Chemical Information Review Document

for

Artificial Butter Flavoring

and Constituents
Diacetyl [CAS No. 431-03-8] and Acetoin [CAS No. 513-86-0]

Supporting Nomination for Toxicological Evaluation by the National Toxicology Program

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Prepared by:
Integrated Laboratory Systems, Inc.
Research Triangle Park, NC
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National Toxicology Program
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Abstract

Artificial butter flavoring and two important constituents, diacetyl and acetoin, were nominated by the United Food and Commercial Workers International Union for long-term testing via inhalation for respiratory and other toxicity and for cancer-causing properties. There is growing concern that workers in the microwave popcorn manufacturing industry may be at risk of developing the lung disease bronchiolitis obliterans from exposure to vapors from artificial butter flavoring. The first case of bronchiolitis obliterans in a popcorn manufacturing worker was reported in 2000 in Missouri. Since then several devastating outbreaks of severe and even fatal lung disease, including bronchiolitis obliterans, have been documented among workers in microwave popcorn manufacturing plants who have been exposed to the vapors of butter flavoring. To date, limited toxicological studies are available for artificial butter flavoring and its constituents. Inhalation studies in male rats showed that exposure to vapors from artificial butter flavoring caused necrosis of nasal and airway epithelium. Necrotizing bronchitis was observed in the lung, and necrosuppurative rhinitis and inflammation were seen at all nasal levels. An inhalation study with diacetyl also produced significant necrosis of nasal epithelium and significant necrosis of tracheal epithelium in male rats. However, no significant effects in the lung were reported. In another inhalation study, necropsy revealed general congestion, focal hyperemia of the lungs, atelectasis and bloody edema of the lungs, bronchial edema, and intensified hydrothorax in rats that did not survive a four hour treatment with diacetyl. Histopathological examination showed emphysema, hyperemia of the lungs, peripheral or centrilobular swelling of hepatocytes, and necrosis in the proximal tubules of the kidney. In rats, [^{14}C]-diacetyl given by intragastric gavage was rapidly metabolized and excreted, primarily as carbon dioxide in the breath and in urine. Metabolism of acetoin *in vivo* was mainly by oxidation at low concentrations and by reduction to 2,3-butanediol at higher concentrations. Diacetyl was not a reproductive or teratological toxicant in studies with pregnant mice, rats, or hamsters. Intraperitoneal injections of diacetyl or acetoin did not induce tumors in mice. Diacetyl, but not acetoin, was mutagenic in some strains of bacteria. It was negative in a micronucleus test but induced sister chromatid exchanges in Chinese hamster ovary AUXB1 cells and unscheduled DNA synthesis in various organs of laboratory animals. Diacetyl exhibited activating and deactivating effects on several enzymes and metabolic processes. It has been postulated that oxidative stress may play a role in diacetyl-induced lung damage.

Executive Summary

Basis for Nomination
Artificial butter flavoring and two important constituents, diacetyl and acetoin, were nominated by the United Food and Commercial Workers International Union (UFCW) for long-term testing via inhalation for respiratory and other toxicity and for cancer-causing properties. After the first incidence of bronchiolitis obliterans associated with microwave popcorn manufacturing in Jasper, MO was reported in 2000, several devastating outbreaks of severe and even fatal lung disease, including bronchiolitis obliterans, have been documented among workers in other microwave popcorn plants who had been exposed to the vapors of butter flavoring. These outbreaks have been reported in the scientific literature and the popular press. Since butter flavoring mixtures consist of more than 100 different chemicals, the most prominent being diacetyl and acetoin, the UFCW also recommends that the flavoring mixture as a whole be tested and that the National Toxicology Program (NTP) explore the effects of compounds with chemical and physical properties similar to diacetyl and acetoin.

Nontoxicological Data
Diacetyl is naturally found in foods and is also used as a synthetic flavoring agent and an aroma carrier in foods, including butter, caramel, vinegar, dairy products, and coffee. Acetoin is used as a fragrance carrier and in the preparation of flavors and essences; it is found in many of the same foodstuffs as diacetyl (e.g., butter, corn, wine, and cocoa). In the United States, diacetyl and acetoin are regulated by the Food and Drug Administration as substances directly added to human food and are generally recognized as safe. Both compounds can be detected using gas chromatography, usually coupled with flame ionization detection. Diacetyl is available form several U.S. suppliers and is produced in different ways, including converting methyl ethyl ketone to an isonitroso compound, followed by hydrolyzation with hydrochloric acid or by oxidation of 2-butanone over a copper catalyst. Diacetyl is also a product of fermentation of glucose via methylacetylcarbinol and of lactic acid bacteria activity during the production of beer. Acetoin is prepared from diacetyl by partial reduction with zinc and acid. It is also produced by the action of sorbose bacteria or *Mycoderma aceti* on 2,3-butanediol or by the action of fungi on sugar cane juice. It is a by-product of fermentation and preparation of cream for churning. Diacetyl has been identified in aroma components of tobacco smoke and in several plants. It photodegrades quickly in the atmosphere and is not likely to absorb significantly in soil or sediment or to bioconcentrate in fish. Acetoin gradually oxidizes to diacetyl in air and forms a solid dimer on standing or treatment with granulated zinc. It has high mobility if released to soil and is not expected to absorb to suspended solids or sediments or to undergo direct photolysis. Like diacetyl, it has a low potential for bioconcentration in aquatic organisms.

Human Data
Diacetyl and acetoin are formed endogenously in humans from decarboxylation of pyruvate. They are important volatile organic compounds (VOCs) emitted from butter flavoring and are of concern to workers in the microwave popcorn production industry. Case reports of severe bronchiolitis obliterans syndrome in eight former workers at a Missouri microwave popcorn plant (Gilster-Mary Lee Corporation) sparked public interest in May 2000. NIOSH reported that workers from three other microwave popcorn plants had been exposed to diacetyl and other VOCs form butter flavoring mixtures and had developed occupational lung disease; at least three deaths were reported among these individuals. Bronchiolitis obliterans has therefore been called "popcorn worker's lung" or "popcorn packers' workers' lung." Of the eight workers at the Missouri plant, four worked in the plant's production area (included a mixing room) and four worked in the packaging areas. Workers in these sections were exposed to 800x and 15x the mean atmospheric concentration of diacetyl, respectively, compared to office, warehouse, and outside areas. Workers in the production areas also had significantly higher rates of shortness of breath on exertion, breathing problems, a combination of respiratory symptoms, unusual fatigue, other systemic symptoms, and rashes or other skin problems. As cumulative exposure to diacetyl increased, the

incidence of airway obstruction increased. The data suggested diacetyl as a cause of respiratory disease or a marker of the causative exposure. However, workers in the production areas were also exposed to the highest concentrations of ketones, other VOCs, and respirable dust. Bronchiolitis obliterans has also been reported in workers of other industries (baking industry, flavoring manufacturing plants, and snack food manufacturer using flavorings or spices).

Workers exposed to butter flavoring vapors have also reported eye (chemical burns), skin, and nasal irritations. Patch testing and maximization testing with diacetyl produced no irritation or sensitization, respectively, in volunteers. Tests with acetoin also resulted in no irritation or sensitization reactions.

Toxicological Data

No short-term/subchronic or chronic inhalation studies were available for artificial butter flavorings, diacetyl, or acetoin. Additionally, data regarding initiation/promotion, anticarcinogenicity, and immunotoxicity were not found.

Chemical Disposition, Metabolism, and Toxicokinetics

Metabolic interconversion between diacetyl, acetoin and 2,3-butanediol has been observed using rat liver extracts.

When administered to male Fischer 344 rats via intragastric gavage, a single dose of radiolabeled [^{14}C]-diacetyl (1.58, 15.8, or 158 mg/kg [0.0184, 0.184, or 1.84 mmol/kg]) resulted in excretion of 82.0, 72.7, and 54.3% of the administered doses, respectively, as carbon dioxide at 72 hours. In urine, the excreted amounts were 6.86, 15.7, and 34.1%, respectively. At all tested levels, total excretion of radioactivity in urine, feces, and expired breath accounted for 86-87% of the dose recovered within 24 hours. In normal rat liver mitochondria, diacetyl uncoupled oxidative phosphorylation, totally eliminated respiratory control with substrates, and partially eliminated it with succinate.

Metabolism of acetoin *in vivo* is mainly by oxidation at low concentrations and by reduction to 2,3-butanediol at high concentrations. When acetoin (doses not provided) was i.p. injected into albino rats, ^{12}C-carbon dioxide was found in expired air (average of 15% during 12 hours). When acetoin was administered orally or subcutaneously to rats, no diacetyl and very little acetoin were detected in the urine; 2,3-butanediol was the major excretion product. In rabbits orally given acetoin and in rabbit liver homogenate incubated with acetoin, acetylation was increased. In male guinea pigs, acetoin was an intermediary metabolite in the reduction of methyl ethyl ketone to 2,3-butanediol. In rat and rabbit liver extracts, >95% of radiolabeled [2,3-^{14}C]-acetoin was detected as a mixture of 2,3-butanediol stereoisomers.

Acute Toxicity

The LC_{50} value for diacetyl in rats was reported between 2.25 and 5.2 mg/L (639 and 1477 ppm) for a four-hour period. Diacetyl was a severe skin and eye irritant in rabbits. Acetoin was a moderate irritant on intact and abraded skin of rabbits; an LD_{50} value >5000 mg/kg (56.75 mmol/kg) was calculated.

Male rats exposed to vapors from artificial butter flavoring (average diacetyl concentrations: 203, 285, 352 [constant], or 371 [pulsed] ppm; range 72-940 ppm) for six hours exhibited necrosis of nasal and airway epithelium. At levels of 285-371 ppm diacetyl, necrotizing bronchitis was observed in the lung; at 203-371 ppm diacetyl, necrosuppurative rhinitis and inflammation were seen at all nasal levels.

Inhalation of diacetyl (99, 198, or 295 ppm [349, 697, or 1039 mg/m^3]) for six hours also produced significant necrosis of nasal epithelium at ≥198 ppm and significant necrosis of tracheal epithelium at the high dose in male rats. No significant effects in the lung were reported. When tested in male and female Wistar rats, inhalation of diacetyl FCC (2.25, 5.2, and 23.9 mg/L [639.0, 1477, or 6788 ppm]) for four hours resulted in deaths at the mid and high doses. Necropsy showed general congestion in dead rats, focal hyperemia of the lungs and empty gastrointestinal tract in mid-dose animals, and atelectasis and bloody edema of the lungs, bronchial edema, and intensified hydrothorax in high-dose rats. Histopathological examination revealed moderate emphysema and focal hyperemia of the lungs as well as peripheral swelling of hepatocytes at the mid dose, and widespread hyperemia of the lung, necrosis in the proximal tubules of the kidney, and centrilobular swelling of hepatocytes at the high dose.

No acute inhalation studies for acetoin were available.

<u>Synergistic/Antagonistic Effects</u>
When acetoin and ethanol were i.p. administered simultaneously in rats to cause loss of righting reflex and respiratory failure, the concentrations of both chemicals were additive in blood.

<u>Cytotoxicity</u>
Diacetyl (0.001, 0.1, or 1 mM [0.086, 8.6, or 86 µg/mL]) inhibited cell growth in ascites sarcoma cells by 37% at the mid dose and by 100% at the high dose.

<u>Reproductive and Teratological Effects</u>
When given via oral intubation to pregnant mice for ten days, diacetyl (1.6 g starter distillate/kg) had no effects on maternal or fetal survival or nidation and caused no statistically significant changes in the number of fetal abnormalities compared to controls. Tests in hamsters and rats gave similar results.

<u>Carcinogenicity</u>
When given i.p. to mice once weekly for 24 weeks, diacetyl (1.70 or 8.40 mg/kg [0.0197 or 0.0976 mmol/kg]) did not induce any lung tumors. Acetoin (total doses of 12.0 or 60.0 g/kg [136 or 681 mmol/kg] given i.p. 3x/week for 6-7 weeks) also showed no carcinogenic activity.

<u>Genotoxicity</u>
In several bacterial assays, diacetyl generally showed mutagenic activity in *Salmonella typhimurium* strains TA100, 102, and 104 with and without metabolic activation but none against strain TA98. Conflicting results were obtained in *Escherichia coli* strain WP2 uvra, but nonmutagenicity was demonstrated in the SOS-chromotest using *E. coli* PQ37. Diacetyl was also negative in a micronucleus test using mouse bone marrow cells. It induced sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) AUXB1 cells and unscheduled DNA synthesis in various organs of laboratory animals.

Acetoin (up to 4500 mg/plate [51.08 mmol/plate]) was generally nonmutagenic in bacteria *in vitro*.

<u>Cogenotoxicity</u>
Diacetyl induced mitotic chromosome loss in *Saccharomyces cerevisiae* in the presence of propionitrile.

<u>Antigenotoxicity</u>
In CHO AUXB1 cells, bisulfite significantly reduced the frequency of SCEs and proportion of endoreduplicated cells when diacetyl was administered. Sodium sulfite and heterocyclic amines inactivated the mutagenicity of diacetyl in *S. typhimurium* strain TA100.

<u>Other Data</u>
Effects on Enzymes
When administered to rats via gastric intubation, diacetyl (300 or 1500 mg/kg bw [3.48 or 17.42 mmol/kg bw]) produced increases in ornithine decarboxylase activity and DNA synthesis in the pyloric mucosa. Diacetyl has activating and deactivating effects on several enzymes and metabolic processes, including inactivating estradiol 17β-dehydrogenase in human placenta under ultraviolet light. In the lysosomal

enzyme α-*L*-iduronidase, it reduced the internalization of the enzyme into human diploid fibroblasts by 50% without affecting enzyme activity and reduced binding to the fibroblast membranes by 90%.

Possible Mechanism for Lung Damage by Diacetyl

Diacetyl may induce lung damage by oxidative stress. Lung injury was induced by phosgene and mustard via several processes, including free radical generation. Lung damage caused by ozone has also been suggested from the formation of reactive free radicals. Reduction potentials for diacetyl and its iminium derivatives were found to be in the range favorable for catalytic electron transfer *in vivo*, which can cause oxidative stress via reaction oxygen species as a result of redox cycling.

Structure-Activity Relationships

Genetic and carcinogenic effects for several diacetyl analogs, such as methylglyoxal and acetaldehyde, are included in an earlier NTP chemical background document for diacetyl (http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/431-03-8.pdf).

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Tables:

1.0 Basis for Nomination

Artificial butter flavoring and two important constituents, diacetyl and acetoin, were nominated by the United Food and Commercial Workers International Union (UFCW) for long-term testing via inhalation for respiratory and other toxicity and for cancer-causing properties (http://defendingscience.org/case_studies/upload/Union_petitionto_NTP.pdf). After the first incidence of bronchiolitis obliterans associated with microwave popcorn manufacturing in Jasper, MO was reported in 2000, several devastating outbreaks of severe and even fatal lung disease, including bronchiolitis obliterans, have been documented among workers in other microwave popcorn plants who had been exposed to the vapors of butter flavoring. These outbreaks have been reported in the scientific literature and the popular press. Since butter flavoring mixtures consist of more than 100 different chemicals, the most prominent being diacetyl and acetoin, the UFCW also recommends that the flavoring mixture as a whole be tested and that the National Toxicology Program (NTP) explore the effects of compounds with chemical and physical properties similar to diacetyl and acetoin.

2.0 Introduction

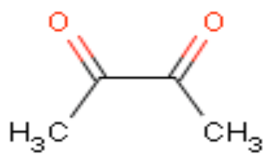
Microwave popcorn is a popular snack food in the United States (U.S.) that is consumed by millions of people in their homes, work place, and at many types of recreational events. It was estimated in 2005 that 156 million bags (39 million pounds) of microwave popcorn are consumed each year in the U.S. (Science News, 2005). The consumption of all types of popcorn in 2001 was estimated at one billion pounds per year, of which a large portion was microwaveable. This is equivalent to ~17.5 billion quarts per year or an average of ~ 70 quarts per person per year (Food History, 2001). In order to keep up with consumer demand in the U.S. alone, the microwave popcorn manufacturing industry has to produce over 100 thousand pounds of popcorn per day (assuming a six day work week). One manufacturer alone reported production levels of 150 million bags of microwave and stove-top popcorn in 2006 (Northwest Indiana Times, 2006).

<u>Public Health Concern</u>

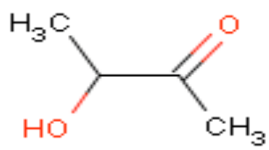
Although there is currently no indication that the general public is at risk of developing lung disease from exposure to vapors released from microwaved popcorn, there is growing concern about the risk microwave popcorn producers face. Increased incidences of fixed airway obstruction, including bronchiolitis obliterans, have been recently reported among workers in the microwave popcorn industry. Bronchiolitis obliterans is also called "popcorn worker's lung" or "popcorn packers' lung" and is a rare inflammatory disease that affects the small airways. Its main respiratory symptoms are cough and shortness of breath; the latter may become severe and persistent. The first case of bronchiolitis obliterans in a microwave popcorn packaging worker was seen in 1994, but it was the case reports of eight former workers from the same Missouri plant (Gilster-Mary Lee Corporation) with severe bronchiolitis obliterans syndrome that sparked public interest in May 2000 (Kanwal et al., 2006b [HETA 2000-0401-2991]; Kreiss et al., 2002). Several studies suggest that exposure to volatile organic compounds (VOCs) released from butter flavorings used in production processes is the greatest risk factor. Two prominent VOCs believed to be the major contributors are diacetyl and acetoin (Kreiss et al., 2002). Diacetyl is one of the main components in butter flavoring that gives it its buttery taste and has been identified as a prominent VOC in air samples from microwave popcorn plants (Akpinar-Elci et al., 2004; Kanwal, 2003 lett. [HETA 2002-0089]; Kanwal et al., 2004 [HETA 2001-0474-2943], 2006a; Kanwal and Martin, 2003 lett. [HETA 2001-0517]; Parmet and Von Essen, 2002 lett.).

Diacetyl is also naturally found in foods and is used as a synthetic flavoring agent and aroma carrier in butter, caramel, vinegar, dairy products, and coffee. Acetoin is used in the preparation of flavors and essences and found in many of the same foodstuffs as diacetyl (HSDB, 2002, 2005a). The results from toxicological studies published in the open literature and reported in the Hazardous Substance Database (HSDB) for butter flavoring and two of its primary components, diacetyl and acetoin, are reviewed here.

Diacetyl
[431-03-8]



Acetoin
[513-86-0]



2.1 Chemical Identification and Analysis

Diacetyl ($C_4H_6O_2$; mol. wt. = 86.09) is also called:

- 2,3-Butadione
- 2,3-Butanedione
- 2,3-Diketobutane
- 2,3-Dioxobutane
- Biacetyl
- Butadiene
- Butanedione
- Diacetyl (natural)
- Dimethyl diketone
- Dimethyl glyoxal
- Dimethylglyoxal
- Glyoxal, dimethyl-
- NSC 8750

Source(s): ChemIDplus, 2004a; Registry (1984)
PubChem CID: 650

Acetoin ($C_4H_8O_2$; mol. wt. = 88.10) is also called:

- 1-Hydroxyethyl methyl ketone
- 2-Butanone, 3-hydroxy-
- 2-Hydroxy-3-butanone
- 2,3-Butanolone
- 3-Hydroxy-2-butanone
- Acethoin

Acetoin (natural)
Acetyl methyl carbinol
Acetylmethylcarbinol
Dimethylketol
γ-Hydroxy-β-oxobutane
Methanol, acetylmethyl-
NSC 7609

Source(s): ChemIDplus (2004b); HSDB (2005a)
PubChem CID: 179

Methods that have been used to analyze diacetyl in foodstuffs (e.g., beer, wine, butter, and butter flavoring) include the National Institute of Occupational Safety and Health (NIOSH) Method #1300, Ketones I: use of gas-chromatography (GC) flame ionization detection (FID) with limit of detection (LOD) at 0.02 mg/sample; NIOSH Method #1301, Ketones II; use of GC-FID with LOD at 0.05 mg/sample; and the Association of Official Agricultural Chemists Method 978.11. Calorimetric methods are also listed as analytical methods for diacetyl analysis (HSDB, 2002). Details of a GC-FID method developed for detecting diacetyl, acetoin, and other ketones in microwave popcorn manufacturing plants are described in Pendergrass (2004 [PMID:14968874]). Sample stability studies using spiked samples collected on Anasorb CMS solid sorbent tubes and stored for seven days at room temperature reported 87-92% (diacetyl) and 63-83% (acetoin) recoveries (Pendergrass, 2004; PMID:14968874). Recoveries up to 95 % were reported for diacetyl samples stored at room temperature for 14 days based on GC-FID analysis (Shah, 2006). GC-FID may also be used to identify VOCs, including diacetyl, in human blood (Houeto et al., 2001).

Thermal desorption with GC-mass spectrometry (GC/MS) has been used to detect diacetyl, acetoin, 2-nanonone, and other VOCs in butter flavorings and air samples from microwave popcorn manufacturing plants (Kanwal and Kullman, 2004 [HETA 2003-0112-2949]; Kanwal et al., 2004 [HETA 2001-0474-2943], 2006b [HETA 2000-0401-2991]; Sahakian et al., 2003 [HETA 2002-0408-2915]). A list of VOCs identified in room air and head space samples from butter flavoring mixtures is given in **Appendix C, Table 1**. Headspace volatiles from unsalted sweet butter heated at 100, 150 or 200 °C for 5 hours were also collected by simultaneous purging and solvent extraction and are included in the table. Analysis by GC-FID, nitrogen-phosphorus, or flame photometric detectors and GC/MS identified 21 aldehydes, 12 fatty acids, 11 ketones (including diacetyl and 2-nonanone; no levels given), 10 nitrogen- and/or sulfur-containing compounds, 7 alkanes, 6 δ-lactones, and 4 furans comprising 85% of total volatiles recovered when heated at 200 °C (Lee et al., 1991).

Diacetyl in foodstuffs can also be indirectly determined by a differential pulse polarographic method; the technique is based on derivatization with *o*-phenylendiamine to yield quinoxaline (Rodrigues et al., 1999; PMID:10552634). In wine, diacetyl can be determined by solid-phase microextraction followed by GC/MS; the detection limit ranged from 0.01 with linearity to 10 µg/mL (Hayasaki and Bartowsky, 1999; PMID:10563940).

Beer samples passed through octadecyl solid-phase extraction column, derivatized with 2,3-diaminonaphthalene and analyzed by high-performance liquid chromatography (HPLC)