N/A
Single pulse	N/A	75	N/A	single 15-minute pulse[1]
Low	100	100	continuous	a. four 15-minute pulses b. continuous
Middle	200	200	continuous	a. four 15-minute pulses b. continuous
High	300	300	continuous	a. four 15-minute pulses b. continuous

Note: TWA, time-weighted average concentration over 6 hours; N/A, not applicable

[1] The single 15-minute pulse exposure was designed to produce an exposure comparable to one of the 15-minute pulse exposures in the high-exposure group.

component of the lung disease seen in popcorn workers, a condition commonly called Popcorn Workers' Lung (Schachter 2002). This newly described disease is the subject of two recent reviews (Harber et al. 2006; Kreiss 2007).

With continued investigation, this newly described disease has been identified in additional workers, with cases being reported in other plants producing microwave popcorn, flavorings, or diacetyl (CDC 2007; Kanwal et al. 2006; Lockey et al. 2002; van Rooy et al. 2007). Although exposures in these workplaces are complex, experimental exposures of rats to vapors of butter flavorings alone provide clues to the cause of this condition. Butter flavoring vapors containing 203 ppm–371 ppm of the diacetyl component caused necrosis and inflammation of the epithelium lining the nasal passageways. Damage to intrapulmonary airways in rats inhaling butter flavoring vapors occurred after six-hour exposures to concentrations of butter flavoring vapors containing 285 ppm or greater of the diacetyl component (Hubbs et al. 2002). Although these studies demonstrated nasal and intrapulmonary airway damage from butter flavoring vapors, these vapors were themselves a mixture.

Among the components of artificial butter flavoring, diacetyl is of particular concern. Exposure measurement data and its chemical reactivity support the conclusion that diacetyl is a respiratory hazard. Diacetyl is a low molecular weight, organic, four-carbon compound with two adjacent carbonyl groups and is easily vaporized at temperatures historically used in microwave popcorn production, resulting in potentially high gas-phase concentrations in the workplace (Harber et al. 2006).

Peak diacetyl concentrations in the head space above heated butter flavoring in ventilated vats have been reported to reach 1230 ppm, suggesting very high peak diacetyl exposures in workers who mix flavorings in the mixing rooms during microwave popcorn production (Kanwal et al. 2006; Kreiss et al. 2002). The time-weighted average (TWA) diacetyl concentration measured over an entire working day was as high as 98 ppm (Kreiss et al. 2002). As an α-dicarbonyl compound, electron sharing between the adjacent carbonyl groups makes diacetyl and related α-dicarbonyl compounds particularly reactive (Wondrak et al. 2002). In vivo, like related α-dicarbonyl compounds, diacetyl can form Schiff bases. In vitro, diacetyl is directly mutagenic in *Salmonella* TA100 and can react directly with guanine residues of organic acids (Rodriguez Mellado and Ruiz Montoya 1994). Diacetyl also reacts with proteins in vitro, causing protein cross-linking via the Maillard reaction (Miller and Gerrard 2005).

Because damage to the lining epithelium is believed to be the cause of bronchiolitis obliterans (King 1989), in this study we investigated the hypothesis: *Diacetyl vapors cause necrosis of airway epithelium.* Because high peaks of diacetyl exposure characterize the environment of mixing rooms in microwave popcorn production plants, we have further investigated the hypothesis: *Peak diacetyl exposure concentration is a greater hazard than the time-weighted-average diacetyl exposure over a six-hour period.*

MATERIALS AND METHODS

Animals

Male Hla:(SD)CVF rats (Hilltop Lab Animals, Scottdale, PA) were 200 g–250 g on arrival. Rats were housed in individually ventilated microisolator units supplied with HEPA-filtered laminar flow air (Thoren Caging Systems, Hazleton, PA), with autoclaved Alpha-Dri™ virgin cellulose chips (Shepherd Specialty Papers, Watertown, TN) and hardwood Beta-chips (NEPCO, Warrensburg, NY) for bedding, and provided tap water and autoclaved Harlan Teklad Global 18% protein rodent diet (Harlan Teklad, Madison, WI) ad libitum. The animal care program was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, and the research proposal was approved by the Institutional Animal Care and Use Committee. Rats were acclimatized in the facility for 7 to 16 days before exposure.

Experimental Design

The experimental design is summarized in Table 1. Two diacetyl inhalation experiments were conducted as part of this study. The first experiment, a diacetyl inhalation toxicity experiment using six-hour continuous diacetyl exposures, was designed to address the hypothesis: *Diacetyl vapors cause death of airway epithelium.* For this experiment, exposure concentrations measured in the chambers were very close to the target concentrations. The rats were exposed to air (n = 18), 99.3 ppm diacetyl, 198.4 ppm diacetyl (n = 6), or 294.6 ppm diacetyl (n = 6) for six hours and necropsied the following morning (18 to 20 hours after removal from the exposure chamber). Thus there were four exposure groups: control, low, medium, and high.

TABLE 2.—Measured time-weighted exposure average (TWA) diacetyl concentrations over six hours (Experiments 1 and 2).

	Experiment 1: continuous exposure TWA (ppm)	Experiment 2: four ~15-minute pulse exposures TWA (ppm)	Experiment 2: Single ~15-minute pulse exposure TWA (ppm)	Experiment 2: continuous exposure TWA (ppm)
Low exposure	99.3	122	N/D	120
Middle exposure	198.4	225	N/D	224
High exposure	294.6	365	92.9	356

The second experiment, the comparative toxicity of different diacetyl exposure patterns using six-hour continuous and pulsed diacetyl exposures, was designed to address the hypothesis: *Peak diacetyl exposure concentration is a greater hazard than the time-weighted-average diacetyl exposure over a six-hour period.* This experiment was designed to provide data for assessing the risk of peak exposures that could be useful when establishing short-term exposure limits (ACGIH 2006; Ferguson 1976; NRC 1995). For this experiment, exposures included pulsed exposures designed to last 15 minutes, four times in a six-hour time period. Owing to difficulties in obtaining a precise beginning and end to each pulsed exposure, these pulsed exposures were more likely to differ from the target exposure concentration than those in the first experiment. The following day, a separate group of rats was exposed to diacetyl as a continuous six-hour exposure designed to produce a comparable TWA to the pulsed exposure. One group of rats was exposed to a single-pulse exposure comparable to one of the four pulses in the high-exposure group (Tables 1 and 2). Although there were some technical difficulties in ending all pulse exposures after exactly 15 minutes owing to residual diacetyl in the chamber, the sharp pulses that were produced mimicked those in mixing rooms (Kanwal et al. 2006). Because of these difficulties in precise control of the pulse exposures, the actual measured diacetyl concentrations in the pulsed exposures exceeded the target concentration, but the matched continuous exposures were very similar (Table 2). The highest diacetyl concentrations in this diacetyl experiment were 356 to 365 ppm, higher than in the previous exposures to diacetyl alone, but similar to the 352 to 371 ppm concentration of the diacetyl component in the previous study of diacetyl-containing butter flavoring vapors (Hubbs et al. 2002). This procedure produced eight exposure groups, each containing six rats: control, low pulsed, low continuous, medium pulsed, medium continuous, high pulsed, high continuous, and single pulse (Table 1).

For both experiments, the target concentration for the low diacetyl exposure was 100 ppm, approximately half of the lowest diacetyl concentration in the study with butter flavoring vapors. The target concentration for the high diacetyl exposure, 300 ppm, was lower than the diacetyl concentration in the highest exposures to butter flavoring vapors, because of the deaths of two rats in that study (Hubbs et al. 2002). The target concentration for the middle diacetyl exposure, 200 ppm, was between the low and high exposures. Air was the control exposure.

Exposures

The rats were exposed to diacetyl vapors in whole-body inhalation chambers using a modification of the system developed for exposures to butter flavoring vapors (Hubbs et al. 2002). Diacetyl (Sigma product number D3634, purity by titration 97%–101.5%, purity by gas chromatography 97%–99.8%, Sigma-Aldrich, St. Louis, MO) was dripped with a computer-controlled syringe pump into a glass vessel, electronically stirred continuously, and maintained at 55°C with a water bath. Air was conditioned and blown across the heated diacetyl, and dilutent air flow was adjusted to produce the desired diacetyl exposure concentration. At the concentrations used in these experiments (less than 400 ppm), air temperatures in the diacetyl exposure chamber were 27.0°C to 27.8°C, and the vapor was generated in a manner similar to the generation of diacetyl vapors in the workplace (Kanwal et al. 2006). This procedure also minimized the potential for aerosol formation, although formation of ultrafine aerosols through the process of nucleation is always possible within both the chamber and the respiratory tract. Measurements were taken with gravimetric filters, a scanning mobility particle sizer (SMPS, TSI, St. Paul, MN), and an aerodynamic particle sizer (APS, TSI, St. Paul, MN) to determine if any of the vapor was in an aerosol form. All results were negligible, which led us to conclude that no aerosol was present during the exposures.

The exposure chamber diacetyl concentration was determined using a volatile organic meter (VOC, PGM-7600, RAE Systems). The VOC used a photoionization technique to estimate the electrons ejected as organic vapors passed by an ultraviolet lamp. The VOC was calibrated by injecting a known amount of diacetyl into a heated jar of known volume. Since the volume was fixed and all diacetyl vaporized, the concentration of diacetyl present in the jar was calculated. We sampled the VOC monitor around the concentrations of interest and adjusted the internal calibration of the device to match the calculated concentrations. The VOC was recalibrated before each exposure, with no changes needed. During exposures, the calibrated VOC sampled air from the exposure chamber at a rate of 0.5 L/min throughout the entire exposure period. The output of the VOC was sampled in real time with a computer at a rate of 1 sample/second. These results were also displayed in real time, which allowed adjustments by the technician to keep the concentration at the desired level. The formula used to calculate the amount of diacetyl to inject into the jar during calibration was:

$$\text{diacetyl volume } (\mu L) = \frac{(\text{ppm diacetyl})(1000\ \text{mg/g})(86\text{g/mol})(2.25\ \text{L})}{(24.4\ \text{L/mol})(0.99\text{g/mol}) \times 10^6}$$

The diacetyl exposure concentration and time were monitored within the chamber using the direct reading VOC PID, and the TWA for diacetyl exposure was calculated for the six-hour time period. For the exposure pattern experiment (Experiment 2), the recorded concentrations of diacetyl are

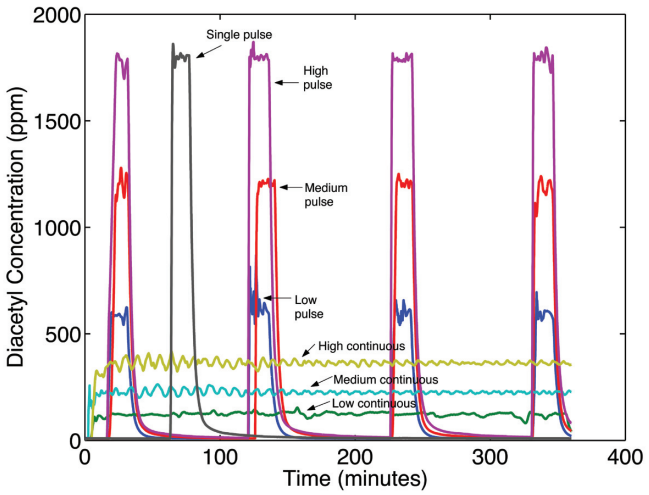

FIGURE 1.—Continous monitoring of diacetyl concentration over the six-hour exposure period comparing dose patterns (Experiment 2).

shown in Figure 1. Control rats were exposed to room air in a separate inhalation chamber.

Necropsies

Rats were euthanized with an intraperitoneal injection of an overdose of Sleepaway (≥ 100 mg/kg pentobarbital), followed by transection of the aorta 24 to 26 hours after the start of exposure (18 to 20 hours after the end of exposure). In the diacetyl inhalation toxicity experiment, lungs were preserved by intratracheal instillation of 6 mL of Karnovsky's fixative (Karnovsky 1965). Noses were immersion fixed in Karnovsky's fixation, decalcified using 13% formic acid, and sectioned at four standard levels (Young 1981). The tracheal bifurcation was sampled for scanning electron microscopy. In the exposure pattern experiment, necropsies were performed in the same manner, except that the larynx and upper half of the trachea were immersed in Karnovsky's fixative and the lung was pressure perfused with Karnovsky's fixative at 20 cm for 30 minutes via the lower trachea and then immersion fixed in Karnovsky's fixative to ensure unaltered tracheal epithelial morphology.

Scanning Electron Microscopy

Because the mucous thickness and cell types of the rat trachea are similar to the mucous thickness and cell types in the human bronchioles, because the rat trachea is a similar diameter to the human fifth-generation intrapulmonary airway, and because bifurcations of airways are sites of injury from impaction of liquids formed by vapor condensation within the respiratory tract (Mercer et al. 1991; Yeh et al. 1976; Yeh et al. 1979), the tracheal bifurcation was selected as the standard site for scanning electron microscopy.

The bifurcations were post-fixed in osmium tetroxide. They were dehydrated in an ethanol series, dried using hexamethyldisalizane, mounted onto aluminum stubs, and sputter-coated with gold/palladium. The samples were then imaged on a JEOL 6400 scanning electron microscope at 20 kv.

Histopathology

The histopathology findings from the left lung lobe, the right lung (the right cardiac lobe in Experiment 1 and each of the 4 right lung lobes in Experiment 2), and four standard levels of the nose were evaluated, and semiquantitative pathology scores reflecting the severity and distribution of morphologic changes were assigned as previously described (Hubbs et al. 2002). The same scoring system was used to additionally evaluate trachea and larynx in Experiment 2. Scores for severity were: none = 0, minimal = 1, mild = 2, moderate = 3, marked = 4, and severe = 5. Scores for distribution were: none = 0, focal = 1, locally extensive = 2, multifocal = 3, multifocal and coalescent = 4, and diffuse = 5. The pathology score was the sum of the severity and distribution scores.

Within the spectrum of necrosuppurative morphologic changes observed in diacetyl-exposed rats, a few changes were best classified as necrotizing and a few changes best classified as suppurative, but they appeared to be within the spectrum of necrosuppurative changes seen throughout the study. Indeed, in a recut section classified as having suppurative inflammation, a focus of epithelial necrosis was identified. For these reasons, pathology scores for necrosuppurative changes included findings classified as necrotizing, suppurative and/or necrosuppurative. Based on the sites of histopathologic changes in rats inhaling butter flavoring vapors and the ventral pathway for the main air flow pathway through the nose (Frederick et al. 1998; Hubbs et al. 2002), the semiquantitative pathology scores for nasal sections T3 and T4 were for the ventral portion of those nasal sections, which is the septal window and the nasopharyngeal duct.

Digital Light Photomicroscopy

All photomicrographs were taken using an Olympus AX70 photomicroscope (Olympus, Melville, NY). Routine color digital light photomicrographs were taken using a Retiga 2000R color digital camera (QImaging, Surrey, BC, Canada). High-resolution color photomicrographs for larger pictures were taken using a digital color tuner with a Quantix cooled digital camera (Photometrics, Tucson, AZ) with QED Camera Plug-in software (QED Imaging, Pittsburgh, PA) to produce 28.4 x 28.4 inch 72 dpi RGB images, which were converted to CMYK images in Corel Photo-Paint and resized to 6.82 x 6.82 inch 300 dpi images without increasing pixel number and cropped as needed to produce images of the desired size.

Transmission Electron Microscopy

Karnovsky's fixed tissues were post-fixed in osmium tetroxide, mordanted in tannic acid, stained with uranyl acetate, dehydrated in alcohol, embedded in Epon, and stained with uranyl acetate and lead citrate. A JEOL 1220 transmission

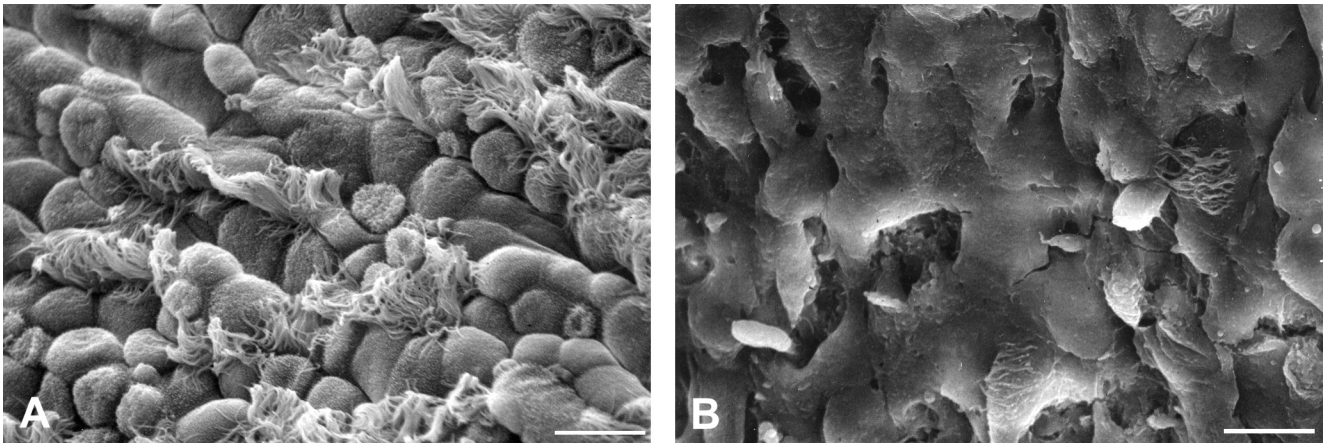

FIGURE 2.—Scanning electron microscopy showing the surface morphology of the tracheal bifurcation of rats exposed to (A) air (control) (B) 294.6 ppm diacetyl as a continuous six-hour exposure. The diacetyl-exposed epithelium is characterized by flattening of cells, loss of microvilli and cilia, and fissure formation. Bar = 10 μm (Experiment 1).

electron microscope was used to evaluate ultrastructural changes. The proximal trachea and the right mainstem bronchus were evaluated in rats from the high-pulsed (365 ppm) and continuous (356 ppm) diacetyl exposures.

Statistics

The data were analyzed using SAS/STAT software, Version 9.1 of the SAS system for Windows (SAS Institute, Inc., Cary, NC). At each airway level, the effects of dose were analyzed using the nonparametric Kruskal-Wallis test and followed with Wilcoxon rank sum tests to make pairwise comparisons. Multiple analyses were conducted to answer the questions of interest. First, for the dose response study, the effect of the different diacetyl exposure concentrations on the histopathology of the airway epithelium was compared at each airway level. Then, for the exposure pattern comparison experiment, we analyzed the continuous and the multiple-pulse diacetyl exposure patterns separately for effect of the different exposure concentrations on the histopathology of the airway epithelium. The fourth analysis compared the comparable exposure groups (low, medium, and high) for the effect of the exposure pattern—continuous or multiple pulses—on airway histopathology. The final analysis compared the single-pulse diacetyl exposure with air controls and the comparable multiple diacetyl exposure group (high-pulsed exposure group) to determine if a single brief exposure could damage airway epithelium histopathology and if repeated pulse exposures to the same concentration were more damaging than the single brief exposure.

The significance level was set at .05 and presented as Wilcoxon p values unless otherwise specified. The exact p value was also calculated and is specifically noted and designated as the exact p value, only when the two different p values (Wilcoxon and exact) affected whether or not a finding was statistically significant.

RESULTS

Diacetyl Inhalation Toxicity Experiment (Experiment 1): Scanning Electron Microscopy after a Six-Hour Continuous Exposure to Diacetyl

Scanning electron microscopy revealed consistent changes in the surface morphology of the tracheal bifurcation of rats in the high-exposure groups. These changes consisted of loss of microvilli, decreased numbers of ciliated and mucous cells, flattening and expansion of remaining epithelial cells, and foci of denuded basement membrane (Figure 2).

Diacetyl Inhalation Toxicity Experiment (Experiment 1): Histopathology of the Nose and Lung after a Six-Hour Continuous Exposure to Diacetyl

The epithelium lining the nasal passageways of all levels of the rat nose was significantly damaged in rats in the middle- and high-exposure groups. The principal morphologic change was necrosuppurative rhinitis (Figures 3A and 3B). In the two sections farthest from the external nares, sections T3 and T4, epithelial changes were limited to the septal window and nasopharyngeal duct, the sites of greatest air flow in this region of the nose (Frederick et al. 1998). The rhinitis in levels T3 and T4 (Figure 3C) was also necrosuppurative. At all levels of the nose, the pathology scores for necrosuppurative rhinitis were significantly greater in rats in the middle- and high-exposure groups than in controls ($p \leq .0001$ for all levels at both exposures). At all levels of the nose, the necrosuppurative rhinitis showed a general dose-responsive trend (Figure 4). In some animals, an occasional hair was seen amidst the necrosuppurative debris, presumably as a result of impaired clearance in the presence of epithelial necrosis.

In the lungs, diacetyl-associated changes were limited to the airways of two rats continuously inhaling the highest diacetyl

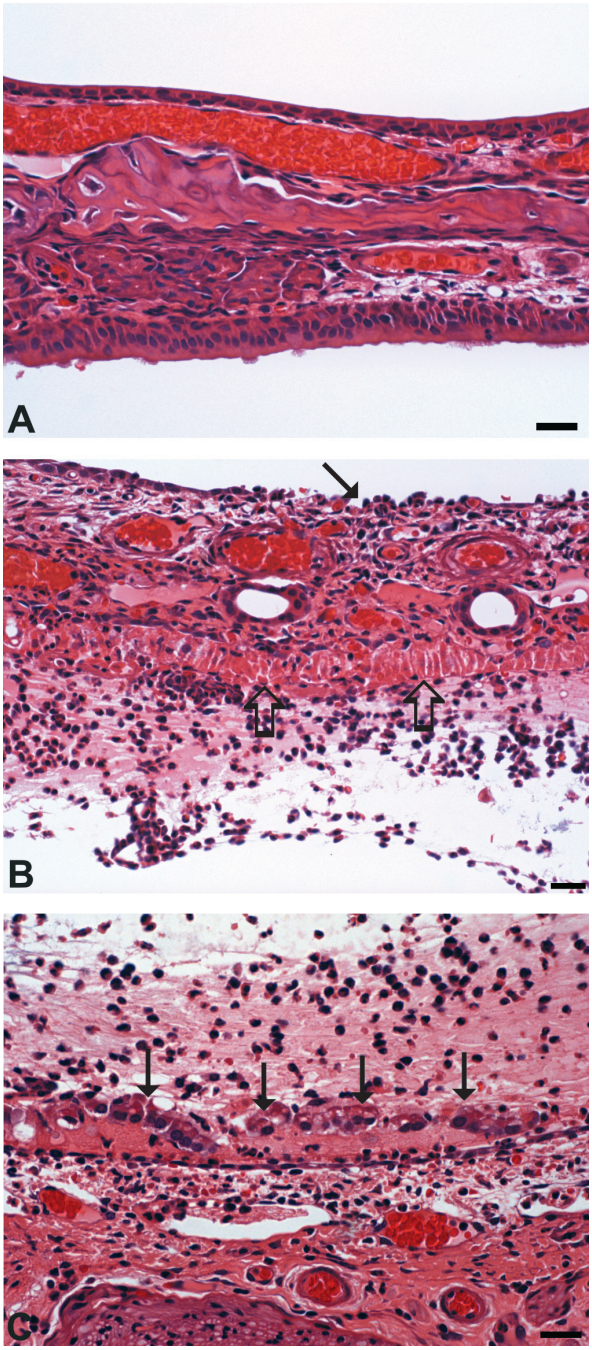

FIGURE 3.—Morphologic changes in the noses of rats inhaling diacetyl vapors in the diacetyl inhalation toxicity experiment (Experiment 1). (A) Histopathology of the nasal turbinate at level T1 of a control rat. Bar = 20 µm. (B) Histopathology of the nasal turbinate at level T1 in a rat inhaling 294 ppm diacetyl as a continuous six-hour exposure. Epithelial cells are frequently intensely eosinophilic and devoid of nuclei (open arrows). In other foci, the basement membrane is denuded of epithelium (solid arrrow). These morphologic changes indicate necrosis of the epithelium. Bar = 20 µm. (C) A higher magnification of the nasopharyngeal duct in a rat inhaling 294 ppm diacetyl showing detachment of the epithelial layer (arrows) and subepithelial accumulation of eosinophilic material. Bar = 20 µm.

concentration, 294.6 ppm. Multifocal, mild, necrosuppurative bronchitis affected the left lung lobe of one rat and focal, minimal suppurative bronchitis affected the left lung of a second rat. At this exposure concentration, the effects of diacetyl on the intrapulmonary airways were statistically significant ($p = .0150$). However, using the exact p value as a measure of significance, the differences only bordered on statistical significance (exact p value = .054). These histopathology findings are summarized in Table 3.

Diacetyl Exposure Pattern Experiment (Experiment 2): Scanning Electron Microscopy

Changes in the surface morphology of the tracheal bifurcation were consistently observed in the high-exposure group, irrespective of pulsed or continuous patterns of exposure. These changes included loss of microvilli, loss of cilia and mucous cells, detachment of epithelial cells, deposition of acellular fibrinous material consistent with fibrin, and deposition of cellular debris (Figure 5).

Diacetyl Exposure Pattern Experiment (Experiment 2): Histopathology Following Pulsed Versus Continuous Diacetyl Inhalation

Table 4 summarizes the histopathology findings from the comparison of pulsed versus continuous diacetyl exposure patterns. As with the previous experiment, in this experiment, diacetyl caused necrosuppurative rhinitis (Figure 6). Necrosuppurative rhinitis pathology scores were statistically significant in the section closest to the external nares, T1, in the low, middle, and high continuous-exposure groups and pulsed diacetyl in the middle- and high-exposure groups (Figure 6). In the low-exposure group, the multiple-pulsed exposure pattern did not cause significant rhinitis in section T1 relative to controls ($p = .171$) and caused significantly less rhinitis in section T1 than the constant exposure pattern ($p = .029$). In nasal sections T2 and T3, continuous or pulsed patterns of diacetyl exposures caused necrosuppurative rhinitis in the middle- and high-exposure groups. In nasal section T4, continuous or pulsed exposure patterns caused necrosuppurative rhinitis in the high-exposure group. In addition, in section T4, the middle diacetyl exposure caused significant necrosuppurative rhinitis when administered continuously ($p = .010$). When administered as four pulses, the middle exposure did not significantly alter the T4 pathology score compared with controls ($p = 0.195$). However, differences between the T4 pathology scores for the pulsed and continuous patterns of exposure in the middle-exposure group were not significant. The larynx and trachea were not affected by the low exposures but were significantly damaged by the middle- and high-diacetyl exposure, irrespective of continuous or pulsed administration (Figures 7 and 8). The intrapulmonary airways were not affected by the low and middle exposures but were significantly damaged by the high-diacetyl exposure (Figure 9), irrespective of continuous or pulsed administration ($p < .001$ and $p = .003$ for continuous and pulsed exposures,

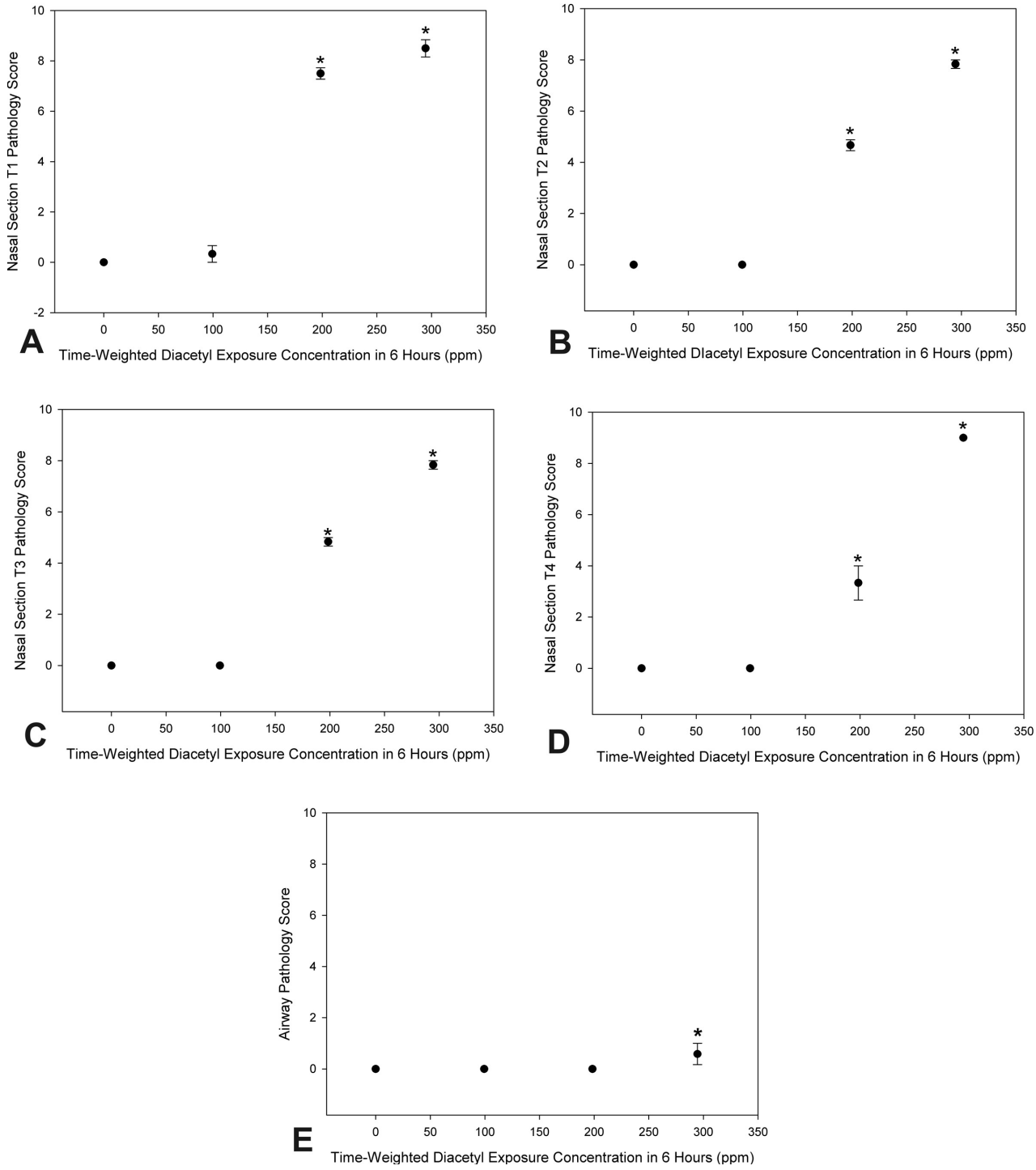

FIGURE 4.—Dose response for necrosuppurative morphologic alterations in the lining epithelium of (A) the nose at level T1; (B) the nose at level T2; (C) the nose at level T3; (D) the nose at level T4; and (E) large intrapulmonary airways in the inhalation toxicity experiment (Experiment 1).

respectively). In the most severely affected rat, acute suppurative bronchopneumonia developed with involvement of the deep lung of all lung lobes. A few hairs and fiberlike structures were seen amidst the inflammation in this rat and were interpreted as being the result of periods of mouth breathing and impaired airway clearance.

The single-pulse diacetyl exposure was comparable to one of the pulses in the multiple high-pulse exposure, with a peak

TABLE 3.—Prevalence and mean histopathology scores for necrosuppurative changes in the respiratory tract of rats inhaling air (n = 18), 99.3 ppm diacetyl, 198.4 ppm diacetyl (n = 6), or 294.6 ppm diacetyl (n = 6) for 6 hours (Experiment 1).

	Exposure concentration (time-weighted average)			
	Control (air)[1]	Low (99.3 ppm)[1]	Middle (198.4 ppm)[1]	High (294.6 ppm)[1]
Nose (T1)	0/18 (0 ± 0)	1/6 (0.33 ± 0.33)	6/6 (7.5 ± 0.22*)	6/6 (8.5 ± 0.34*)
Nose (T2)	0/18 (0 ± 0)	0/6 (0 ± 0)	6/6 (4.7 ± 0.21*)	6/6 (7.8 ± 0.17*)
Nose (T3)[2]	0/18 (0 ± 0)	0/6 (0 ± 0)	6/6 (4.8 ± 0.41*)	6/6 (7.8 ± 0.17*)
Nose (T4)[2]	0/18 (0 ± 0)	0/6 (0 ± 0)	5/6 (3.3 ± 0.67*)	6/6 (9.0 ± 0*)
Intrapulmonary airways[3]	0/18 (0 ± 0)	0/6 (0 ± 0)	0/6 (0 ± 0)	2/6 (0.58 ± 0.42*)

[1] Affected rats/total rats (mean pathology score ± SE for the exposure group). Necrosuppurative pathology scores are the scores for lesions designated as necrosuppurative, necrotizing, and/or suppurative.
[2] For sections T3 and T4, these are the scores for the ventral air passageways of the nose (nasopharyngeal duct and septal window).
[3] Scores are for the mainstem bronchus and the largest non-cartilaginous airways.
* Significantly different from air-exposed controls ($p \leq .05$, Wilcoxon).

diacetyl exposure of 1949 ppm but a six-hour TWA of 92.9 ppm, approximately one fourth of the TWA concentration in the pulsed and continuous high-diacetyl exposure groups. In rats with the single-pulse exposure, findings from the first nasal section demonstrated that a brief pulse exposure to high concentrations of diacetyl could damage the respiratory epithelium of the nose (Table 4, Figure 6D). Specifically, the single-pulse exposure, with a target concentration of 1800 ppm (real-time readouts revealed a peak of 1949 ppm, Figure 1) and a target duration of 15 minutes, produced a TWA of 92.9 ppm over six hours and produced significant necrotizing and/or suppurative changes in section T1 relative to controls ($p = .039$). In this single-pulse exposure group, two rats had karyorrhectic and pyknotic nuclei without cell swelling in the epithelium of the nasoturbinates. Because these changes were more consistent with apoptosis than necrosis, this change was not scored as a necrotizing change but was noted. This single-pulse exposure did not cause damage to the respiratory epithelium at other sites in the nose (levels T2, T3, and T4) or to the larynx, trachea, or intrapulmonary airways. Four pulse exposures of the comparable diacetyl concentration caused significant damage to the respiratory epithelium at each of these sites. This damage was significantly greater than damage caused by the single-pulse exposure in the same site (Wilcoxon $p = .004$, .003, .003, .003, .003, .003, and .028 for T1, T2, T3, T4, larynx, trachea, and intrapulmonary airways, respectively).

Diacetyl Exposure Pattern Experiment (Experiment 2): Ultrastructural Changes in the Trachea and Bronchus after a Six-Hour Inhalation Exposure to High Diacetyl Concentrations

In the trachea, the high diacetyl exposure (356–365 ppm) caused ultrastructural changes in the trachea in rats exposed with either pulsed or continuous exposure patterns. Ultrastructural changes included cellular degeneration and death in the epithelial layer (Figures 10A and 10B) with foci of denuded basement membrane (Figure 11A). Degenerative changes within cells included dilation and whorling of the endoplasmic reticulum, chromatin clumping beneath the nuclear membrane, vacuolation, and increased intercellular space. Edema and hemorrhage extended into the lamina propria (Figure 11A). In some foci, a single layer of epithelial cells was composed of poorly differentiated, loosely associated epithelial cells with tonofilaments, a change suggestive of spreading and migration of epithelial cells during attempted repair (Figure 11B). One focus of bronchial epithelial necrosis was observed in the sections of right mainstem bronchi examined ultrastructurally. The bronchial epithelial necrosis was from a rat in the high continuous-exposure group and provides some clues regarding the ultrastructural features which may characterize the multiple foci of bronchial epithelial necrosis demonstrated by light microscopy. Ultrastructurally, this focus demonstrated cell degeneration and necrosis in conjunction with denudation and rupture of the basement membrane, edema, and neutrophilic inflammation of the lamina propria, and a fibrinonecrotic membrane (Figure 12).

DISCUSSION

These experiments demonstrate cellular degeneration and death in the epithelium lining the nose, larynx, trachea, and intrapulmonary airways of rats inhaling diacetyl vapors as a single-agent exposure. Epithelial damage was accompanied by a principally neutrophilic inflammatory response. Histopathology indicated that the changes in the lining epithelium were dependent on the TWA exposure concentration in each affected region of the respiratory tract. The exposure pattern used to produce a TWA influenced the pathology score only at the first level of the nose (T1), where the low continuous diacetyl exposure caused significantly greater injury to the nasal epithelium than the low multiple-pulsed diacetyl exposure at a comparable TWA. However, a single pulse exposure for approximately 15 minutes resulted in a lower six-hour TWA of 92.9 ppm diacetyl but caused significant necrosuppurative changes in the T1 level of the nose. This TWA is comparable to the highest TWA measured in the workplace, 98 ppm (Kreiss et al. 2002). Thus, our data suggest that the no observable adverse effect level (NOAEL) for inhaled diacetyl is less than 93 ppm, but the exact value still needs to be established. In addition, our data suggest that even

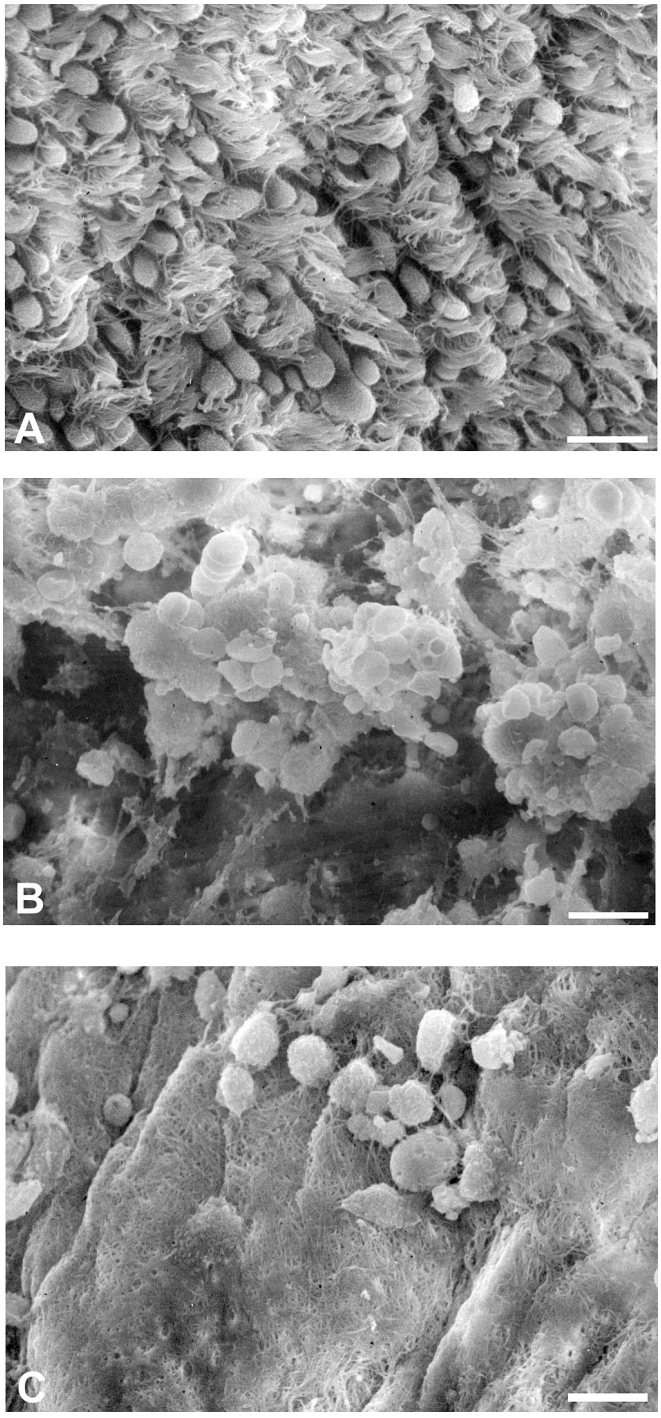

FIGURE 5.—Scanning electron microscopy showing the surface morphology of the tracheal bifurcation of rats in the exposure pattern experiment (Experiment 2). (A) Tracheal bifurcation from an air-exposed (control) rat. (B) Tracheal bifurcation from a rat exposed to 365 ppm diacetyl as a TWA delivered by four pulsed exposures over a six-hour period. Cellular debris is lifting off of the denuded basement membrane. (C) Tracheal bifurcation from a rat exposed to 356 ppm diacetyl as a TWA delivered by a continuous six-hour exposure. The fibrillar surface containing cellular debris is consistent with a fibrinonecrotic membrane. Bar = 10 μm.

brief diacetyl exposures can damage airway epithelium if these exposures are very high, as may be possible in workers mixing flavorings (Kanwal et al. 2006). However, our data also suggest that during acute exposures producing TWAs of more than 100 ppm, pulsatile and continuous exposures have similar potentials to cause acute airway injury.

Epithelial damage in this study occurred in the nose, larynx, trachea, and mainstem bronchi of the diacetyl-exposed rats. In considering the toxicity of diacetyl, both intrapulmonary and extrapulmonary airway injury should be evaluated. Studies of workers have generally concentrated on the intrapulmonary airway injury (Akpinar-Elci et al. 2004; Kreiss et al. 2002). However, nasal irritation is also noted in reports of popcorn workers and declines following implementation of exposure controls (NIOSH 2003, 2004, 2006).

It is expected that the rat may experience greater nasal epithelial damage than humans from vapor exposures. The gas-phase mass transport coefficients of the rat nose are one to two orders of magnitude higher than in the human nose (Frederick et al. 1998). Epithelial necrosis in intrapulmonary airways of diacetyl-exposed rats principally involved the mainstem bronchus. These intrapulmonary airways and the trachea of the rat have dimensions similar to airways in the deep lung of humans. For example, the diameter of the rat trachea, ~0.35 cm, is similar to the diameter of the 5th generation human intrapulmonary airway (Yeh et al. 1976; Yeh et al. 1979). This finding is important because small airway diameter decreases the lumen volume to mucous surface area ratio, increases resistance, and decreases air flow (Frederick et al. 1998; Mauroy et al. 2004). Mucosal deposition of vapors increases as air flow decreases (Morris 1997). Thus, the smaller diameter of the rat nasal passages, trachea, and bronchi would be expected to produce increased resistance, decreased air flow, and increased mucosal deposition of vapors when compared with the corresponding structures in the human respiratory tract. This would be expected to shift the site of mucosal absorption of vapors higher up in the respiratory tract of rats as compared to in humans. In addition, high-resolution computer tomography scans of severely affected workers demonstrate damage in the bronchi, indicating large airway as well as small airway damage in at least some of the workers with Popcorn Workers' Lung (Akpinar-Elci et al. 2004). It is certainly possible that the shift in site of epithelial injury in the rat relative to the site of epithelial injury in diacetyl-exposed workers can be explained by known differences in rat and human respiratory tract anatomy with resulting changes in sites of vapor absorption.

Thus, this study of diacetyl inhalation in rats demonstrates damage to the airway epithelium, which is believed to be the initiating injury for the general pathologic entity known as bronchiolitis obliterans (King 1989). Diacetyl-exposed workers in the popcorn industry have clinical signs consistent with bronchiolitis obliterans (Kreiss et al. 2002). In addition, some of the diacetyl-exposed workers have biopsy changes consistent with bronchiolitis obliterans (Akpinar-Elci et al. 2004). Diacetyl-exposed workers also have damage to the bronchi, the cartilaginous intrapulmonary airways (Akpinar-Elci et al. 2004).

TABLE 4.—Prevalence and mean histopathology scores for necrosuppurative changes in the respiratory tract of rats exposed to air or diacetyl. Diacetyl exposures produced a time-weighted average over 6 hours, which was delivered continuously throughout the 6 hours, in four ~15 minute pulses, or in a single ~15 minute pulse (Experiment 2).

	Exposure group (time-weighted average) and pattern							
	Control air[1]	Low (122 ppm), four pulses[1]	Low (120 ppm), continuous[1]	Middle (225 ppm), four pulses[1]	Middle (224 ppm), continuous[1]	High (365 ppm), four pulses[1]	High (356 ppm), continuous[1]	Single pulse (92.9 ppm), one pulse[1]
Nose (T1)	1/12 (0.17 ± 0.17)	2/6 (1.2 ± 0.75)	5/6 (4.33 ± 0.96[*,**])[1]	6/6 (6.7 ± 0.49[*])	6/6 (7.8 ± 0.31[*])	6/6 (8.3 ± 0.33[*])	6/6 (8.8 ± 0.75[*])	3/6 (1.8 ± 0.83[*,***])
Nose (T2)	0/12 (0 ± 0)	0/6 (0 ± 0)	0/6 (0 ± 0)[1]	6/6 (4.5 ± 0.43[*])	6/6 (5.8 ± 0.65[*])	6/6 (7.5 ± 0.43[*])	6/6 (7.7 ± 0.33[*])	0/6 (0 ± 0)
Nose (T3)[2]	0/12 (0 ± 0)	0/6 (0 ± 0)	0/6 (0 ± 0)[1]	2/6 (1.5 ± 1.0[*])	5/6 (4.0 ± 0.97[*])	6/6 (8.3 ± 0.42[*])	6/6 (8.3 ± 0.42[*])	0/6 (0 ± 0)
Nose (T4)[2]	0/12 (0 ± 0)	0/6 (0 ± 0)	0/6 (0 ± 0)[1]	1/6 (0.83 ± 0.83)	5/6 (4.1 ± 1.1[*])	6/6 (8.3 ± 0.42[*])	6/6 (9.5 ± 0.22[*])	0/6 (0 ± 0)
Larynx	0/12 (0 ± 0)	0/6 (0 ± 0)	0/6 (0 ± 0)[1]	5/6 (6.0 ± 1.3[*])	5/6 (5.7 ± 1.2[*])	6/6 (9.2 ± 0.40[*])	6/6 (9.5 ± 0.34[*])	0/6 (0 ± 0)
Trachea	0/12 (0 ± 0)	0/6 (0 ± 0)	0/6 (0 ± 0)[1]	2/6 (1.7 ± 1.1[*])	5/6 (3.5 ± 0.92[*])	6/6 (7.8 ± 0.60[*])	6/6 (8.7 ± 0.33[*])	0/6 (0 ± 0)
Intrapulmonary Airways[3]	0/12 (0 ± 0)	0/6 (0 ± 0)	1/6 (0.07 ± 0.07)	0/6 (0 ± 0)	1/6 (0.13 ± 0.13)	4/6 (0.80 ± 0.43[*])	6/6 (2.0 ± 0.51[*])	0/6 (0 ± 0)

[1] Affected rats/total rats (mean pathology score ± SE for the exposure group). Necrosuppurative pathology scores are the scores for lesions designated as necrosuppurative, necrotizing, or suppurative.
[2] For sections T3 and T4, these are the scores for the ventral air passageways of the nose (nasopharyngeal duct and septal window).
[3] Scores are for the mainstem bronchus and the largest non-cartilaginous airways.
[*] Significantly different from air-exposed controls ($p \leq .05$, Wilcoxon).
[**] Significantly different from the low multiple pulse group.
[***] Significantly different from the high multiple pulse group.

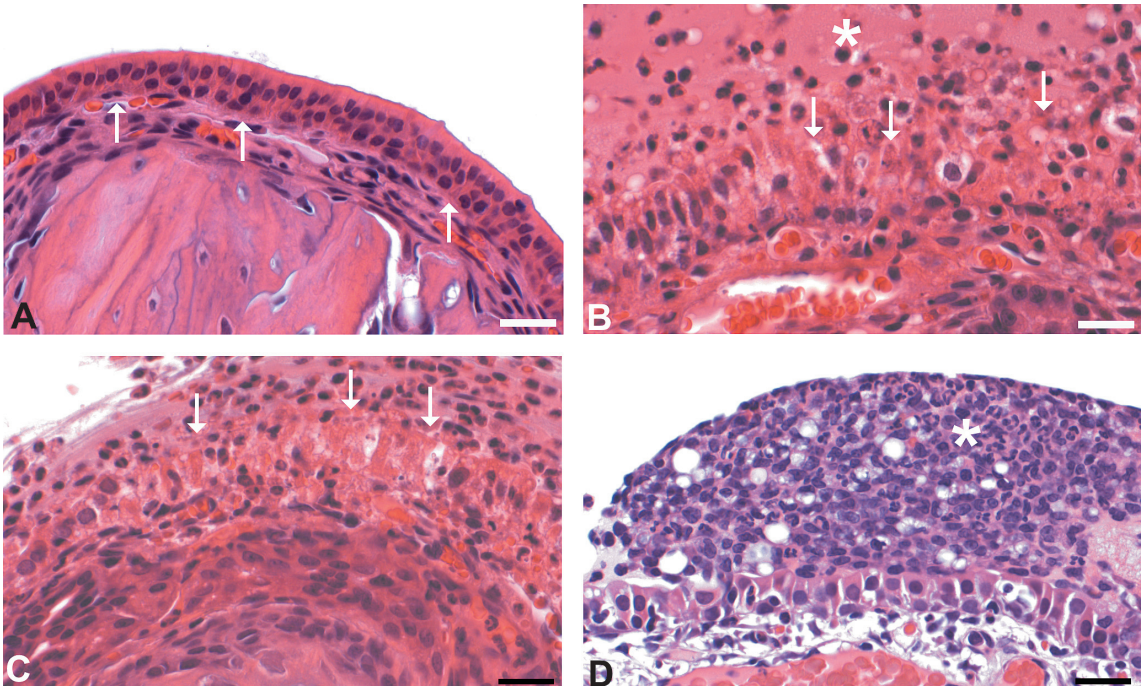

FIGURE 6.—Histopathology of the nose in diacetyl-exposed rats from the exposure pattern experiment (Experiment 2). (A) Intact epithelium (solid arrows) covers basement membrane in a nasoturbinate at level T1 of a control rat. (B) Necrotic epithelium (solid arrows) in a nasoturbinate at level T1 of a rat receiving 365 ppm diacetyl as a TWA delivered by four pulsed exposures over a six-hour period. Eosinophilic proteinaceous material, cellular debris, and neutrophils (*) are above the epithelium. (C) Necrotic epithelium (solid arrows) in a nasoturbinate at level T1 of a rat receiving 356 ppm diacetyl as a TWA delivered at a continuous rate over a six-hour period. (D) Neutrophils, macrophages, and cellular debris above the epithelium in the nasoturbinate at level T1 of a rat inhaling a single-pulse exposure to diacetyl, which produced a six-hour diacetyl TWA of 92.9 ppm. H&E stain. Bar = 20 µm.

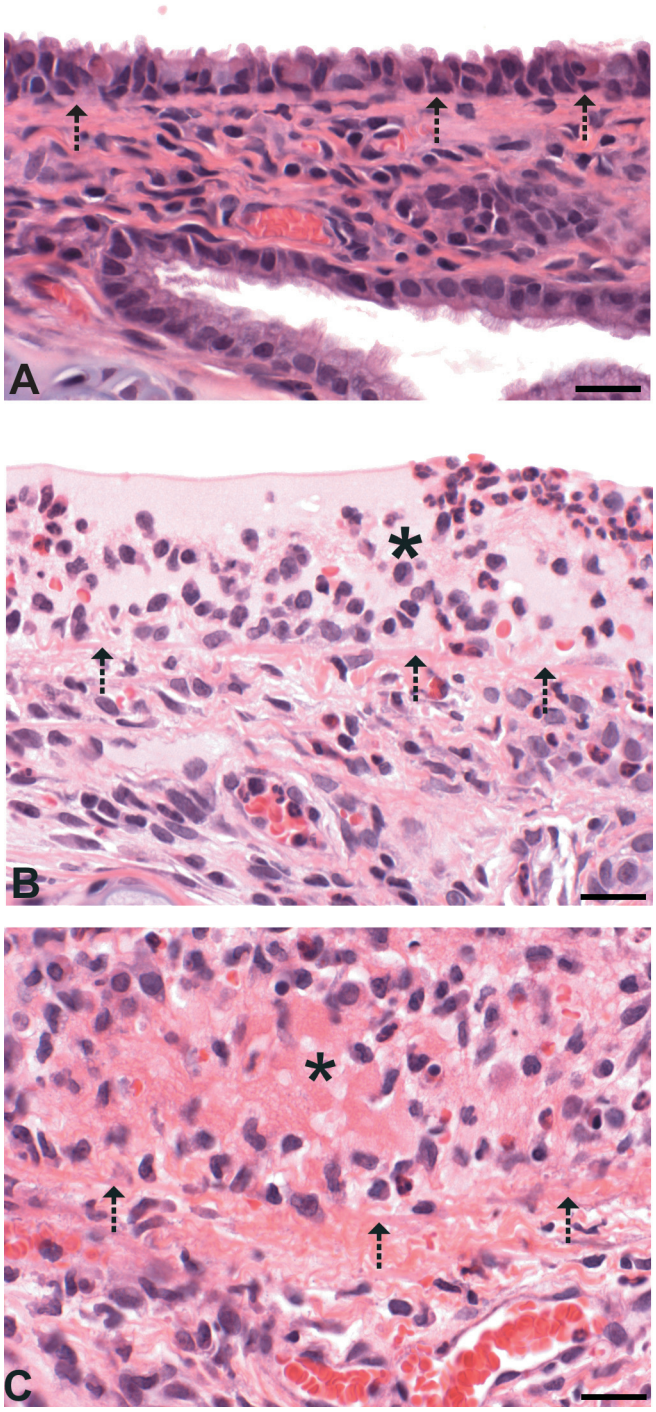

FIGURE 7.—Histopathology of the trachea in diacetyl-exposed rats in the control and middle-exposure groups from the exposure pattern experiment (Experiment 2). (A) Intact epithelium lines basement membrane (dashed arrows) in the trachea of a control rat. (B) Denuded basement membrane (dashed arrows) in the trachea of a rat inhaling 225 ppm diacetyl as a TWA delivered by four pulsed exposures over a six-hour period. Mucus, fibrin, neutrophils, and necrotic epithelial cells (*) line the luminal surface. (C) Denuded basement membrane (dashed arrows) in the trachea of a rat inhaling 224 ppm diacetyl as a TWA delivered at a continuous rate over a six-hour period. Fibrin and cellular debris (*) line the luminal surface. H&E stain. Bar = 20 µm.

We documented necrosuppurative changes in the cartilaginous airways of rats in this study, although the cartilaginous airways of the rat are largely extrapulmonary. As noted earlier, the mucous thickness and cell types of the rat trachea are similar to the mucous thickness and cell types in the human bronchioles (Mercer et al. 1991), suggesting many similarities in the targets of diacetyl-induced airway injury in rats and man. Future studies are planned to investigate whether the site of diacetyl-induced epithelial injury shifts where decreased nasal absorbance of diacetyl would be predicted in guinea pigs and in a rat model of mouth breathing.

Foci of denuded basement membrane identified in the nose, larynx, and trachea were frequently large and accompanied by fibrinous exudation. Repair of the airway epithelium has principally been studied after mechanical damage to the trachea (White 2003). The SEM images of the tracheal bifurcation of diacetyl-exposed rats show a loss of ciliated and secretory cells with flattening and loss of microvilli in remaining epithelial cells. These findings are reminiscent of the elongated and rounded epithelial cells seen at sites of mechanical damage to the tracheal epithelium (Gordon and Lane 1976; White 2003). Repair in the first day after mechanical damage to airway epithelium generally involves spreading and migration of epithelial cells resembling basal cells to cover exposed basement membrane, generally within six hours of injury (Gordon and Lane 1976). We saw ultrastructural changes consistent with epithelial spreading and migration. However, we also saw locally extensive areas of basement membrane which remained denuded and were sometimes associated with fibrin and cellular debris. The transmission electron microscopy images indicated that cell degeneration and cell death were still present in the tracheas from the highest exposure groups 18 hours after diacetyl exposures were discontinued.

The presence of dilated and whorled endoplasmic reticulum in the TEM images of diacetyl-exposed epithelial cells is particularly interesting. It raises the possibility of alterations in secreted proteins, because diacetyl causes protein cross-linking in vitro, and abnormal protein tertiary structure can cause accumulation of nascent proteins in the endoplasmic reticulum and trigger caspase activation (Kumar et al. 2005; Miller and Gerrard 2005). This finding deserves additional investigation to evaluate caspase activation and ultrastructural changes at lower diacetyl exposures. Irrespective of the mechanism of diacetyl-induced epithelial cell death, the extensive tissue destruction and the accompanying fibrinous exudation observed in the diacetyl-exposed epithelium are both important, because each of these findings is classically associated with the development of fibrosis (Kumar et al. 2005). Thus, this study of the inhalation toxicity of diacetyl is consistent with the conclusion that diacetyl is a respiratory hazard. In view of the somewhat greater damage to intrapulmonary airways noted in rats inhaling butter flavoring vapor mixtures (Hubbs et al. 2002), we cannot exclude the possibility that other vapors in butter flavoring contribute to the lung disease seen in workers exposed to butter flavoring.

Wheezing is a symptom commonly reported by popcorn workers (Kanwal et al. 2006). In vitro experiments suggest that

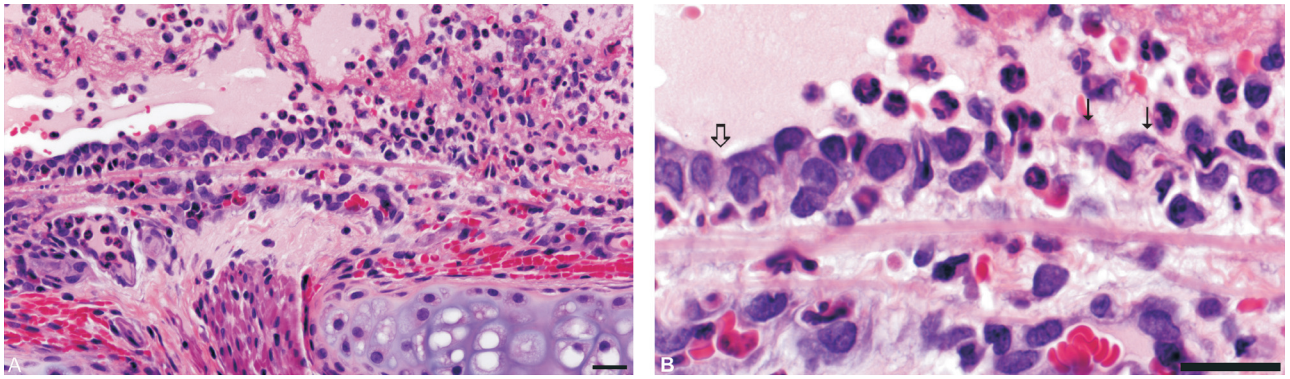

FIGURE 8.—Histopathology of the trachea in a rat inhaling 356 ppm diacetyl as a TWA delivered by a continuous six-hour exposure in the exposure pattern experiment (Experiment 2). (A) The epithelium is attenuated and mildly disorganized to absent. The lumenal surface contains abundant fibrin, cellular debris, and neutrophils. (B) A higher magnification of the epithelial junction in the trachea shown in A. On the left side of the photomicrograph, the epithelium is attenuated (open arrow), and neutrophils are infiltrating between and below epithelial cells. Recognizable epithelial cells are absent on the right side of the photomicrograph (solid arrows). H&E stain. Bar = 20 μm.

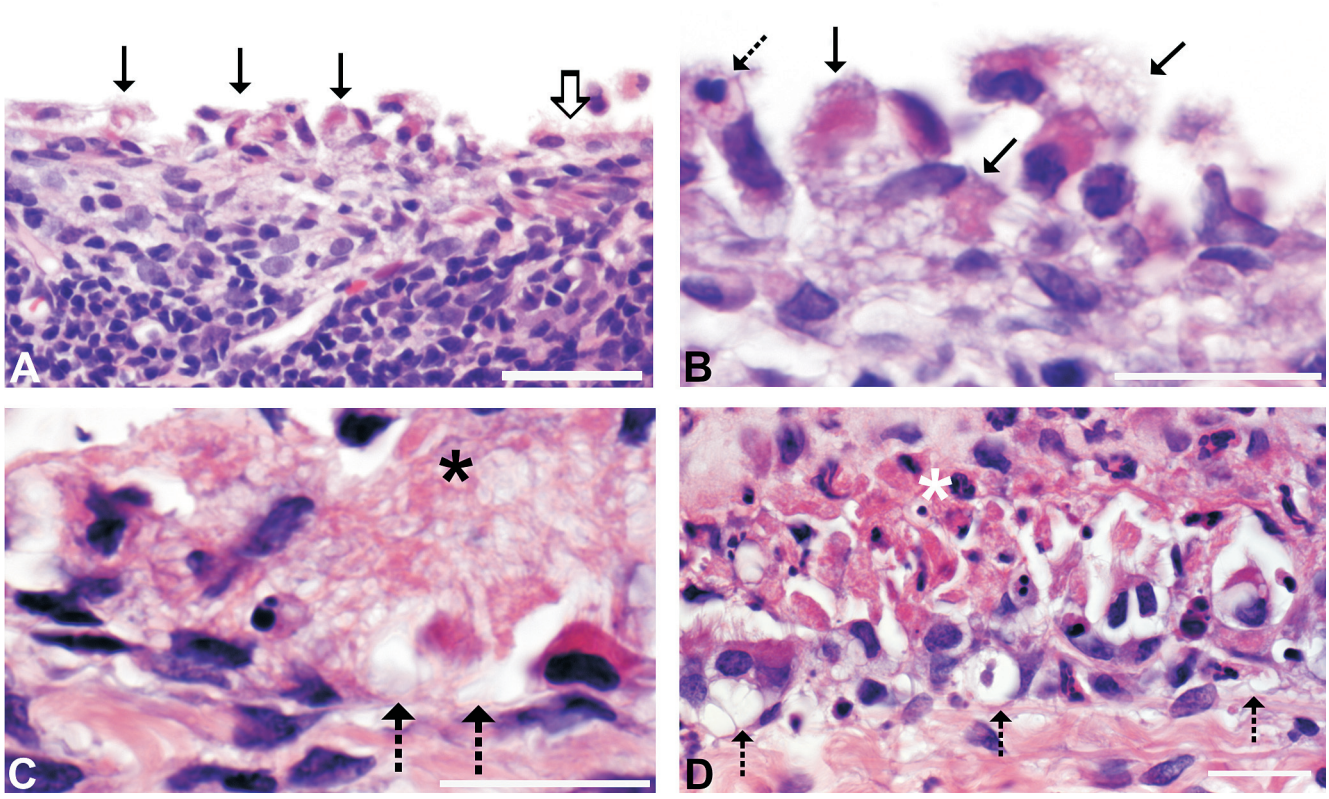

FIGURE 9.—Intrapulmonary airways of rats inhaling diacetyl. (A) The epithelium covering bronchus-associated lymphoid tissue focally changes from organized and attached (open arrow) to necrotic and detaching (solid arrows) in a rat inhaling 365 ppm diacetyl as a TWA delivered by four pulsed exposures over a six-hour period. (B) A higher magnification of the bronchus in A showing cytoplasmic vacuolation and eosinophilia (solid arrows) in the detaching epithelial cells. Nuclear changes include pyknosis (dashed arrow) and loss of visible nuclei. (C) Mainstem bronchus showing denuded basement membrane (dashed arrows), fibrin (*), and cellular debris in a rat inhaling 356 ppm diacetyl as a TWA delivered by a continuous six-hour exposure. (D) Necrosuppurative bronchitis in the mainstem bronchus with spaces above basement (dashed arrows) and abundant cellular debris (*). H&E stain. Bar = 20 μm.

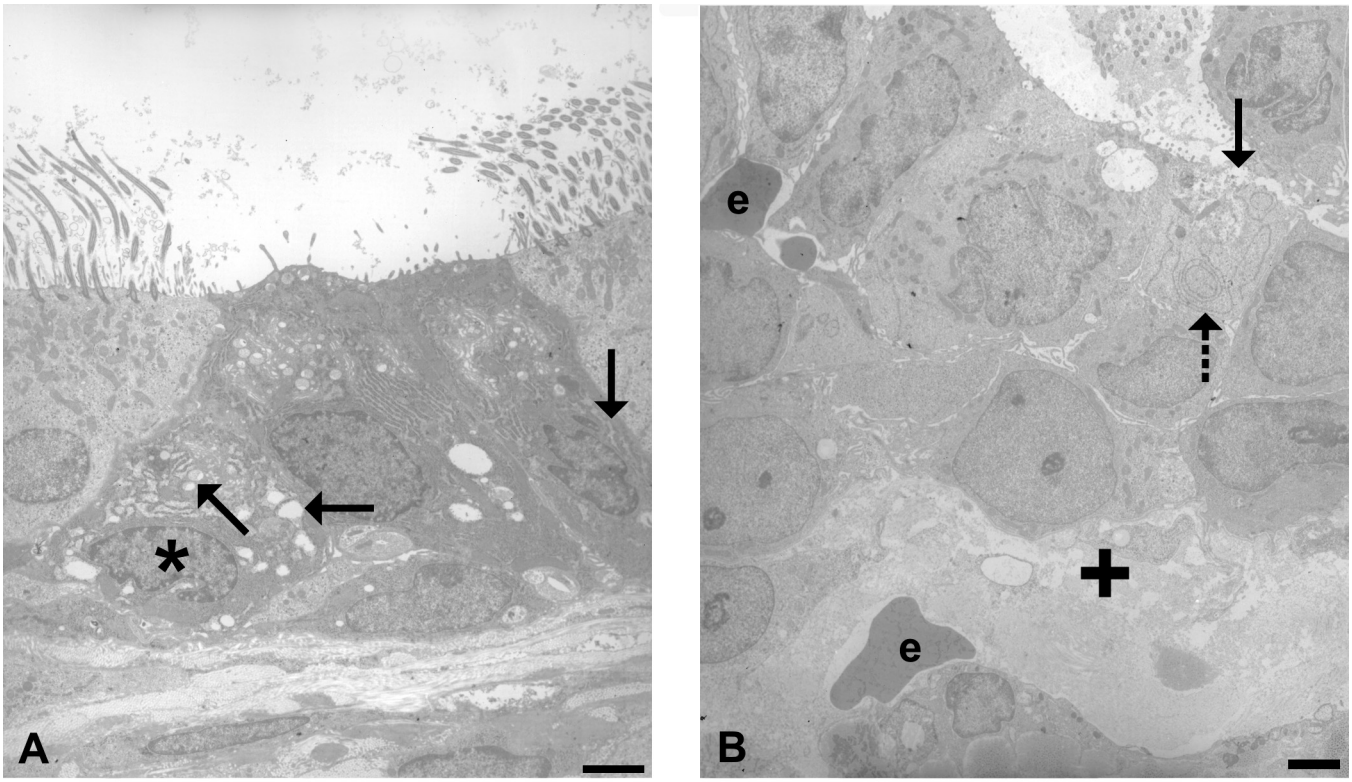

FIGURE 10.—Diacetyl-induced changes in the trachea in the exposure pattern experiment (Experiment 2): ultrastructural characterization of degeneration and necrosis of the tracheal epithelium of rats inhaling a TWA of 365 ppm diacetyl administered in four pulse exposures over six hours. (A) Degenerative changes in epithelial cells include dilation of the rough endoplasmic reticulum (arrows) and clumping of chromatin beneath the nuclear membrane(*). Bar = 2 μm. (B) Necrosis is characterized by loss of cell membrane integrity (solid arrow), which is accompanied in this cell by whorling of the rough endoplasmic reticulum (dashed arrow). Additional ultrastructural changes include edema of the lamina propria (+) and free erythrocytes (e) in the lamina propria and epithelial layer. Bar = 2 μm.

diacetyl can increase airway reactivity (Fedan et al. 2006). Diacetyl is also a sensitizing agent (Anderson et al. 2007; Roberts et al. 1999), certainly a concern given the wheezing reported by workers. Although fixed airways obstruction is the most distinguishing pulmonary function change noted in severely affected workers, some workers have a reversible component to their disease (Akpinar-Elci et al. 2004).

Thus, the role of diacetyl in Popcorn Workers' Lung (flavorings-related lung disease) is likely to include the damage to respiratory epithelium described in this report, since that injury is believed to play a role in bronchiolitis obliterans etiology, and epithelial damage clearly disrupts the barrier function of the epithelium as well as contributing to the development of airway reactivity (Fedan et al. 2006; King 1989). The effects of exposures to vapor mixtures containing diacetyl, chronic diacetyl exposure, the mechanisms of diacetyl-induced airway epithelial injury, and the role of diacetyl sensitization in airways obstruction in flavorings workers are areas of ongoing and future research.

In terms of our first hypothesis that *diacetyl vapors cause necrosis of airway epithelium,* both the diacetyl inhalation toxicity experiment and the diacetyl exposure pattern experiment demonstrate diacetyl-induced airway epithelial necrosis. This necrosis is concentration dependent and affects the nasal passageways, larynx, trachea, and the large intrapulmonary airways of rats inhaling diacetyl vapors. In terms of our second hypothesis that *peak diacetyl exposure concentration is a greater hazard than the time-weighted-average diacetyl exposure,* the diacetyl exposure pattern experiment did not support this hypothesis. However, when a single-pulse diacetyl exposure lasting slightly more than 15 minutes was used as the method for delivering a six-hour TWA of 92.9 ppm, the resulting significant changes in the nose indicated that the six-hour NOAEL for diacetyl must be below this concentration, whereas significant changes were not detected with four lower-concentration pulses that produced a TWA of 122 ppm or a continuous exposure pattern producing a TWA of 99.3 ppm. This suggests that for reactive vapors such as diacetyl, NOAELs that may be predicted with a continuous exposure rate should also be evaluated using single short-term, higher-level exposures that reproduce exposure patterns in the workplace. Our exposure pattern experiment indicates that the NOAEL for inhaled diacetyl is less than 92.9 ppm.

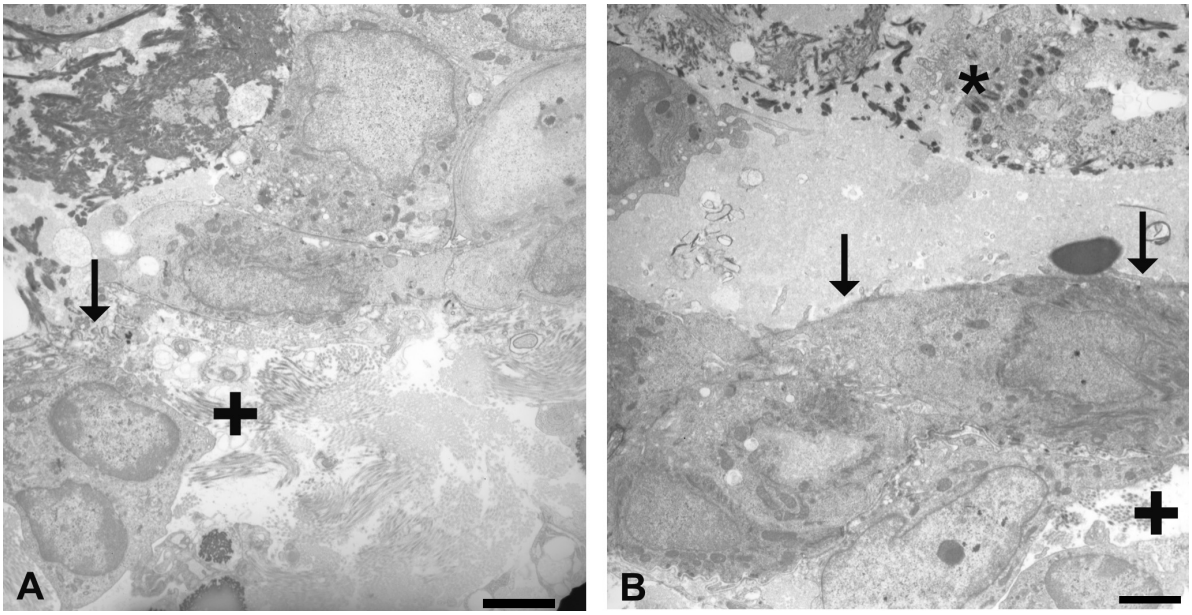

FIGURE 11.—Changes beneath the epithelium of the trachea in the exposure pattern experiment (Experiment 2): ultrastructural evidence of damage extending beneath the respiratory epithelium of the trachea in rats inhaling a TWA of 365 ppm diacetyl administered in four pulse exposures over six hours. (A) Denuding of basement membrane (arrow) and edema of the lamina propria (+). Bar = 2 μm. (B) The normal respiratory epithelium of the trachea has been replaced by an attenuated, simple epithelium (arrows), suggesting migration and spreading of epithelial cells to cover epithelial defects. The subjacent lamina propria is edematous (+). A fibrinonecrotic membrane (*) is above the attenuated epithelium. Bar = 2 μm.

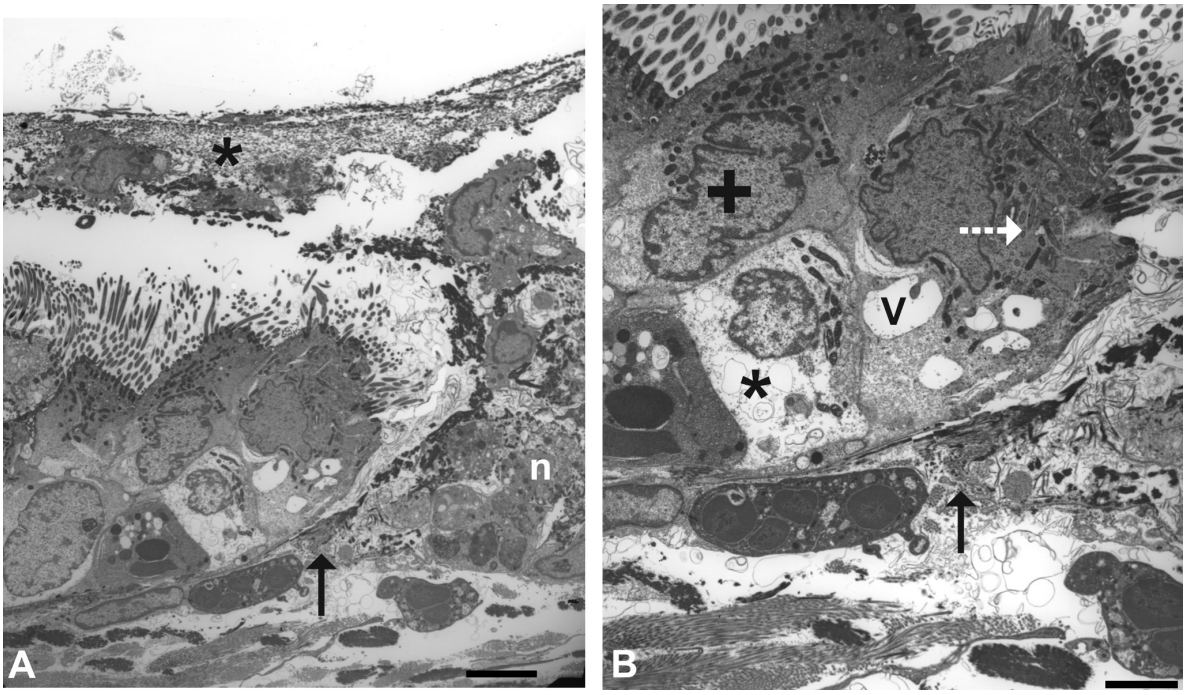

FIGURE 12.—Changes in a bronchus in the exposure pattern experiment (Experiment 2): ultrastructural changes in the right mainstem bronchus of a rat after inhaling 356 ppm diacetyl as a continuous exposure for six hours. (A) Ultrastructural changes in the bronchus include a fibrinonecrotic membrane (*), epithelial necrosis (n), and rupture of basement membrane (arrow) with edema and inflammation of the subjacent lamina propria (bar = 5 μm). (B) A higher magnification of the ruptured basement membrane (black arrow) and degenerative changes in epithelial cells, including vacuolation (v), internalization of cilia (dashed white arrow), cytoplasmic rarefaction (*), and condensation of chromatin beneath the nuclear membrane (+). Bar = 2 μm.

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