4 Toxicology of Diacetyl and 2,3-Pentanedione

4.1 Chemistry and Metabolism

Chemical and physical properties of diacetyl and 2,3-pentanedione are presented in section 1.4. As mentioned there, diacetyl is an α-dicarbonyl. Endogenous α-dicarbonyl compounds are among the reactive carbonyl species implicated in the formation of advanced glycation end products [Nakagawa et al. 2002a; Wondrak et al. 2002]. Like other α-dicarbonyl compounds, diacetyl is reactive, with a tendency to cause protein cross-links [Miller and Gerrard 2005]. The reactivity of the α-dicarbonyl compounds is enhanced by electron attracting groups and decreased by electron donors [Roberts et al. 1999]. Thus, diacetyl is a reactive compound, but its alkyl components are electron donors that may somewhat decrease the reactivity of the adjacent carbonyl groups [Roberts et al. 1999].

Diacetyl and related α-dicarbonyl compounds can inactivate proteins, principally through reactions with the amino acid, arginine [Epperly and Dekker 1989; Mathews et al. 2010; Saraiva et al. 2006]. The related α-dicarbonyl flavoring, 2,3-pentanedione, has been reported to be even more reactive with arginine groups than diacetyl [Epperly and Dekker 1989]. Recently, in vitro studies indicate that diacetyl can cause β-amyloid aggregates and covalently modify β-amyloid at 5th arginine (Arg^5), although the in vivo relevance of this finding remains to be investigated [More et al. 2012b]. Thus, existing studies indicate that diacetyl is a reactive compound that can modify proteins and suggest that diacetyl-associated protein modification may occur in vivo.

4.1.1 Diacetyl and 2,3-Pentanedione in Food

Diacetyl and 2,3-pentanedione have a long history as components of food, suggesting that exposures can occur in diverse workplaces. They occur as natural products in many foods [Jiang et al. 2013; Majcher and Jelen 2005; Majcher et al. 2013; Rincon-Delgadillo et al. 2012; Santos et al. 2013]. Diacetyl imparts the flavor and aroma of butter to many common foods and drinks including butter, cheese, yogurt, beer, and wine [Jang et al. 2013; Rincon-Delgadillo et al. 2012]. Diacetyl in food plays an important role in food preference [Liggett et al. 2008]. While less extensively studied in foods than diacetyl, 2,3-pentanedione is a common flavoring in margarine and vegetable spreads [Rincon-Delgadillo et al. 2012]. Roasted coffee also contains appreciable amounts of diacetyl [CDC 2013; Daglia et al. 2007a; Daglia et al. 2007b]. Because diacetyl is not a component of green coffee beans, it appears to be a product of the roasting process [Daglia et al. 2007a].

Bacteria and yeast produce diacetyl during fermentation [Chuang and Collins 1968]. It can be produced by metabolism of an acetaldehyde-thiamine pyrophosphate complex in the presence of acetyl-coenzyme A [Speckman and Collins 1968]. Microbes can also produce diacetyl from pyruvate in the presence of acetyl-coenzyme A [Chuang and Collins 1968].

Microbial culture conditions, such as pH, can influence the relative amount of diacetyl produced during fermentation [Garcia-Quintans et al. 2008; St-Gelais et al. 2009]. In addition, the steam distillate of several bacterial cultures grown on skim milk is known as "starter distillate" and is also considered a flavoring [FASEB 1980; FDA 1983]. Major components of some starter distillate samples include diacetyl and acetic acid [FASEB 1980; Rincon-Delgadillo et al. 2012]. A recent study demonstrated that diacetyl remains a frequent component of commercial starter distillate samples, and diacetyl concentrations exceeded 20 mg/g in one sample [Rincon-Delgadillo et al. 2012]. Starter distillate can also contain 2,3-pentanedione as well as butyric acid, which inhibits the metabolism of diacetyl and 2,3-pentanedione [Morris and Hubbs 2009; Nakagawa et al. 2002a; Rincon-Delgadillo et al. 2012]. Thus, diacetyl and 2,3-penanedione can occur naturally in food, may be added to food as flavorings, may be produced during the roasting process, and can be anticipated components when starter distillate is added as a flavoring.

4.1.2 Metabolism in Mammalian Cells

In the rat and hamster liver, diacetyl is metabolized principally by reduction to acetoin in an enzymatic reaction catalyzed by dicarbonyl/L-xylulose reductase (DCXR), the enzyme is also known as diacetyl reductase, with either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as coenzymes [Nakagawa et al. 2002a; Otsuka et al. 1996; Sawada et al. 1985]. Acetoin can be further reduced to 2,3-butanediol in an NADH-dependent manner [Otsuka et al. 1996]. This diacetyl reductase activity is higher in the liver than in the kidney, and kidney activity is higher than in the brain. However, after oral administration of diacetyl, the levels of acetoin are much higher in the brain than in the kidney or liver. 2,3-Butanediol accumulates in liver, kidney, and brain after the administration of diacetyl, acetoin, or 2,3-butanediol. Oral administration of acetoin and, to a lesser extent, 2,3-butanediol, in rats also causes diacetyl accumulation in the liver and brain [Otsuka et al. 1996]. However, liver homogenates do not produce significant diacetyl from acetoin or 2,3-butanediol [Otsuka et al. 1996]. Thus, the metabolic interconversion of the 4-carbon compounds, acetoin, diacetyl, and 2,3-butanediol occurs in mammalian systems in vivo and in vitro but the full spectrum of metabolic pathways remains incompletely investigated.

As mentioned above, in mammalian cells, the predominant metabolic pathway for diacetyl is catalyzed by DCXR, a tetrameric protein that is comprised of four subunits, each 244 amino acids long [El-Kabbani et al. 2005; Ishikura et al. 2001; Nakagawa et al. 2002a; Sawada et al. 1985]. In addition to the reductive metabolism of diacetyl, DCXR catalyzes the metabolism of several other dicarbonyl compounds, including 2,3-pentanedione, 2,3-hexanedione, 2,3-heptanedione, and 3,4-hexanedione [Nakagawa et al. 2002a]. In addition, DCXR catalyzes the reductive metabolism of a number of monosaccharides, including L-xylulose, and plays a role in the glucuronic acid/uronate cycle of glucose metabolism as well as the metabolism of carbonyl compounds [Carbone et al. 2005; El-Kabbani et al. 2005; Nakagawa et al. 2002a]. Inhibitors of DCXR are well described and include *n*-butyric acid, 2-furoic acid, benzoic acid, and nicotinic acid [Carbone et al. 2005; Nakagawa et al. 2002a]. At least one of these DCXR inhibitors, n-butyric acid, is well absorbed in the nose, and its presence in vapor mixtures causes small but significant decreases in diacetyl absorption in the nasal mucosa and, thereby, leaves more diacetyl in the vapor stream of the nasal airways for delivery to the lung [Morris and Hubbs 2009].

Table 4-1. Experimental respiratory toxicology studies

Reference	Test subject	Exposure	Effects of exposure
Fedan et al. [2006]	In vitro preparations of guinea pig trachea	Diacetyl 1 mM 3 mM 10 mM 30 mM	Direct effect of diacetyl: Very weak tracheal contractions with threshold at 1 mM, relaxation at exposures above 3 mM. Diacetyl effect on response to intraluminal (mucosal) methacholine: 4-hour perfusion with 3 mM diacetyl increased methacholine reactivity 10 times; 10 mM diacetyl completely inhibited the methacholine response. Depolarization of transepithelial potential difference at 3 and 10 mM. Decrease in transepithelial potential difference at 10 mM.
Gloede et al. [2011]	F344 rats	Respiratory uptake of diacetyl in F344 rats was used to validate a model of respiratory tract uptake of diacetyl	At a given inhaled diacetyl dose, the predicted dose to the bronchiolar epithelium of a lightly exercising employee is predicted to be more than 40 times the dose to the bronchiole of a rat. Describes a low affinity, high capacity and a high affinity, low capacity pathway for diacetyl metabolism in the rat respiratory epithelium. The high affinity pathway is inhibited by sodium benzoate, indicating that it is DCXR.
[Goravanahally et al. 2014]	Male Sprague-Daley Rats	6-hr diacetyl inhalation with sacrifice 18–20 hr post-exposure 0 ppm 25 ppm 249 ppm 346 ppm	Diacetyl increased substance P positive neurons in the jugular ganglia in a dose-dependent manner. PGP9.5 nerve fiber density was decreased in foci with denuded tracheal epithelium after inhaling 249 or 346 ppm diacetyl. Substance P-positive nerve fiber density was increased in foci of intact epithelium adjacent to denuded epithelium.
Hubbs et al. [2002]	Male Sprague-Dawley rats	Control (n=16) 0 ppm Diacetyl exposures were to vapors of a complex mixture of diacetyl-containing butter flavoring Exposure for 6 hr: Low constant exposure (n=6) 203 ppm of the diacetyl component	Airway epithelial damage with necrosis and inflammation in nose

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Table 4-1 (Continued). Experimental respiratory toxicology studies

Reference	Test subject	Exposure	Effects of exposure
		Middle constant exposure (n=3) 285 ppm of the diacetyl component	Airway epithelial damage with necrosis and inflammation in nose and lungs (bronchi)
		High constant exposure (n=3) 352 ppm of the diacetyl component	Airway epithelial damage with necrosis and inflammation in nose and lungs (bronchi)
		High pulsed exposure (n=3) 371 ppm (range 72–940) of the diacetyl component	Airway epithelial damage with necrosis and inflammation in nose and lungs (bronchi and bronchioles)
Hubbs et al. [2008]	Male Sprague-Dawley rats	Control (n=18) 0 ppm Diacetyl inhalation for 6 hr:	
		99.3 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in the nose (1/6)
		198.4 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (6/6)
		294.6 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (6/6) and bronchi (2/6)
		Four ~15 min diacetyl inhalation pulses in 6-hr (TWA):	
		122 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (2/6)
		225 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (6/6) and trachea (2/6)
		365 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in the nose (6/6), trachea (6/6) and bronchi (6/6)
		Continuous diacetyl inhalation exposure for 6 hr to match pulse TWA:	
		120 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (5/6) and bronchus (1/6)
		224 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (6/6), trachea (5/6) and bronchus (1/6)
		356 ppm (n=6)	Airway epithelial damage with necrosis and inflammation in nose (6/6), trachea (6/6) and bronchi

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Table 4-1 (Continued). Experimental respiratory toxicology studies

Reference	Test subject	Exposure	Effects of exposure
		Single ~15 min pulse exposure 1949 ppm (92.9 6 hr TWA) (n=6)	Airway epithelial damage with necrosis and/or inflammation in nose (3/6)
Hubbs et al. [2012]	Male Sprague-Dawley rats	6-hr inhalation exposures to air, 2,3-pentanedione (PD) or diacetyl; sacrifice 18–20 hr after exposure.	
		Six exposure groups: Air 111.7 ± 0.12 ppm PD 241.2 ± 0.15 ppm PD 318.4 ± 0.17 ppm PD 354.2 ± 0.20 ppm PD 240.3 ± 0.26 ppm diacetyl	Apoptosis and necrosis of respiratory and transitional epithelium of the nose in all 2,3-pentanedione- and diacetyl-exposed rats; necrosis of olfactory neuroepithelium and necrotizing tracheitis in diacetyl exposed rats and rats inhaling ≥ 241.2 ppm 2,3-pentanedione; necrotizing bronchitis in rats inhaling ≥ 318.4 ppm 2,3-pentanedione
		6-hr inhalation exposures to air or 319 (317.9 to 318.9) ppm PD. Air exposed rats sacrificed at 18 hr post-exposure; 2,3-pentanedione exposed rats were sacrificed at 3 different post-exposure time points: 0–2 hr post-exposure 12–14 hr post-exposure 18–20 hr post-exposure	In the proximal nose, severity of damage increased between the 0–2 hr post-exposure and 12–14 hr post-exposure, suggesting delayed toxicity; disorganization of the neuroepithelium with a loss of the organized expression of DCXR at the air/olfactory neuroepithelium interface; activation of caspase-3 in olfactory axons
Larsen et al. [2009]	Male BALB/cJ mice	Diacetyl inhalation exposures to 191, 334, 79, or 1154 ppm	Pulmonary irritation at 790 and 1154 ppm, decrease in respiratory rate, increase in "time of break"
		Challenge after acute exposure 555 ppm	Higher diacetyl concentrations at the acute exposure reduced sensitivity to challenge exposure, lower acute exposure increased sensitivity; challenge exposure did not alter lung inflammation
Morgan et al. [2008]	Male C57BI/6 mice	Subacute diacetyl inhalation, whole body 6 hr/day, 5 days	
		0 ppm	
		200 ppm	Necrosis and inflammation in mucosa of the nose and larynx
		400 ppm	Necrosis and inflammation in the mucosa of the nose, larynx and bronchi

See footnotes at end of table.

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Table 4-1 (Continued). Experimental respiratory toxicology studies

Reference	Test subject	Exposure	Effects of exposure
		Intermittent diacetyl inhalation, nose only	
		0, 100, 200, or 400 ppm 1 hr/day, 5 days/week, 2 weeks	Chronic-active inflammation in the nose of all diacetyl exposed mice (100, 200 and 400 ppm), squamous metaplasia of respiratory epithelium of the nose (1/5 at both 100 and 200 ppm); 4/4 at 400 ppm; necrosis and ulceration of respiratory epithelium of the nose at 400 ppm (3/4); atrophy of olfactory epithelium of the nose (1/4 at 100 ppm and 2/2 at 400 ppm; lymphocyte infiltrates around bronchi in the lung (4/5 at 100 ppm; 5/5 at 200 and 400 ppm)
		0, 100, 200, or 400 ppm 1 hr/day, 5 days/week, 4 weeks	Chronic-active inflammation in the nose (2/5 at 100 ppm; 5/5 at 200 and 400 ppm); squamous metaplasia of the respiratory epithelium of the nose (1/5 at 100 ppm; 5/5 at 200 and 400 ppm). Atrophy of olfactory epithelium (1/5 at 200 ppm; 5/5 at 400 ppm). Lymphocytic infiltrates around bronchi (2/3 at 100 ppm; 5/5 at 200 and 400 ppm).
		0 or 1200 ppm 15 min twice a day, 5 days/week, 4 weeks	Chronic-active inflammation in the nose (5/5); necrosis and ulceration of the respiratory epithelium of the nose (3/5); squamous metaplasia of the respiratory epithelium of the nose (5/5); atrophy of the olfactory epithelium (4/5); both peribronchial and peribronchiolar lymphocytic infiltrates (5/5)
		Subchronic inhalation, whole body	
		0, 25, 50, or 100 ppm 6 hr/day, 5 days/week, 6 weeks	Necrosis and ulceration of the respiratory epithelium of the nose (2/5 at 50 ppm and 5/5 at 100 ppm); squamous metaplasia of the respiratory epithelium of the nose (1/4 at 25 ppm; 3/5 at 50 ppm; 5/5 at 100 ppm); atrophy of the olfactory epithelium (3/5 at 50 and 1/5 at 100 ppm); peribronchial lymphocytic inflammation (3/5 at 25 ppm; 5/5 at 50 ppm; 5/5 at 100 ppm); denudation and atrophy of bronchial epithelium (5/5 at 100 ppm).

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Table 4-1 (Continued). Experimental respiratory toxicology studies

Reference	Test subject	Exposure	Effects of exposure
		0, 25, 50, or 100 ppm 6 hr/day, 5 days/week, 12 weeks	Necrosis and ulceration of the respiratory epithelium of the nose (1/5 at 50 ppm and 5/5 at 100 ppm); squamous metaplasia of the respiratory epithelium of the nose (2/5 at 25 ppm; 4/5 at 50 ppm; 5/5 at 100 ppm); atrophy of the olfactory epithelium (5/5 at 100 ppm); peribronchial lymphocytic infiltrates (2/5 at 25 ppm; 4/5 at 50 ppm; 5/5 at 100 ppm); denudation and attenuation of bronchial epithelium (5/5 at 100 ppm)
		Oropharyngeal aspiration of diacetyl 0, 100, 200, or 400 mg/kg, single dose 4 days post-aspiration	Foci of fibrohistiocytic proliferation with little or no inflammation present at the junction of the terminal bronchioles and alveolar ducts (400 mg/kg)
Morgan et al. [2012]	Male and female Wistar-Han rats and B6C3F1 mice	2,3-Pentanedione 0, 50, 100 or 200 ppm 6 hr/day, 5 days/week, Up to 12 exposures	In rats, 12-day 2,3-pentanedione exposure cause necrosis of respiratory epithelium and atrophy of olfactory neuroepithelium in the nose; intramural and intraluminal fibrosis of intrapulmonary airways. In rats, 5 or 10 days of inhaling 200 ppm 2,3-pentanedione increased bronchoalveolar lavage fluid concentrations of monocyte chemotactic protein-1, monocyte chemotactic protein-3, C-reactive protein, fibroblast growth factor-9, and fibrinogen. In mice, 12-day 2,3-pentanedione exposure caused necrosis of nasal turbinates, suppurative exudate and atrophy of olfactory neuroepithelium in the nose; inflammation in intrapulmonary airways with necrosis, ulceration and regeneration at 200 ppm exposure.
Morris and Hubbs [2009]	Male Sprague-Dawley rats	Diacetyl 100 or 300 ppm in airstream flows of 100–400 mL/min	A hybrid computational fluid dynamic-physiologically based pharmacokinetic model (CFD-PBPK) was used to extrapolate diacetyl and butyric acid uptake in rat airways epithelium to human airways epithelium. The CFD-PBPK suggests that nasal injury in rats can predict a risk to deep lung tissue in humans. Diacetyl damages airway epithelium when it reaches a critical concentration in the target cells; the CFD-PBPK indicates that more diacetyl will be absorbed in the nose of rats than in humans. Butyric acid is shown to shift diacetyl absorption from the nose and trachea into deeper lung tissue.

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Table 4-1 (Continued). Experimental respiratory toxicology studies

Reference	Test subject	Exposure	Effects of exposure
Palmer et al. [2011]	Male Sprague-Dawley Rats	200 µl of a 188 mg/mL diacetyl solution by intratracheal instillation (125 mg/kg)	Initial airway epithelial injury was followed by the development of obliterative bronchiolitis
Zaccone et al. [2013]	In vitro preparation of rat trachea after exposure of animals	Diacetyl 6-hr inhalation exposures to 100, 200, 300, or 360 ppm (n=6–11)	Small increase in basal airway compliance at 360 ppm Increase in reactivity at 320 and 360 ppm
		2,3-Pentanedione 6-hr inhalation exposures to 120, 240, 320, or 360 ppm (n=5–8)	No effect on basal airway resistance or compliance Increase in reactivity at 320 and 360 ppm
	In vivo exposure of male Sprague-Dawley rats	Diacetyl 6-hr inhalation exposures to 100, 200, 300, or 360 ppm	Decrease in reactivity at 360 ppm
		2,3-Pentanedione 6-hr inhalation exposures to 120, 240, 320, or 360 ppm (n=4–10)	Decrease in reactivity at 240 and 320 ppm

In the rat kidney, DCXR is localized in the distal tubules and collecting ducts and colocalizes with carboxylmethyllysine, an advanced glycation end product [Nakagawa et al. 2002a]. In the mouse kidney, DCXR is localized to the brush border of the proximal renal tubules [Nakagawa et al. 2002a]. In the human prostate epithelial cells and in normal human skin, DCXR is associated with the cell membrane [Cho-Vega et al. 2007a, b; Cho-Vega et al. 2007b]. In human skin, DCXR is located near the adhesion molecules, e-cadherin and β-catenin [Cho-Vega et al. 2007b]. Similarly, in the vascular endothelium in the dermis, DCXR localizes near intercellular junctions, suggesting a potential role for DCXR in cell adhesion [Cho-Vega et al. 2007b]. In addition, DCXR activity is present in the respiratory mucosa of the rat, with the highest activity in the olfactory epithelium [Morris and Hubbs 2009]. DCXR knockout mice are not well characterized phenotypically but are reported to be fertile [Nakagawa et al. 2002b]. People without DCXR excrete pentose in their urine but are otherwise believed to be healthy, indicating that DCXR and the major diacetyl metabolic pathway are not essential for life [Flynn 1955; Lane and Jenkins 1985]. Importantly, the metabolism of diacetyl is not exclusively by DCXR. For example, aldo-keto reductase 1C15 is a newly-described aldo-keto reductase expressed in rat lung that can metabolize α-diketones [Endo et al. 2007]. Recently, a low affinity, high capacity and a high affinity, low capacity pathway for diacetyl metabolism have been described in the respiratory tract of the rat [Gloede et al. 2011]. The high affinity pathway was inhibited by sodium benzoate indicating that it is DCXR. The low affinity pathway is not believed to play a major role at diacetyl concentrations associated with most occupational exposures.

4.2 In Vivo and in Vitro Toxicology Studies

Diacetyl and 2,3-pentanedione may be consumed in food, the vapors may be inhaled, and direct skin contact is possible. In vivo studies have modeled these routes of human exposure.

Table 4-1 summarizes key respiratory toxicology findings for diacetyl and 2,3-pentanedione through 2013. Studies of acute oral toxicity have used gavage exposures in rats. Based upon gavage administration of a 20% diacetyl solution in water, the LD_{50} for a single oral dose of diacetyl is estimated to be 3 g/kg in female rats and 3.4 g/kg in male rats [Colley et al. 1969].

4.2.1 In Vivo Toxicology of Orally Administered Diacetyl

Subchronic (90-day) gavage administration of 540 mg diacetyl/kg/day caused multiple changes in exposed rats, including decreased body weight, increased water consumption, increased adrenal weight, increased relative kidney and liver weights (in females absolute kidney and liver weights were also increased), decreased blood hemoglobin concentration and gastric ulceration [Colley et al. 1969]. No adverse effects were noted at the next highest dose level, which was 90 mg/kg/day. On a mg/kg basis, the 90 mg/kg/level was estimated to be roughly 500-fold greater than the estimated human maximum daily intake of diacetyl from foods consumed at that time, with 50 ppm diacetyl being the highest estimated concentration in any food [Colley et al. 1969].

4.2.2 Effects of Topically Applied Diacetyl and 2,3-Pentanedione in Vivo

Sensitization following topical application of diacetyl is predicted on the basis of results of a murine local lymph node assay [Anderson et al. 2013; Anderson et al. 2007; Roberts et al. 1999].

On the basis of the results of the local lymph node assay and immune cell phenotyping, it is suggested that diacetyl and 2,3-pentanedione function as T-cell mediated chemical sensitizers [Anderson et al. 2013]. Cutaneous sensitization by diacetyl and 2,3-pentanedione may be initiated through haptenation with proteins containing the amino acids lysine and arginine [Roberts et al. 1999]. Diacetyl is corrosive to the cornea of the eye using the Draise test [Sugai et al. 1990].

4.2.3 Toxicology of Inhaled Diacetyl in Vivo

In rats, acute exposures to diacetyl or diacetyl-containing butter flavoring vapors cause necrosis in the epithelial lining of nasal and pulmonary airways. Rats inhaling vapors of butter flavoring that contained diacetyl developed multifocal necrotizing bronchitis one day after a 6-hour exposure. The mainstem bronchus was the most affected intrapulmonary airway. However, nasal airways were more affected than intrapulmonary airways. Necrosuppurative rhinitis was seen in rats inhaling butter flavoring vapors at concentrations of butter flavoring that did not cause damage in intrapulmonary airways [Hubbs et al. 2002]. As a single agent acute exposure in rats, a 6-hour diacetyl inhalation exposure caused epithelial necrosis and inflammation in bronchi at concentrations of ≥ 294.6 ppm and caused epithelial necrosis and inflammation in the trachea and larynx at concentrations of ≥224 ppm [Hubbs et al. 2008]. The airway epithelial damage in rats inhaling 356 ppm was remarkable, with an average pathology score of 9.5 on a 10-point scale in the nasopharyngeal duct and larynx, while damage in the tracheal epithelium averaged 8.7 on a 10-point scale. In a pattern reminiscent of airway damage from butter flavoring vapors, diacetyl causes greater damage to nasal airways than to intrapulmonary airways [Hubbs et al. 2008]. The data from the National Toxicology Program 90-day inhalation study are available online and was used for the NIOSH animal-based risk assessment (see Chapter 6). Airway damage in mice one day after diacetyl inhalation was found to correlate with markers of increased protein turnover, implicating protein damage in the etiology of diacetyl-induced airway damage [Hubbs et al. 2016].

Eighteen hours after a 6-hour exposure to inhaled diacetyl (100, 200, 300, or 360 ppm), in anesthetized rats 360 ppm elevated slightly lung resistance and dynamic compliance [Zaccone et al. 2013]. Subsequent inhalation of methacholine aerosol (0.3–10 mg/mL) revealed that airway reactivity was decreased after exposure to diacetyl at 360 ppm. It had been predicted, based on extensive epithelial damage noted after diacetyl inhalation, that reactivity to inhaled methacholine would be increased. Eighteen hours after a 6-hour exposure to inhaled 2,3-pentanedione (120, 240, 300, or 360 ppm), in anesthetized rats basal lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were not affected.

Following inhalation of 346 ppm diacetyl by rats, foci in the trachea that demonstrate epithelial denudation also appear to have loss of sensory nerves, as reflected in a decreased density of PGP9.5-immunoreactive nerve fibers. However, in the epithelium adjacent to denuded foci, increased numbers of nerve fibers contain immunoreactive substance P, a neuropeptide important in neurogenic airway inflammation. In addition, the number of substance P immunoreactive neurons increase in the ganglia supplying the trachea in a dose-dependent manner in exposed rats. These findings suggest that diacetyl-induced airway epithelial damage may be accompanied by changes in the sensory nerves [Goravanahally et al. 2014]. In mice, inhaling diacetyl at concentrations of 200 or 400 ppm for 6 hours/day for up to 5 days causes respiratory tract changes similar to those seen in rats inhaling diacetyl or butter flavoring vapors [Morgan et al. 2008]. At both 200 and 400 ppm, diacetyl caused necrotizing rhinitis in mice that

was most prominent in the front portion of the nose. At 400, but not at 200 ppm, the olfactory epithelium demonstrated vacuolar degeneration and apoptosis. Necrotizing laryngitis was consistently observed in all mice inhaling 400 ppm diacetyl, while only one mouse inhaling 200 ppm diacetyl had comparable necrotizing laryngitis, but erosive laryngitis was present in 9 of 10 mice inhaling the 200 ppm concentration.

Exposing mice to diacetyl for only 1 hour/day at 100, 200, or 400 ppm diacetyl, 5 days per week, for 2 to 4 weeks rather than 6 hours/day for the same number of days eliminated epithelial necrosis in mice inhaling 200 ppm diacetyl and decreased the severity of epithelial necrosis in mice inhaling 400 ppm diacetyl. Lymphocytic inflammation was seen around the bronchi in some mice inhaling 100 ppm and in all mice inhaling 200 or 400 ppm diacetyl [Morgan et al. 2008]. Exposing mice to diacetyl for 15 minutes per day at 1,200 ppm diacetyl, 5 days/week for 2 weeks also caused lymphocytic infiltrates around bronchi, and lymphocytic infiltrates extended deeper into the lung, reaching the level of the preterminal bronchioles [Morgan et al. 2008].

Subchronic, 12-week, diacetyl inhalation for 6 hours/day, 5 days/week caused significant histopathologic changes in mice at all concentrations studied. Peribronchial lymphocytic infiltrates were seen at terminal sacrifice at 12 weeks in all subchronically-exposed mice inhaling 100 ppm diacetyl and in some mice inhaling 25 or 50 ppm diacetyl. In mice inhaling 100 ppm diacetyl, bronchial epithelial changes included denudation, attenuation, and hyperplasia [Morgan et al. 2008]. Chronic active nasal inflammation was seen in all mice inhaling 50 or 100 ppm and in four of five mice inhaling 25 ppm diacetyl for 12 weeks, an exposure that also caused minimal to mild lymphocytic bronchitis in two of five mice. This suggests that the no observable adverse effect level in mice for subchronic inhalation may be less than 25 ppm diacetyl.

Butyric acid caused a small but significant reduction in nasal uptake of diacetyl in the rat nose, and, thereby, increased the diacetyl exposure to the lung due to a reduced "scrubbing" effect [Morris and Hubbs 2009].

Oropharyngeal aspiration permits exposures that bypass the rodent nose and, hence, scrubbing at that site [Foster et al. 2001; Rao et al. 2003]. A single aspiration exposure to 400 mg/kg diacetyl produced a fibrohistiocytic response at the bronchioloalveolar junction of mice after 4 days. While oropharyngeal aspiration of diacetyl delivers a high bolus dose of diacetyl, the unusual fibrohistiocytic response could suggest that the smallest airways may be particularly susceptible to diacetyl-induced epithelial injury and fibrosis [Morgan et al. 2008]. A subsequent study demonstrates development of obliterative bronchiolitis in rats after intratracheal instillation of diacetyl [Palmer et al. 2011]. In this model, diacetyl-induced obliterative bronchiolitis was associated with abnormal repair of the injured bronchiolar epithelium. The reports of the induction of obliterative bronchiolitis and obliterative bronchiolitis-like changes in the deep lung of laboratory animals following aspiration of diacetyl are important because no prior animal model of obliterative bronchiolitis existed, and it is a technique that bypasses the rodent nose, which CFD-PBK models have demonstrated to absorb more diacetyl than will be absorbed in the upper airways of employees (section 4.2.6). However, as noted in the study, the very large single dose used in these studies may have limitations for the use of single exposure intratracheal instillations for risk assessment purposes [Palmer et al. 2011]. A study demonstrated bronchial fibrosis in rats inhaling 150 or 200 ppm diacetyl for 2 weeks [Morgan et al. 2016].

Pulmonary function changes have been investigated in mice after acute or subchronic diacetyl exposure. In mice, acute 2-hour diacetyl inhalation at concentrations from 191 to 1154 ppm

caused a decrease in respiratory rate and an increase in the "time of break" between inhalation and exhalation, an indicator of sensory irritation [Larsen et al. 2009]. In addition, acute diacetyl inhalation in mice caused decreases in tidal volume and mid-expiratory flow rate. Mice previously exposed to high diacetyl concentrations were *less* sensitive to the sensory irritation effects of a diacetyl challenge exposure, while mice previously exposed to low diacetyl concentrations were *more* sensitive to a diacetyl challenge exposure. Extrapolation of the mouse dose-response relationship to humans suggested no sensory irritation to warn employees during acute diacetyl exposures at concentrations less than 20 ppm [Larsen et al. 2009]. As mentioned earlier, a recent study suggests that acute diacetyl inhalation exposures can actually increase the number of substance P-positive neurons in the jugular ganglia and the number of nerve fibers containing substance P in the epithelium adjacent to sites of epithelial damage, but decreases the sensory innervation at the actual sites of greatest epithelial damage [Goravanahally et al. 2014]. As a group, these studies suggest dysregulation of airway sensory innervation and responses. Additional studies support the potential for diacetyl to alter pulmonary function in exposed rodents. Mice inhaling 100 ppm diacetyl for 12 weeks had concentration-dependent decreases in respiratory rate and minute volume after 3 and 6 weeks of exposure; mice inhaling 50 ppm diacetyl had decreased respiratory rates after 6 weeks exposure, but pulmonary function improved with time with continued exposure at these concentrations [Morgan et al. 2008]. However, after 18 weeks of exposure, respiratory rates in mice inhaling 25 ppm diacetyl were significantly lower than in controls [Morgan et al. 2008].

The effects of diacetyl inhalation may not be limited to the respiratory tract. Inhaling 2,500 ppm diacetyl for 45 minutes increased 2-deoxyglucose uptake in foci in the posterior portion of the rat brain olfactory bulb [Johnson et al. 2007]. While this finding has generally been interpreted as being related to olfaction [Johnson et al. 2007], the potential exists for toxicity to olfactory neurons that radiate into the olfactory bulb. Phagocytosis of olfactory nerve material and increases in Tnfα mRNA were recently demonstrated in the olfactory bulb of mice one day after a 6-hr diacetyl inhalation exposure. By immunofluorescence, the multifunctional scaffolding protein sequestosome-1 accumulated in the olfactory bulb of these mice and often congregated in the microglial cells that contained phagocytized olfactory neuronal material [Hubbs et al. 2016].

Although powdered butter flavoring can produce fewer vapors than liquid butter flavorings, the powders have a major respirable component [Boylstein et al. 2006; Rigler and Longo 2010]. If powdered butter flavorings are substituted for liquid butter flavorings, diacetyl and 2,3-pentanedione vapor concentrations may well be below exposure limits. In particular, encapsulated flavoring powders are designed to contain diacetyl or 2,3-pentanedione vapors. However, inhalable particulates can be deposited in the nose, the conducting airways, and deep lung. No peer reviewed studies have investigated the potential for encapsulated flavorings to release diacetyl and/or 2,3-pentanedione directly to the target cells lining airways. However, a recent study indicates that more than a quarter of particulates in flavoring powders are less than 10 μm in diameter. Therefore, powders have the potential to reach the intrapulmonary airways [Rigler and Longo 2010].

4.2.4 In Vitro Toxicology of Diacetyl and 2,3-Pentanedione

Diacetyl is mutagenic in the *Salmonella typhimurium* tester strains TA100 and TA104 [Kim et al. 1987; Marnett et al. 1985]. However, diacetyl also reacts with mutagenic heterocyclic amines and suppresses the mutagenicity of

heterocyclic amines in *Salmonella typhimurium* tester strain TA98. Diacetyl also enhances chromosome loss by proprionitrile in *Saccharomyces cerevisiae*. Recently, diacetyl in the presence of human S9 demonstrated a high degree of mutagenicity in a mouse lymphoma mutation assay [Whittaker et al. 2008]. Consistent with these findings, recent in vitro studies of direct interactions between diacetyl and single-stranded oligonucleotides under acellular conditions indicate that diacetyl can form adducts with 2-deoxyguanosine [More et al. 2012a]. Additional studies on the genotoxicity of diacetyl have been reviewed in the background documents available online as part of the National Toxicology Program [National Toxicology Program 2007].

In isolated mitochondria, diacetyl closes the mitochondrial permeability transition pore and renders it insensitive to Ca^{2+} [Eriksson et al. 1998]. This effect of diacetyl occurs at concentrations that could occur in tissues of diacetyl-exposed individuals, with half-maximal inhibition of the mitochondrial transition reported to be at a diacetyl concentration of 1 mM [Eriksson et al. 1998]. This concentration has been shown to have pharmacological activity in airways and has been modeled to be achieved in the airway wall after inhalation (see below). The effect of diacetyl on the mitochondrial permeability transition pore appears to be caused by arginine modification (see above) [Eriksson et al. 1998]. In addition, diacetyl can be metabolized by pig heart mitochondrial pyruvate kinase to form acetate and acetyl-CoA with a K_m value of 0.46 mM. Diacetyl is also a competitive inhibitor of pyruvate metabolism by pyruvate dehydrogenase with a K_i of 0.43 mM [Sumegi et al. 1982].

The isolated, perfused trachea system employing tracheas from unexposed guinea pigs has been used to investigate the effects of diacetyl in vitro [Fedan et al. 2006]. In this model, the direct, potentially toxic effects of the agent on epithelium may be examined. Agents such as diacetyl may be applied to the epithelium (mucosal surface) or separately to the smooth muscle (serosal surface) of the trachea while measuring contractile or relaxant responses of the airway smooth muscle. An advantage of this model is that the effects of the diketone do not involve an inflammatory response, inasmuch as the trachea has been removed from the animal and there is no source for the recruitment of inflammatory cells into the wall of the airway.

In unstimulated tracheas, diacetyl or 2,3-pentanedione applied to the mucosal surface in concentrations 1 mM and higher dissolved in a physiological salt solution elicited small contractions; in concentrations higher than 3 mM (i.e., 10 and 30 mM), contractions to diacetyl and 2,3-pentanedione were followed by relaxations. The relaxation responses were larger than the contractile responses. Exploring these phenomena further, adding the flavorings to the mucosa of tracheas that were first contracted with methacholine, a bronchoconstrictor agonist, resulted in full relaxation of the smooth muscle over the same range of diacetyl concentrations. These findings indicate that diacetyl is a weak contractile agent when applied to the epithelial surface, but that it is capable of eliciting strong relaxant responses. Thus, a diversity of responses in the airway that depend on the diacetyl concentration is produced.

Investigation of inhaled diacetyl effects (60, 100, 200, 300, and 360 ppm) and inhaled 2,3-pentanedione effects (120, 240, 320, and 360 ppm) on reactivity of tracheas removed from exposed rats and studied in the isolated, perfused trachea model showed that reactivity to methacholine applied to the mucosal surface was increased slightly after inhalation of 300 and 360 ppm diacetyl and 320 and 360 ppm 2,3-pentanedione. Based on epithelial damage in airways after exposure to diacetyl, a larger

increase in airway reactivity had been anticipated [Zaccone et al. 2013].

Diacetyl inhalation elicits substantial histopathologic changes to airway epithelium, including denudation and necrosis (section 4.2.3). Commonly, damage to respiratory epithelium leads to airway hyperreactivity. For example, after ozone inhalation, airway reactivity of guinea pigs to inhaled methacholine is increased; likewise, reactivity to methacholine applied to the mucosa of isolated, perfused trachea is also increased [Fedan et al. 2000]. Incubation of perfused trachea with diacetyl dissolved in a physiological salt solution and applied to the mucosal surface led to no effect (1 mM diacetyl), an approximately 10-fold increase in reactivity to methacholine (3 mM), or full suppression of contraction to methacholine (10 mM) [Fedan et al. 2006]. The effects of diacetyl in isolated airways from naïve animals does not involve the airway epithelium [Zaccone et al. 2013].

Damage to the epithelium after diacetyl inhalation suggests that epithelial ion transport and electrical resistance could be affected by the diketone. In rat tracheal segments investigated in vitro with Ussing chambers, diacetyl dissolved in physiological salt solution at 3 mM decreased transepithelial potential difference (V_t, mV), indicative of a decrease in electrogenic ion transport and/or an effect on paracellular ion transport involving tight junctions, whereas 10 mM diacetyl reduced V_t further and decreased transepithelial resistance (R_t, $\Omega \cdot cm^2$). R_t is an index of tight junction permeability. Thus, ion transport and epithelial integrity are affected directly by diacetyl.

The diacetyl concentrations observed to affect tracheal diameter and elicit bioelectric responses, i.e., 1 to 3 mM, are within the range estimated to occur in the rat tracheal mucosa after diacetyl inhalation. Using a CFD-PBK model, Morris and Hubbs [Morris and Hubbs 2009] calculated that inhalation levels of 100, 200, and 300 ppm diacetyl could yield concentrations in the mucosa of 1.1 to 1.2, 2.3 to 2.5, and 3.7 to 3.8 mM diacetyl, respectively. This suggests that some or all of the observed in vitro effects may occur in the airways during vapor inhalation.

The mechanism(s) of the effects of diacetyl on trachea in vitro are not known at present. However, a related structure, 2,3-butanedione monixime, has been reported to inhibit contraction of smooth muscle, perhaps as a result of inhibiting phosphorylation of myosin light chains [Lizarraga et al. 1998; Siegman et al. 1994; Stowe et al. 1997; Waurick et al. 1999]. Bioelectric responses of neurons also have been reported to be inhibited by 2,3-butanedione monixime [Lizarraga et al. 1998].

2,3-Pentanedione is not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA102, or TA104 [Aeschbacher et al. 1989; Marnett et al. 1985].

A recent study demonstrates that in vitro diacetyl exposure increases shedding of the epidermal growth factor ligand, amphiregulin. These findings are further supported by evidence that amphiregulin transcripts and protein are also increased in an in vivo model of obliterative bronchiolitis induced by repeated intratracheal instillation of diacetyl [Kelly et al. 2014]. Amphiregulin has previously been reported to play a role in pulmonary fibrosis, although the nature of that role is not fully understood [Zhou et al. 2012].

4.2.5 Toxicology of Inhaled Diacetyl Substitutes

Diacetyl is the compound largely responsible for the flavor of butter in butter [FASEB 1980; FDA 1983]. However, exposures in workplaces that make or use butter flavoring and emissions from heated butter flavoring involve

multiple volatile compounds [Boylstein et al. 2006; Kullman et al. 2005]. Among the potential replacements for diacetyl, starter mix contains high concentrations of diacetyl [FASEB 1980; FDA 1983]. Another chemical that adds the flavor of butter to food is acetoin, and it was present along with diacetyl in many of the workplaces where obliterative bronchiolitis occurred in employees who make or use diacetyl [Kullman et al. 2005; van Rooy et al. 2007]. Acetoin is structurally very similar to diacetyl but an α-hydroxyketone in acetoin replaces the reactive α-diketone implicated in the toxicity of diacetyl and 2,3-pentanedione [Hubbs et al. 2012]. In a National Toxicology Program (NTP) 90-day study on acetoin, the chosen maximum exposure level (generally representing the maximum tolerated dose) was 800 ppm, whereas in the NTP 90-day diacetyl study the maximum exposure level was 100 ppm (Chapter 6). In 90-day inhalation exposures, diacetyl produced statistically significant respiratory tract lesions from exposures as low as 25 ppm, in both rats and mice (Chapter 6). Statistically significant histopathology changes in the 25 ppm diacetyl exposures were nasal respiratory epithelial metaplasia (in both male and female rats), nasal olfactory epithelial degeneration in female rats; nasal respiratory epithelial necrosis (in male and female mice) plus chronic inflammation and respiratory epithelial hyperplasia of the larynx in female mice (Chapter 6). In addition acetoin (150 ppm), after inhalation for 6 hours by rats, had no effect on reactivity to inhaled methacholine while inhaled acetic acid (27 ppm for 6 hours), another component of flavoring mixture, increased airway reactivity to methacholine [Zaccone et al. 2013]. Thus, current data, while limited, indicate that acetoin is considerably less hazardous than diacetyl.

2,3-Pentanedione is structurally very similar to diacetyl because it is a 5-carbon α-diketone, and diacetyl is a 4-carbon α-diketone. Eighteen hours after a 6-hour inhalation exposure to 2,3-pentanedione (120, 240, 320, and 360 ppm), R_L and C_{dyn} in anesthetized rats were unaffected [Zaccone et al. 2013]. Subsequently, airway reactivity to inhaled methacholine aerosol was decreased after inhalation of 120, 240, and 320 ppm 2,3-pentanedione. 2,3-Pentanedione affected methacholine reactivity more than diacetyl. Airway hyperreactivity to methacholine had been anticipated, in view of the epithelium damage caused by the flavoring.

Following inhalation exposure of rats to these same concentrations of 2,3-pentanedione and perfusion of the trachea in vitro, reactivity to mucosally-applied methacholine was increased by 240, 320, and 360 ppm 2,3-pentanedione [Zaccone et al. 2013]. The magnitude of this effect surpassed that caused by diacetyl inhalation.

Morphologic data suggest that 2,3-pentanedione can cause airway epithelial damage similar to the damage caused by diacetyl [Hubbs et al. 2012; Morgan et al. 2012; Morgan et al. 2016]. Rats repeatedly inhaling 2,3-pentanedione at concentrations ≥ 150 ppm for up to 2 weeks develop fibrosis of intrapulmonary airways, a morphologic change similar to obliterative bronchiolitis in humans [Morgan et al. 2016]. Recently, more than 3500 genes were found to be upregulated in RNA isolated from the fibrotic bronchi of 2,3-pentanedione exposed rats [Morgan et al. 2015]. Some of the up-regulated genes were ones previously implicated in fibrosis, including transforming growth factor-β2, interleukin-1α, interleukin-18, interleukin-33, and fibronectin. In addition, at high exposure concentrations, messenger RNA changes were noted in the brain of rats after acute 2,3-pentanedione inhalation [Hubbs et al. 2012].

4.2.6 Diacetyl and 2,3-Pentanedione in Cigarette Smoke

A recent study suggests that mainstream tobacco smoke collected by a smoking machine

contained significant amounts of diacetyl that varied with the smoking parameters set by different organizations. The average for seven types of cigarettes ranged between 285 μg diacetyl/cigarette and 42.8 μg 2,3-pentanedione/cigarette for the International Organization for Standardization (ISO) parameters to 778 μg diacetyl/cigarette and 83.5 μg 2,3-pentanedione/cigarette by the Health Canada Intensive (HCI) parameters. In mainstream cigarette smoke, the calculated diacetyl concentration ranged from 250 ppm with ISO parameters to 361 ppm with HCI parameters, while the concentration of 2,3-pentanedione ranged from 32.2 ppm with ISO parameters to 50.1 ppm with HCI parameters [Pierce et al. 2014]. Unfortunately, the analytical technique used in the study (high performance liquid chromatography with ultraviolet detection analyses of 2,4-dintrophenylhydrazine derivatives) is not especially selective and would be prone to interferences in complex mixtures. Nevertheless, two other recent studies support the presence of significant diacetyl in mainstream cigarette smoke [Fujioka and Shibamoto 2006; Polzin et al. 2007]. Other recent studies have identified diacetyl and 2,3-pentanedione in electronic cigarette liquids and aerosols [Allen et al. 2016; Farsalinos et al. 2015].

In one study, after derivatization with 1,2-phenylenediamine, quantification by GC-NPD, and confirmation by GC-MS, 301–433 μg diacetyl/cigarette was measured in the total mainstream smoke withdrawn from each burning cigarette from 15 different commercial reference cigarettes [Fujioka and Shibamoto 2006]. However, that study did not use a smoking machine to simulate actual smoking behavior, so that the amount of diacetyl observed may not be representative of the amount produced under realistic smoking conditions. In another study, using GC/MS and a smoking machine with ISO parameters, a range of 12.7–145 μg diacetyl/cigarette was measured from 41 different types of cigarettes [Polzin et al. 2007]. Thus, several studies suggest that significant diacetyl is present in mainstream cigarette smoke, although the concentrations vary [Fujioka and Shibamoto 2006; Pierce et al. 2014; Polzin et al. 2007]. Finally, electronic cigarette liquids often contain diacetyl and 2,3-pentanedione [Allen et al. 2016; Farsalinos et al. 2015].

The Pierce et al. [2014] report is the first to calculate diacetyl concentrations in mainstream cigarette smoke in ppm. However, it should be noted that the calculated concentration in ppm such as the reported average concentration of 250 ppm diacetyl using the ISO parameters is actually not comparable to workplace exposures [Pierce et al. 2014]. Using ISO parameters an average of 0.3255 liters of mainstream cigarette smoke was inhaled, but this would not be the only air inhaled by a smoker. Roughly 6.75 liters per minute (L/min) of air is inhaled by the average person at rest, while 19.5 L/min is inhaled under conditions of light work [ICRP 1975]. Assuming that all of the air an employee inhales contains the concentration of diacetyl measured in the workplace, it is critical to recognize that the mainstream smoke of a smoker is not the only source of air; they are also breathing air with much lower diacetyl concentrations. Assuming that the other air breathed by smokers does not contain diacetyl, the workplace equivalent exposure concentration for a smoker during the smoking time period is roughly 190- to 560-fold lower than the concentration measured in the mainstream cigarette smoke using the ISO parameters of one 0.035 L puff/min (6.75/0.035 is 193 and 19.5/.035 is 557). Using the ISO parameters, this would be an average equivalent of a workplace concentration of 0.45 to 1.3 ppm for diacetyl and 58 to 170 ppb for 2,3-pentanedione during the smoking process. If the smoking machine parameters of 9.3 minutes/cigarette are used to calculate the duration of exposure, a smoker who smokes one pack of 20 cigarettes/day spends 186 minutes a day smoking, but the employee is working for 8 hours. Therefore,

the 8-hour time-weighted average equivalent concentration for the smoker is 2.6-fold lower to reflect the time period they are not exposed, which would be 170 to 500 ppb for diacetyl and 22 to 65 ppb for 2,3-pentanedione, depending upon exercise level.

Calculations of workplace equivalent exposures are similar using total mass of diacetyl and 2,3-pentanedione reported by Pierce et al. [Pierce et al. 2014]. The total mass of diacetyl and 2,3-pentanedione in an average cigarette measured with ISO parameters was 285 and 42.8 µg, respectively [Pierce et al. 2014]. For a one pack a day smoker that results in 5,700 µg of diacetyl and 856 µg of 2,3-pentanedione inhaled each day. If that mass was contained in the amount of air inhaled in a work day under light exercise conditions, at 25°C and 760 torr, the one pack/day smoker inhales the approximate equivalent of an occupational exposure to 169 ppb diacetyl and 22 ppb 2,3-pentanedione during an 8-hour work day.

Although it is important to recognize that breathing patterns differ between cigarette smokers or electronic cigarette users ("vapers") and employees, which may affect the pharmacokinetics of inhaled diacetyl and 2,3-pentanedione [Hubbs et al. 2015], these concentrations of diacetyl would be predicted to decrease FEV_1 in some individuals if inhaled for a working lifetime (see Chapter 5). Indeed, many smokers do demonstrate significant decreases in FEV_1 [Barnes 2004; Fletcher and Peto 1977; Wright et al. 1987; Xu et al. 1994]. Additionally, cigarette smoking is a major risk factor for chronic obstructive pulmonary disease, and a decrease in FEV_1 is a characteristic feature of this disease. In addition, bronchiolar fibrosis is part of the airway remodeling response that characterizes chronic obstructive pulmonary disease [Kim et al. 2008; Sohal et al. 2013]. Decreases in FEV_1 and fibrosis of bronchioles are features that also characterize obliterative bronchiolitis [Schlesinger et al. 1998]. However, smokers with chronic obstructive pulmonary disease have additional morphologic changes in their lungs, including emphysema, that are not seen in obliterative bronchiolitis [Snider 2003]. While it has been hypothesized that the failure to diagnose diacetyl-induced obliterative bronchiolitis as a cigarette smoker-associated disease suggests that diacetyl does not cause obliterative bronchiolitis in exposed employees [Pierce et al. 2014], the data when corrected for the actual corresponding occupational exposure concentrations may instead suggest the hypothesis that diacetyl and related reactive carbonyl compounds in cigarettes could potentially contribute to chronic obstructive pulmonary disease. Because chronic obstructive pulmonary disease is a leading cause of morbidity and mortality [Halbert et al. 2006; Lopez et al. 2006], the role of diacetyl and other reactive carbonyl compounds in cigarette smoke in contributing to chronic obstructive pulmonary disease is a worthy topic for future studies. As extensively discussed in Chapter 3, airway obstruction and decreased FEV_1 can, nevertheless, be identified in smokers who are exposed to diacetyl. Most importantly, because diacetyl causes obstructive lung disease and because smoking causes obstructive lung disease, the presence of diacetyl in cigarette smoke does not diminish the need to control diacetyl exposures in employees. In fact, it highlights the greater risks occurring in employees who are exposed to diacetyl in the workplace and who also smoke.

4.2.7 Relevance of Diacetyl Animal Studies to Humans

Four converging lines of evidence support the relevance of diacetyl inhalation studies in rats and mice to humans. First, diacetyl inhalation causes damage to respiratory epithelium in rats and mice. This finding is important because injury to the respiratory epithelium of the deep lung is the accepted cause for obliterative bronchiolitis. Second, dosimetry calculations indicate that diacetyl

concentration in respiratory epithelium of the human deep lung under working conditions may be much higher than diacetyl concentrations in laboratory animals. Third, another organic compound, sulfur mustard, implicated in causing obliterative bronchiolitis in humans, produces a similar pattern of predominantly nasal injury in rats exposed by nose-only inhalation [Weber et al. 2010]. Fourth, repeated inhalation exposure to 2,3-pentanedione causes fibrosis of intrapulmonary airways [Morgan et al. 2012; Morgan et al. 2016]. Each of these findings supports the conclusion that with appropriate dosimetry studies, damage to the respiratory epithelium of the upper airways of rodents should be considered when evaluating risk for humans.

Animal exposure studies have revealed that the upper airways of rodents are sensitive to flavoring-induced toxicity, whereas the lower airways of humans are most affected by these agents. Importantly, diacetyl exposures in rodents caused extensive damage to the respiratory epithelium lining the nose and the trachea [Hubbs et al. 2008; Morgan et al. 2008]. The cell types that are injured in the nose and trachea in rodents are very similar to the respiratory epithelium lining the airways of the deep lung of humans that are involved pathophysiologically in the development of obliterative bronchiolitis [Borthwick et al. 2009; King 1989]. In addition, the bronchi were damaged at high concentrations in acute exposures and at lower concentrations in subchronic exposures in mice. Thus, inhalation toxicology studies showed that diacetyl could damage respiratory epithelium, providing biological plausibility for its etiologic role in obliterative bronchiolitis. Indeed, at the time of the first inhalation toxicology studies of diacetyl, no accepted cause of obliterative bronchiolitis in humans had been demonstrated to cause obliterative bronchiolitis in rodents. Recently, repeated inhalation exposures to 2,3-pentanedione have been shown to cause fibrosis of intrapulmonary airways in rats, demonstrating a pathologic change in the rodent model that is very similar to obliterative bronchiolitis in humans [Morgan et al. 2012]. Interpretation of the species difference in the anatomic location of diacetyl-induced damage to respiratory epithelium may be explained by species differences in respiratory tract anatomy, breathing patterns, and diacetyl dosimetry.

Rodents are obligate nasal breathers while humans are oronasal breathers. Inhaled air may bypass the human nose, particularly during exertion [Conolly et al. 2004]. In addition, the rodent nasal passageways and the rodent trachea are much narrower than the corresponding human nasal passageways and trachea. Small airway diameter increases the percentage of the airstream that is in contact with the mucous layer, increases resistance, and thereby increases mucosal absorption of water-soluble vapors [Frederick et al. 1998; Morris 1997]. Thus, the dimensions of the rodent nose predict much greater absorption of diacetyl vapors in the rodent than in the human nose. However, the surface area of the airways within the human lung is 100 times greater than the surface area of airways in the rat lung [Mercer et al. 1994]. These anatomic differences predict that, at a given exposure concentration, the mucosa in the rodent nose receives a much higher diacetyl dose than does the human nose and that the human lung receives a much higher dose than the rodent lung.

To investigate these dosimetry predictions, a CFD-PBPK model of diacetyl uptake was developed [Morris and Hubbs 2009]. The CFD-PBPK model also predicted greater intrapulmonary diacetyl concentrations in the lung of humans than in rats at a given exposure concentration, especially during mouth breathing [Morris and Hubbs 2009]. Under resting conditions at an exposure concentration of 100 ppm, the rat nose and trachea are predicted to

remove 39% of the inhaled diacetyl, while the trachea of a mouth breathing human would remove 4% of the inhaled diacetyl. This same study suggests the potential importance of nasal lesions in rats for predicting pulmonary toxicity in humans [Morris and Hubbs 2009]. Because the respiratory epithelium of the terminal bronchiole of humans is believed to be the target tissue for the development of obliterative bronchiolitis [King 1989], and because diacetyl doses reaching the respiratory epithelium in the nose of rodents can be similar to diacetyl doses reaching the respiratory epithelium of the deep lung in humans, it may be appropriate to consider toxicity to the respiratory epithelium lining the nose of rodents in evaluating the risk of diacetyl to mouth-breathing employees. In addition, butyric acid, which is a component of some butter flavorings, caused a small but statistically significant reduction in nasal uptake of diacetyl in the rat nose, and thereby increased the diacetyl exposure to the lung due to a reduced "scrubbing effect" [Morris and Hubbs 2009]. A subsequent CFD-PBK model indicates that with low levels of exercise that could occur in the workplace, diacetyl dose to the bronchiolar epithelium of humans may be more than 40-fold greater than the dose received by the bronchiolar epithelium of experimentally exposed rodents [Gloede et al. 2011].

Damage to the nose of rodents has recently been described for another agent implicated in causing obliterative bronchiolitis in humans, sulfur mustard [bis(2-chloroethyl) sulfide], a chemical warfare agent. Obliterative bronchiolitis is considered a major cause of progressive respiratory disease in survivors of sulfur mustard exposure [Ghanei 2004a,b,c; Ghanei et al. 2008; Rowell et al. 2009]. Nose-only inhalation exposures of F344 rats to sulfur mustard caused severe mucosal damage in the rat nose but the changes in the lung were absent or minimal [Weber et al. 2010]. When the nose was bypassed using intubation with tubing lined by Teflon®, sulfur mustard did indeed cause necrosis of the epithelium lining the proximal airways [Weber et al. 2010]. This suggests that sulfur mustard, an accepted cause of obliterative bronchiolitis in humans, causes a similar pattern of injury to the pattern observed with diacetyl at different levels of the respiratory tract of rodents. Thus, predominantly nasal injury has been seen in rodent inhalation studies with organic agents implicated in causing obliterative bronchiolitis.

4.3 Conclusions

Inhalation toxicity studies in rats and mice, and in vitro studies in guinea pig tracheal preparations, indicate that diacetyl-containing butter flavoring vapors can damage airway epithelium and cause inflammation in the respiratory tract after acute or subchronic exposure. In addition, in vivo local lymph node assays indicate that diacetyl is a sensitizer, and in vitro studies indicate that diacetyl is mutagenic. Diacetyl can react with arginine residues causing structural changes in proteins that influence the function of the altered proteins. These functional changes in proteins include changes in enzyme activity and the mitochondrial permeability pore. Pharmacologic studies in vitro indicate that diacetyl can alter ion transport and reduce epithelial integrity. CFD-PBPK modeling indicates that diacetyl concentrations in the deep airways of humans may be higher than those in laboratory rodents, explaining the tendency for diacetyl-induced airway damage to be more anterior in the respiratory tract of rodents than in humans. Most recently, studies of the related α-diketone, 2,3-pentanedione, suggest that the airway toxicity of diacetyl may be shared with other structurally related, α-diketones, and that inhalation of either diacetyl and 2,3-pentanedione can cause airway fibrosis in rats.

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References

Aeschbacher HU, Wolleb U, Loliger J, Spadone JC, Liardon R [1989]. Contribution of coffee aroma constituents to the mutagenicity of coffee. Food Chem Toxicol *27*(4):227–232.

Allen JG, Flanigan SS, LeBlanc M, Vallarino J, MacNaughton P, Stewart JH, Christiani DC [2016]. Flavoring chemicals in e-cigarettes: diacetyl, 2,3-pentanedione, and acetoin in a sample of 51 products, including fruit-, candy-, and cocktail-flavored e-cigarettes. Environ Health Perspect *124*(6):733–739.

Anderson SE, Franko J, Wells JR, Lukomska E, Meade BJ [2013]. Evaluation of the hypersensitivity potential of alternative butter flavorings. Food Chem Toxicol *62C*:373–381.

Anderson SE, Wells JR, Fedorowicz A, Butterworth LF, Meade BJ, Munson AE [2007]. Evaluation of the contact and respiratory sensitization potential of volatile organic compounds generated by simulated indoor air chemistry. Toxicol Sci *97*(2):355–363.

Barnes PJ [2004]. Mediators of chronic obstructive pulmonary disease. Pharmacol Rev *56*(4):515–548.

Borthwick LA, Parker SM, Brougham KA, Johnson GE, Gorowiec MR, Ward C, Lordan JL, Corris PA, Kirby JA, Fisher AJ [2009]. Epithelial to mesenchymal transition (EMT) and airway remodelling after human lung transplantation. Thorax *64*(9):770–777.

Boylstein R, Piacitelli C, Grote A, Kanwal R, Kullman G, Kreiss K [2006]. Diacetyl emissions and airborne dust from butter flavorings used in microwave popcorn production. J Occup Environ Hyg *3*(10):530–535.

Carbone V, Ishikura S, Hara A, El-Kabbani O [2005]. Structure-based discovery of human L-xylulose reductase inhibitors from database screening and molecular docking. Bioorg Med Chem *13*(2):301–312.

CDC [2013]. Obliterative bronchiolitis in workers in a coffee-processing facility—Texas, 2008-2012. MMWR *62*(16):305–307.

Cho-Vega JH, Tsavachidis S, Do KA, Nakagawa J, Medeiros LJ, McDonnell TJ [2007a]. Dicarbonyl/L-xylulose reductase: a potential biomarker identified by laser-capture microdissection-micro serial analysis of gene expression of human prostate adenocarcinoma. Cancer Epidemiol Biomarkers Prev *16*(12):2615–2622.

Cho-Vega JH, Vega F, Schwartz MR, Prieto VG [2007b]. Expression of dicarbonyl/L-xylulose reductase (DCXR) in human skin and melanocytic lesions: morphological studies supporting cell adhesion function of DCXR. J Cutan Pathol *34*(7):535–542.

Chuang LF, Collins EB [1968]. Biosynthesis of diacetyl in bacteria and yeast. J Bacteriol *95*(6):2083–2089.

Colley J, Gaunt IF, Lansdown AB, Grasso P, Gangolli SD [1969]. Acute and short-term toxicity of diacetyl in rats. Food Cosmet Toxicol *7*(6):571–580.

Conolly RB, Kimbell JS, Janszen D, Schlosser PM, Kalisak D, Preston J, Miller FJ [2004]. Human respiratory tract cancer risks of inhaled formaldehyde: dose-response predictions derived from biologically-motivated computational modeling of a combined rodent and human dataset. Toxicol Sci *82*(1):279–296.

Daglia M, Papetti A, Aceti C, Sordelli B, Spini V, Gazzani G [2007a]. Isolation and determination of α-dicarbonyl compounds by RP-HPLC-DAD in green and roasted coffee. J Agric Food Chem *55*(22):8877–8882.

Daglia M, Papetti A, Grisoli P, Aceti C, Spini V, Dacarro C, Gazzani G [2007b]. Isolation, identification, and quantification of roasted coffee antibacterial compounds. J Agric Food Chem *55*(25):10208–10213.

El-Kabbani O, Carbone V, Darmanin C, Ishikura S, Hara A [2005]. Structure of the tetrameric form of human L-xylulose reductase: probing the inhibitor-binding site with molecular modeling and site-directed mutagenesis. Proteins *60*(3):424–432.

Endo S, Matsunaga T, Horie K, Tajima K, Bunai Y, Carbone V, El-Kabbani O, Hara A [2007]. Enzymatic characteristics of an aldo-keto reductase family protein (AKR1C15) and its localization in rat tissues. Arch Biochem Biophys *465*(1):136–147.

Epperly BR, Dekker EE [1989]. Inactivation of Escherichia coli L-threonine dehydrogenase by

2,3-butanedione. Evidence for a catalytically essential arginine residue. J Biol Chem *264*(31):18296–18301.

Eriksson O, Fontaine E, Bernardi P [1998]. Chemical modification of arginines by 2,3-butanedione and phenylglyoxal causes closure of the mitochondrial permeability transition pore. J Biol Chem *273*(20):12669–12674.

Farsalinos KE, Kistler KA, Gillman G, Voudris V [2015]. Evaluation of electronic cigarette liquids and aerosol for the presence of selected inhalation toxins. Nicotine Tob Res *17*(2):168–174.

FASEB, Life Science Resource Office (Federation of American Societies for Experimental Biology) [1980]. Evaluation of the health aspects of starter distillate and diacetyl and food ingredients. Food and Drug Administration.

FDA (Food and Drug Administration) [1983]. Code of Federal Regulations, Title 21, Volume 3, Part 184, Direct food substances affirmed as generally recognized as safe, 21CFR184.1848, starter distillate. U.S. Government Printing Office.

Fedan JS, Dowdy JA, Fedan KB, Hubbs AF [2006]. Popcorn worker's lung: in vitro exposure to diacetyl, an ingredient in microwave popcorn butter flavoring, increases reactivity to methacholine. Toxicol Appl Pharmacol *215*(1):17–22.

Fedan JS, Millecchia LL, Johnston RA, Rengasamy A, Hubbs A, Dey RD, Yuan LX, Watson D, Goldsmith WT, Reynolds JS, Orsini L, Dortch-Carnes J, Cutler D, Frazer DG (485) [2000]. Effect of ozone treatment on airway reactivity and epithelium-derived relaxing factor in guinea pigs. J Pharmacol Exp Ther *293*(3):724–734.

Fletcher C, Peto R [1977]. The natural history of chronic airflow obstruction. Br Med J *1*(6077):1645–1648.

Flynn FV [1955]. Essential pentosuria. Br Med J *1*(4910):391–395.

Foster WM, Walters DM, Longphre M, Macri K, Miller LM [2001]. Methodology for the measurement of mucociliary function in the mouse by scintigraphy. J Appl Physiol *90*(3):1111–1117.

Frederick CB, Bush ML, Lomax LG, Black KA, Finch L, Kimbell JS, Morgan KT, Subramaniam RP, Morris JB, Ultman JS [1998]. Application of a hybrid computational fluid dynamics and physiologically based inhalation model for interspecies dosimetry extrapolation of acidic vapors in the upper airways. Toxicol Appl Pharmacol *152*(1):211–231.

Fujioka K, Shibamoto T [2006]. Determination of toxic carbonyl compounds in cigarette smoke. Environ Toxicol *21*(1):47–54.

Garcia-Quintans N, Repizo G, Martin M, Magni C, Lopez P [2008]. Activation of the diacetyl/acetoin pathway in Lactococcus lactis subsp. lactis bv. diacetylactis CRL264 by acidic growth. Appl Environ Microbiol *74*(7):1988–1996.

Ghanei M, Fathi H, Mohammad MM, Aslani J, Nematizadeh F [2004a]. Long-term respiratory disorders of claimers with subclinical exposure to chemical warfare agents. Inhal Toxicol *16*(8):491–495.

Ghanei M, Mokhtari M, Mohammad MM, Aslani J [2004b]. Bronchiolitis obliterans following exposure to sulfur mustard: chest high resolution computed tomography. Eur J Radiol *52*(2):164–169.

Ghanei M, Tazelaar HD, Chilosi M, Harandi AA, Peyman M, Akbari HM, Shamsaei H, Bahadori M, Aslani J, Mohammadi A [2008]. An international collaborative pathologic study of surgical lung biopsies from mustard gas-exposed patients. Respir Med *102*(6):825–830.

Gloede E, Cichocki JA, Baldino JB, Morris JB [2011]. A validated hybrid computational fluid dynamics-physiologically based pharmacokinetic model for respiratory tract vapor absorption in the human and rat and its application to inhalation dosimetry of diacetyl. Toxicol Sci *123*(1):231–246.

Goravanahally MP, Hubbs AF, Fedan JS, Kashon ML, Battelli LA, Mercer RR, Goldsmith WT, Jackson MC, Cumpston A, Frazer DG, Dey RD [2014]. Diacetyl increases sensory innervation and substance P production in rat trachea. Toxicol Pathol *42*(3):582–590.

Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, Mannino DM [2006]. Global burden of COPD: systematic review and meta-analysis. Eur Respir J *28*(3):523–532.

Hubbs AF, Battelli LA, Goldsmith WT, Porter DW, Frazer D, Friend S, Schwegler-Berry D, Mercer RR, Reynolds JS, Grote A, Castranova V, Kullman G, Fedan JS, Dowdy J, Jones WG [2002]. Necrosis of nasal and airway epithelium in rats inhaling vapors of artificial butter flavoring. Toxicol Appl Pharmacol *185*(2):128–135.

Hubbs AF, Cummings KJ, McKernan LT, Dankovic DA, Park RM, Kreiss K [2015]. Comment on Farsalinos et al., "Evaluation of electronic cigarette liquids and

vapour for the presence of selected inhalation toxins." Nicotine Tob Res *17*(10):1288–1289.

Hubbs AF, Cumpston AM, Goldsmith WT, Battelli LA, Kashon ML, Jackson MC, Frazer DG, Fedan JS, Goravanahally MP, Castranova V, Kreiss K, Willard PA, Friend S, Schwegler-Berry D, Fluharty KL, Sriram K [2012]. Respiratory and olfactory cytotoxicity of inhaled 2,3-pentanedione in Sprague-Dawley rats. Am J Pathol *181*(3):829–844.

Hubbs AF, Fluharty KL, Edwards RJ, Barnabei JL, Grantham JT, Palmer SM, Kelly F, Sargent LM, Reynolds SH, Mercer RR, Goravanahally MP, Kashon ML, Honaker JC, Jackson MC, Cumpston AM, Goldsmith WT, McKinney W, Fedan JS, Battelli LA, Munro T, Bucklew-Moyers W, McKinstry K, Schwegler-Berry D, Friend S, Knepp AK, Smith SL, Sriram K [2016]. Accumulation of ubiquitin and sequestosome-1 implicate protein damage in diacetyl-induced cytotoxicity. Am J Pathol *186*(11):2887–2908.

Hubbs AF, Goldsmith WT, Kashon ML, Frazer D, Mercer RR, Battelli LA, Kullman GJ, Schwegler-Berry D, Friend S, Castranova V [2008]. Respiratory toxicologic pathology of inhaled diacetyl in Sprague-Dawley rats. Toxicol Pathol *36*(2):330–344.

ICRP [1975]. Report of the task group on reference man. Oxford: Pergamon Press. International Commission on Radiological Protection, ICRP Publication No. 23.

Ishikura S, Isaji T, Usami N, Kitahara K, Nakagawa J, Hara A [2001]. Molecular cloning, expression and tissue distribution of hamster diacetyl reductase. Identity with L-xylulose reductase. Chem Biol Interact *130–132*(1–3):879–889.

Jang HW, Jiang Y, Hengel M, Shibamoto T [2013]. Formation of 4(5)-methylimidazole and its precursors, α-dicarbonyl compounds, in Maillard model systems. J Agric Food Chem *61*(28):6865–6872.

Jiang Y, Hengel M, Pan C, Seiber JN, Shibamoto T [2013]. Determination of toxic α-dicarbonyl compounds, glyoxal, methylglyoxal, and diacetyl, released to the headspace of lipid commodities upon heat treatment. J Agric Food Chem *61*(5):1067–1071.

Johnson BA, Arguello S, Leon M [2007]. Odorants with multiple oxygen-containing functional groups and other odorants with high water solubility preferentially activate posterior olfactory bulb glomeruli. J Comp Neurol *502*(3):468–482.

Kelly FL, Sun J, Fischer BM, Voynow JA, Kummarapurugu AB, Zhang HL, Nugent JL, Beasley RF, Martinu T, Gwinn WM, Morgan DL, Palmer SM [2014]. Diacetyl induces amphiregulin shedding in pulmonary epithelial cells and in experimental bronchiolitis obliterans. Am J Respir Cell Mol Biol *51*(4):568–574.

Kim SB, Hayase F, Kato H [1987]. Desmutagenic effect of α-dicarbonyl and α-hydroxycarbonyl compounds against mutagenic heterocyclic amines. Mutat Res *177*(1):9–15.

Kim V, Rogers TJ, Criner GJ [2008]. New concepts in the pathobiology of chronic obstructive pulmonary disease. Proc Am Thorac Soc *5*(4):478–485.

King TE, Jr. [1989]. Bronchiolitis obliterans. Lung *167*(2):69–93.

Kullman G, Boylstein R, Jones W, Piacitelli C, Pendergrass S, Kreiss K [2005]. Characterization of respiratory exposures at a microwave popcorn plant with cases of bronchiolitis obliterans. J Occup Environ Hyg *2*(3):169–178.

Lane AB, Jenkins T [1985]. Human L-xylulose reductase variation: family and population studies. Ann Hum Genet *49*(Pt 3):227–235.

Larsen ST, Alarie Y, Hammer M, Nielsen GD [2009]. Acute airway effects of diacetyl in mice. Inhal Toxicol *21*(13):1123–1128.

Liggett RE, Drake MA, Delwiche JF [2008]. Impact of flavor attributes on consumer liking of Swiss cheese. J Dairy Sci *91*(2):466–476.

Lizarraga I, Alfaro MJ, Goicoechea C, Lopez F, Martin MI (1275) [1998]. Effect of butanedione monoxime on the contractility of guinea pig ileum and on the electrophysiological activity of myenteric S-type neurones. Neurosci Lett *246*(2):105–108.

Lopez AD, Shibuya K, Rao C, Mathers CD, Hansell AL, Held LS, Schmid V, Buist S [2006]. Chronic obstructive pulmonary disease: current burden and future projections. Eur Respir J *27*(2):397–412.

Majcher MA, Jelen HH [2005]. Identification of potent odorants formed during the preparation of extruded potato snacks. J Agric Food Chem *53*(16):6432–6437.

Majcher MA, Klensporf-Pawlik D, Dziadas M, Jelen HH [2013]. Identification of aroma active compounds of cereal coffee brew and its roasted ingredients. J Agric Food Chem *61*(11):2648–2654.

Marnett LJ, Hurd HK, Hollstein MC, Levin DE, Esterbauer H, Ames BN [1985]. Naturally occurring carbonyl compounds are mutagens in Salmonella tester strain TA104. Mutat Res *148*(1–2):25–34.

Mathews JM, Watson SL, Snyder RW, Burgess JP, Morgan DL [2010]. Reaction of the butter flavorant diacetyl (2,3-butanedione) with N-α-acetylarginine: a model for epitope formation with pulmonary proteins in the etiology of obliterative bronchiolitis. J Agric Food Chem *58*(24):12761–12768.

Mercer RR, Russell ML, Roggli VL, Crapo JD [1994]. Cell number and distribution in human and rat airways. Am J Respir Cell Mol Biol *10*(6):613–624.

Miller AG, Gerrard JA [2005]. Assessment of protein function following cross-linking by alpha-dicarbonyls. Ann N Y Acad Sci Jun; *1043*:195–200.

More SS, Raza A, Vince R [2012a]. The butter flavorant, diacetyl, forms a covalent adduct with 2-deoxyguanosine, uncoils DNA, and leads to cell death. J Agric Food Chem *60*(12):3311–3317.

More SS, Vartak AP, Vince R [2012b]. The butter flavorant, diacetyl, exacerbates β-amyloid cytotoxicity. Chem Res Toxicol *25*(10):2083–2091.

Morgan DL, Flake GP, Kirby PJ, Palmer SM [2008]. Respiratory toxicity of diacetyl in C57BL/6 mice. Toxicol Sci *103*(1):169–180.

Morgan DL, Jokinen MP, Johnson CL, Price HC, Gwinn WM, Bousquet RW, Flake GP [2016]. Chemical reactivity and respiratory toxicity of the alpha-diketone flavoring agents: 2,3-butanedione, 2,3-pentanedione, and 2,3-hexanedione. Toxicol Pathol *44*(5):763–783.

Morgan DL, Jokinen MP, Price HC, Gwinn WM, Palmer SM, Flake GP [2012]. Bronchial and bronchiolar fibrosis in rats exposed to 2,3-pentanedione vapors: implications for bronchiolitis obliterans in humans. Toxicol Pathol *40*(3):448–465.

Morgan DL, Merrick BA, Gerrish KE, Stockton PS, Wang Y, Foley JF, Gwinn WM, Kelly FL, Palmer SM, Ton TV, Flake GP [2015]. Gene expression in obliterative bronchiolitis-like lesions in 2,3-pentanedione-exposed rats. PLoS One *10*(2):e0118459.

Morris JB [1997]. Uptake of acetaldehyde vapor and aldehyde dehydrogenase levels in the upper respiratory tracts of the mouse, rat, hamster, and guinea pig. Fundam Appl Toxicol *35*(1):91–100.

Morris JB, Hubbs AF [2009]. Inhalation dosimetry of diacetyl and butyric acid, two components of butter flavoring vapors. Toxicol Sci *108*(1):173–183.

Nakagawa J, Ishikura S, Asami J, Isaji T, Usami N, Hara A, Sakurai T, Tsuritani K, Oda K, Takahashi M, Yoshimoto M, Otsuka N, Kitamura K [2002a]. Molecular characterization of mammalian dicarbonyl/L-xylulose reductase and its localization in kidney. J Biol Chem *277*(20):17883–17891.

Nakagawa J, Oide K, Uematsu Y, Sudo T, Sakurai T, Asami J, Tada N, Ishikura S, Hara A, Odani H, Maeda K [2002b]. Characterization of a dicarbonyl/L-xylulose reductase (DCXR) in kidney with trangenic and knockout mice. Nephrol Dial Transplant *17*(S 12):52.

National Toxicology Program [2007]. Chemical information review document for artificial butter flavoring and constituents: diacetyl [CAS No. 431-03-8] and acetoin [CAS No. 513-86-0]. http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Artificial_butter_flavoring.pdf. Date accessed: March 2011.

Otsuka M, Mine T, Ohuchi K, Ohmori S [1996]. A detoxication route for acetaldehyde: metabolism of diacetyl, acetoin, and 2,3-butanediol in liver homogenate and perfused liver of rats. J Biochem (Tokyo) *119*(2):246–251.

Palmer SM, Flake GP, Kelly FL, Zhang HL, Nugent JL, Kirby PJ, Foley JF, Gwinn WM, Morgan DL [2011]. Severe airway epithelial injury, aberrant repair and bronchiolitis obliterans develops after diacetyl instillation in rats. PLoS One *6*(3):e17644.

Pierce JS, Abelmann A, Spicer LJ, Adams RE, Finley BL [2014]. Diacetyl and 2,3-pentanedione exposures associated with cigarette smoking: implications for risk assessment of food and flavoring workers. Crit Rev Toxicol *44*(5):420–435.

Polzin GM, Kosa-Maines RE, Ashley DL, Watson CH [2007]. Analysis of volatile organic compounds in mainstream cigarette smoke. Environ Sci Technol *41*(4):1297–1302.

Rao GV, Tinkle S, Weissman DN, Antonini JM, Kashon ML, Salmen R, Battelli LA, Willard PA, Hoover MD, Hubbs AF [2003]. Efficacy of a technique for exposing the mouse lung to particles aspirated from the pharynx. J Toxicol Environ Health A *66*(15):1441–1452.

Rigler MW, Longo WE [2010]. Emission of diacetyl (2,3 butanedione) from natural butter, microwave popcorn

butter flavor powder, paste, and liquid products. Int J Occup Environ Health *16*(3):291–302.

Rincon-Delgadillo MI, Lopez-Hernandez A, Wijaya I, Rankin SA [2012]. Diacetyl levels and volatile profiles of commercial starter distillates and selected dairy foods. J Dairy Sci *95*(3):1128–1139.

Roberts DW, York M, Basketter DA [1999]. Structure-activity relationships in the murine local lymph node assay for skin sensitization: α,β-diketones. Contact Dermatitis *41*(1):14–17.

Rowell M, Kehe K, Balszuweit F, Thiermann H [2009]. The chronic effects of sulfur mustard exposure. Toxicology *263*(1):9–11.

Santos CM, Valente IM, Goncalves LM, Rodrigues JA [2013]. Chromatographic analysis of methylglyoxal and other α-dicarbonyls using gas-diffusion microextraction. Analyst *138*(23):7233–7237.

Saraiva MA, Borges CM, Florencio MH [2006]. Non-enzymatic model glycation reactions: a comprehensive study of the reactivity of a modified arginine with aldehydic and diketonic dicarbonyl compounds by electrospray mass spectrometry. J Mass Spectrom *41*(6):755–770.

Sawada H, Hara A, Nakayama T, Seiriki K [1985]. Kinetic and structural properties of diacetyl reductase from hamster liver. J Biochem *98*(5):1349–1357.

Schlesinger C, Meyer CA, Veeraraghavan S, Koss MN [1998]. Constrictive (obliterative) bronchiolitis: diagnosis, etiology, and a critical review of the literature. Ann Diagn Pathol *2*(5):321–334.

Siegman MJ, Mooers SU, Warren TB, Warshaw DM, Ikebe M, Butler TM (1277) [1994]. Comparison of the effects of 2,3-butanedione monoxime on force production, myosin light chain phosphorylation and chemical energy usage in intact and permeabilized smooth and skeletal muscles. J Muscle Res Cell Motil *15*(4):457–472.

Snider GL [2003]. Nosology for our day: its application to chronic obstructive pulmonary disease. Am J Respir Crit Care Med *167*(5):678–683.

Sohal SS, Ward C, Danial W, Wood-Baker R, Walters EH [2013]. Recent advances in understanding inflammation and remodeling in the airways in chronic obstructive pulmonary disease. Expert Rev Respir Med *7*(3):275–288.

Speckman RA, Collins EB [1968]. Diacetyl biosynthesis in Streptococcus diacetilactis and Leuconostoc citrovorum. J Bacteriol *95*:174–180.

St-Gelais D, Lessard J, Champagne CP, Vuillemard JC [2009]. Production of fresh cheddar cheese curds with controlled postacidification and enhanced flavor. J Dairy Sci *92*(5):1856–1863.

Stowe DF, O'Brien WC, Chang D, Knop CS, Kampine JP (1276) [1997]. Reversal of endothelin-induced vasoconstriction by endothelium-dependent and -independent vasodilators in isolated hearts and vascular rings. J Cardiovasc Pharmacol *29*(6):747–754.

Sugai S, Murata K, Kitagaki T, Tomita I [1990]. Studies on eye irritation caused by chemicals in rabbits–1. A quantitative structure-activity relationships approach to primary eye irritation of chemicals in rabbits. J Toxicol Sci *15*(4):245–262.

Sumegi B, Gyocsi L, Alkonyi [1982]. Diacetyl: a new substrate in the overall reaction of the pyruvate dehydrogenase complex. Biochim Biophys Acta *705*(1):70–75.

van Rooy FG, Rooyackers JM, Prokop M, Houba R, Smit LA, Heederik DJ [2007]. Bronchiolitis obliterans syndrome in chemical workers producing diacetyl for food flavorings. Am J Respir Crit Care Med *176*(5):498–504.

Waurick R, Knapp J, Van Aken H, Boknik P, Neumann J, Schmitz W (1277) [1999]. Effect of 2,3-butanedione monoxime on force of contraction and protein phosphorylation in bovine smooth muscle. Naunyn Schmiedebergs Arch Pharmacol *359*(6):484–492.

Weber WM, Kracko DA, Lehman MR, Irvin CM, Blair LF, White RK, Benson JM, Grotendorst GR, Cheng YS, McDonald JD [2010]. Inhalation exposure systems for the development of rodent models of sulfur mustard-induced pulmonary injury. Toxicol Mech Methods *20*(1):14–24.

Whittaker P, Clarke JJ, San RH, Begley TH, Dunkel VC [2008]. Evaluation of the butter flavoring chemical diacetyl and a fluorochemical paper additive for mutagenicity and toxicity using the mammalian cell gene mutation assay in L5178Y mouse lymphoma cells. Food Chem Toxicol *46*(8):2928–2933.

Wondrak GT, Cervantes-Laurean D, Roberts MJ, Qasem JG, Kim M, Jacobson EL, Jacobson MK [2002]. Identification of α-dicarbonyl scavengers for cellular protection against carbonyl stress. Biochem Pharmacol *63*(3):361–373.

Wright JL, Hobson J, Wiggs BR, Pare PD, Hogg JC [1987]. Effect of cigarette smoking on structure of the small airways. Lung *165*(2):91–100.

Xu X, Weiss ST, Rijcken B, Schouten JP [1994]. Smoking, changes in smoking habits, and rate of decline in FEV1: new insight into gender differences. Eur Respir J *7*(6):1056–1061.

Zaccone EJ, Thompson JA, Ponnoth DS, Cumpston AM, Goldsmith WT, Jackson MC, Kashon ML, Frazer DG, Hubbs AF, Shimko MJ, Fedan JS [2013]. Popcorn flavoring effects on reactivity of rat airways in vivo and in vitro. J Toxicol Environ Health A, Part A *76*:669–689.

Zhou Y, Lee J-Y, Lee C-M, Cho W-K, Kang M-J, Koff JL, Yoon P-O, Chae J, Park H-O, Elias JA [2012]. Amphiregulin, an epidermal growth factor receptor ligand, plays an essential role in the pathogenesis of transforming growth factor-β-induced pulmonary fibrosis. J Biol Chem *287*(50):41991–42000.