

This document was developed by NIEHS/NTP staff to facilitate internal and external review of a proposed research program prior to designing and conducting toxicology studies. The purpose of the research concept document is to outline the general elements of a research program that would address the specific public health concerns that prompted the nomination of the substance or issue for study. It may also encompass substance-specific studies that address larger public health issues or topics in toxicology. Additional information about the nomination, review, and selection of substances for study by the NTP is provided at *Nominations to the NTP Testing Program* (<http://ntp.niehs.nih.gov/go/nom>). A draft version of this research concept was reviewed by the NTP Board of Scientific Counselors at a public meeting on June 22, 2007 (<http://ntp.niehs.nih.gov/go/9741>), subsequently revised, and approved by the NTP Executive Committee.

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## **NTP Research Concept: Artificial Butter Flavoring and Certain Components, Diacetyl and Acetoin**

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### **Nomination Rationale and Background**

Diacetyl was first nominated to the National Toxicology Program (NTP) in 1994 by the National Cancer Institute for mechanistic, metabolism, and carcinogenicity studies by the oral route. Because the chemical was not palatable in dosed water and was too volatile in dosed feed, gavage was considered the only feasible oral route of exposure. The NTP conducted oral chemical disposition studies in rats that showed near complete metabolism of diacetyl to CO<sub>2</sub> (unpublished data). Based upon the limitations in exposure route, the potent irritant properties of this chemical, and its near complete metabolism to CO<sub>2</sub>, it was thought unlikely that effects other than ulceration in the stomach would be detected in short-term gavage studies. Based upon this information, toxicity studies were not recommended and the nomination was formally withdrawn in 1999.

In 2007, artificial butter flavoring (ABF) and two major volatile constituents, diacetyl and acetoin, were nominated by the United Food and Commercial Workers Union for long-term inhalation testing for respiratory toxicity, general toxicity and carcinogenicity (<http://ntp.niehs.nih.gov/go/29287>). Because ingestion is the most common route of human exposure to ABF, little attention has been given to the potential toxicity of inhaled vapors. Inhalation exposure to significant concentrations of ABF vapors occurs primarily in the food industry, and until recently no adverse health effects have been reported. The toxicity of inhaled ABF first came under scrutiny in 2000 after an unusually high percentage of young employees at a microwave popcorn plant were diagnosed with obliterative bronchiolitis (OB), an irreversible airway obstruction disease that is often fatal. Since then OB has been diagnosed in workers in other microwave popcorn manufacturing plants, as well as in the flavoring, baking, and diacetyl manufacturing industries. Occupational exposure to diacetyl, acetoin and artificial butter flavoring are currently not regulated in part because of a lack of inhalation toxicity data.

### **Rationale for Study**

Workers in the microwave popcorn packaging, candy, snack food, baking, and flavoring industries are exposed to artificial butter flavoring and its components. Cases of OB have been identified in each of these industries. Obliterative bronchiolitis is a rare, debilitating, irreversible and often progressive and fatal disease. Currently there is no treatment or cure for OB. In the workplace, OB develops insidiously with no incidents of over exposure. Typically by the time

respiratory symptoms become severe enough to seek medical attention, the disease has already caused irreversible lung damage.

Inhalation toxicity data are needed for artificial butter flavoring, diacetyl and acetoin in order to set safe worker exposure limits to these compounds. Although diacetyl and acetoin are the two major volatile components of butter flavoring, the toxicity of these and many other constituents of butter flavoring are unknown. All cases of OB in the popcorn, flavoring, food, and diacetyl production industries involved mixed exposures, making it difficult to identify a single toxic component. Inhalation studies in animals using controlled exposures to single constituents of ABF or ABF are necessary in order to identify the toxic agent(s). Dose-response and No Observable Effect Level (NOEL) data are needed by regulatory agencies to set exposure limits and protect workers. Mechanistic studies are needed to understand the etiology and progression of airway disease in humans in order to develop prevention and treatment strategies.

### **Proposed Approach**

The overall goal of this research program is to obtain respiratory toxicity data for inhaled ABF, diacetyl and acetoin in rats and mice. Hypotheses to evaluate are:

- Respiratory toxicity of inhaled artificial butter flavoring is due to diacetyl. Acetoin and other components do not contribute to toxicity.
- There is no systemic absorption and toxicity of artificial butter flavoring or its components. Toxicity is limited to the site of contact.
- Butter flavoring induced OB is immune-mediated. Diacetyl is a potent sensitizer and forms adducts with arginine residues on proteins. Adducts with “self-proteins” may result in antibody formation. OB in lung transplant rejection has a strong immune component. Lymphocytic bronchitis is a precursor to OB in lung transplant patients and this lesion has also been observed in diacetyl-exposed mice.

The specific aims of the proposed research program are to:

1. Evaluate the contribution of diacetyl and acetoin to the respiratory toxicity of artificial butter flavoring.
  - Quantify the toxicity of flavoring containing known amounts of diacetyl and acetoin
  - Compare the toxicity of ABF to equal concentrations of diacetyl, acetoin, and diacetyl plus acetoin.
2. Evaluate the systemic distribution and toxicity after inhalation of butter flavoring, diacetyl or acetoin.
  - Histopathology in pulmonary and extrapulmonary tissues
  - Clinical chemistry for systemic toxicity
  - Binding sites using radiolabeled or immunolabeled diacetyl and acetoin
3. Investigate immune-mediated mechanism for OB.
  - Antibody response, splenic subpopulations, NK cell assay, lymphoproliferative response, cytotoxic T lymphocyte assay
  - Dermal sensitization followed by inhalation challenge.

4. Obtain inhalation dose-response and NOEL data.
  - Subchronic and chronic inhalation studies in rats and mice
  - Evaluate other exposure profiles, e.g. intermittent high-concentration exposures.

#### **Preliminary NTP Studies on Diacetyl**

After the initial reports of OB in microwave popcorn workers the NTP conducted subacute and subchronic inhalation studies of diacetyl in mice. These studies were designed after coordination with NIOSH. Because NIOSH was focusing on the toxicity of artificial butter flavoring in rats, the NTP studies evaluated the toxicity of diacetyl in mice.

Results of initial inhalation studies of diacetyl were reported in abstract form (Morgan et al., 2006) and a manuscript is in preparation. In an acute study, male C57BL/6 mice were exposed by inhalation to 0, 200 or 400 ppm diacetyl 6 hr/day for 5 days. Exposure to 400 ppm caused deaths and acute necrotizing rhinitis, laryngitis and bronchitis (proximal large bronchi). Exposure to 200 ppm caused a few deaths and acute necrotizing rhinitis and either erosive or necrotizing laryngitis in all mice. However, there were no lung or bronchiolar lesions in the diacetyl exposed mice. In an attempt to reduce the nasal cavity toxicity, the daily exposure duration was decreased to 1 hr/day, 5 days/week and the study duration increased to 4 weeks at 0, 100, 200, 400 ppm. Chronic bronchitis, laryngitis, and rhinitis were present after 2 and 4 weeks of exposure. The lesion severity was concentration related and ranged from minimal to moderate. To investigate effects of episodic exposures to high diacetyl concentrations, such as occurs during flavor mixing, mice were exposed to 1200 ppm diacetyl for 15 minutes, 2x/day, 5 days/week for 4 weeks. This exposure caused less nasal cavity toxicity than acute exposures, and an increased incidence of lymphocytic bronchitis in exposed mice.

In a subchronic study mice were exposed to 0, 25, 50, or 100 ppm diacetyl (6h/d, 5d/w) for 12 weeks, and a subgroup evaluated at 6 weeks after the last exposure. Toxicity occurred primarily in the 100 ppm group. At 100 ppm minimal necrosis was observed in the nasal cavity and lesions were characterized by suppurative exudates and squamous metaplasia of the septum turbinate. Lymphocytic bronchitis was present in some smaller airway branches of the lung at 100 ppm. Because of the sensitivity of the nasal cavity to inhaled diacetyl, mice (5/group) were administered 100, 200, or 400 mg/kg diacetyl by oropharyngeal aspiration and evaluated 4 days later. In the 400 mg/kg group 2 mice died 2 days after aspiration. By 4 days after aspiration, foci of fibrosis without inflammation were present at the junction of the terminal bronchiole and alveolar duct in all 3 remaining mice. Similar lesions with mild inflammation were noted in 1/5 mice treated with 200 mg/kg, and no lesions were present in the 100 mg/kg group. Although the histological appearance of these lesions is not identical to OB these lesions may progress to OB with continued exposure. These results indicate that in mice most inhaled diacetyl reacts in the nasal cavity and bronchi and toxic concentrations do not reach the distal airways.

A BALB/c mouse hypersensitivity model was used to investigate the mechanisms of diacetyl-induced hypersensitivity. These data were reported in abstract form (Patterson et al., 2007) and a manuscript is in preparation. Diacetyl stimulated lymph node cell proliferation following subcutaneous exposure in a dose dependant manner at concentrations as low as 2.5 mg/kg/day. At 25 mg/kg/day diacetyl, an increase in total cell number of all lymphocyte sub-populations tested (CD3+ T-cell, B220+ B-cell, CD4+ T-helper cell, CD8+ cytotoxic T-cell, Mac3+

macrophage) was observed in both lymph nodes and spleen, indicating possible generalized systemic inflammation. At 10 mg/kg/day diacetyl, only B-cells were altered in the spleen. However, in the draining lymph nodes, both T-cell (CD3+, CD4+ and CD8+) and B-cell numbers were elevated. Diacetyl also regulated synthesis of several inflammatory cytokines, including IL-1 $\alpha$  and IL-4 in lymph nodes and spleen, and IL-2, IL-10, MIG, and TNF- $\alpha$  in lymph nodes. The lymph node cytokine profile suggests alteration of both Th1 and Th2 cytokines. Collectively these data suggest that exposure to diacetyl induces inflammation, systemic hypersensitivity, and functional changes in immune responses.

Diacetyl has been shown to specifically react with arginine residues in proteins suggesting an immunological response to modified proteins may be a contributing factor in the development of obstructive airway disease. The formation of diacetyl-arginine adducts is being studied using C14-labeled diacetyl (unpublished data). Future plans are to isolate diacetyl-arginine adducts for use in preparing antibodies. These antibodies may be used to identify the site(s) of reaction in the respiratory tract of exposed animals.

### **Key Considerations in Study Design**

Artificial butter flavorings are produced by a number of manufacturers each with many different proprietary mixtures. The specific flavoring selected for the proposed studies must be representative of the flavorings with the greatest use and worker exposure. The NTP will seek input from NIOSH and industry in making this selection.

Although diacetyl and acetoin are the two predominant volatile constituents of ABF, other constituents may contribute to the overall toxicity. Artificial butter flavorings contain more than 100 different volatile chemicals. Diacetyl alone has been shown to cause injury to airway epithelium; however, other toxic constituents may have additive or synergistic effects.

Initial studies indicate that the primary target site of ABF (and diacetyl) vapors in rodents is the nasal cavity, whereas the bronchioles are the primary site of toxicity in humans. The rodent nasal cavity is much more efficient than that of humans in removing direct-acting irritants from inhaled air. Rodent nasal turbinates are anatomically more complex and have a larger surface area relative to the human nasal turbinates. For this reason, the rodent nasal cavity receives the highest inhaled dose of diacetyl and the greatest injury while the bronchioles are protected. Although humans have reported some nasal irritation from ABF, the major toxic effect is in the distal airways. Differences in respiration can also account for some of these species differences in the site of toxicity. Rodents are obligate nose breathers, and humans are both mouth and nose breathers. Nasal irritation in humans may lead to increased mouth breathing. In humans, mouth breathing bypasses the scrubbing action of the nose and may allow more diacetyl to reach the distal airways. The aforementioned species differences in target sites must be considered when using rodent toxicity data for setting exposure limits for humans.

Because of these species differences in target sites, rodent toxicokinetic data must be collected to allow extrapolation from rodents to humans. A number of physiologically-based pharmacokinetic models are available for extrapolating toxicity data for reactive gases from rodents to humans.

### **Significance and Expected Outcome**

The primary human health concern for this nomination is OB resulting from exposure to volatile components of ABF during the production of microwave popcorn. However, obstructive airway disease associated with ABF is not limited to the microwave popcorn industry. Thousands of workers in the food flavoring industry may be exposed to diacetyl and other chemicals that make up ABF. Although NIOSH and OSHA have issued alerts and recommended use of respirators and additional ventilation to limit worker exposures, insufficient inhalation toxicity data are available to set workplace exposure standards or short-term exposure limits for artificial butter flavoring, diacetyl, or acetoin. The proposed studies will provide pharmacokinetic and dose-response data for all three compounds, particularly with respect to respiratory toxicity, and also help identify the toxic constituent(s) of ABF. These data will allow public health and regulatory agencies to set safe exposure levels for ABF, diacetyl and acetoin, and to develop guidance to protect the health of workers in occupations where these chemicals are used.

### **References**

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