EXECUTIVE SUMMARY
to the
CALIFORNIA AIR RESOURCES BOARD
(Contract CARB AO-031-31)
Period of July 1980 to February 1982
(3rd Contract Year)

Project Title: In Vivo Fate of Nitrogenous Air Pollutant Derivatives

February 20, 1982

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Report No. 3

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EXECUTIVE SUMMARY

Introduction. This is the third and final report to the California Air Resources Board (CARB No. AO-Ø31-31) of a series (CARB A7-190-3Ø, CARB A8-121-31) of investigations into the fate of nitrate and nitrite introduced into the pulmonary tract. Our present work, combined with earlier CARB sponsored research at Davis (CARB 1116) into the fate of inhaled nitrogen dioxide, and basic chemical research has now revealed that the in vivo metabolism of these compounds are inseparably linked. With our development of new methods for radiochemical engineering in biological research, we have successfully used radioactive Nitrogen-13 as a label to track the metabolic fate of nitrogenous air pollutants.

The combustion of gasoline, oil, coal, and other hydrocarbon fuels in air, produces nitrogen oxides (NO_x) and other gaseous or particulate air pollutants. The composite term, NO_x , refers to a combination of nitric oxide (NO) and nitrogen dioxide (NO_2) gas. In the atmosphere, NO is largely converted to NO_2, which may in turn react with or be adsorbed into the fine, wet respirable particles that exist in the atmospheric aerosol.

Nitrogen dioxide reacts with water to form nitrous (HNO_2) and nitric (HNO_3) acids that can be neutralized to form nitrite (NO_2^-) and nitrate (NO_3^-) ions in solution. Chemical reactions in polluted atmospheres are a matter of current investigation; it is known that nitrogenous aerosols are a large contributor to the particulate pollution problem in California. Gaseous nitrogen dioxide that has not absorbed into or reacted with a particle may be inhaled into the moist environment of the lungs and pulmonary tract. Numerous toxicology experiments have shown that inhaled nitrogen dioxide compromises lung function and increases the likelihood of respiratory infection. However, many of these experiments have used exposure levels above those encountered in a real-world polluted atmosphere, and consequently normal physiological defense mechanisms for dealing with noxious chemicals may have been saturated.

Our understanding of the biochemical fate of inhaled nitrogen dioxide, nitrates, and nitrites has been limited because the powerful tool or radioactive labeling of suspected toxic compounds was not previously available. Unlike radioactive carbon-14 and hydrogen-3 that have half-lives measured in years and can be used to label potentially hazardous hydrocarbons so that their biological fate in living animals can be studied, the only

usable radioactive isotope of nitrogen (N-13) has a 10 minute half-life.

The situation then at the inception of the current research program was as follows. Evidence that nitrates and nitrites existed or could exist in polluted atmospheres was available. Evidence which strongly indicated that a known inhalation hazard, nitrogen dioxide, was converted to nitrates and nitrites after inhalation was available. The pharmacological literature suggested that pollutant organic nitrates would be degraded to nitrite and an organic residue. No biochemical evidence derived from intact, living animals was available to indicate whether the known adverse effects of nitrogen dioxide were caused by direct reactions or reactions of the suspected secondary reaction products, nitrate and nitrite.

In addition, scientists who were concerned about the use of nitrite as a food additive for the prevention of botulism had reported that nitrite-fed animals developed cancers when given dosages about 10-fold higher than used in foods. It was and is generally believed that nitrite combines, in the stomach and possible the intestinal tract, with secondary amines derived from food to produce N-nitrosamines. N-nitrosamines are among the most powerful chemical inducers of cancer that have been observed in animal toxicology research.

Objectives. The primary objective has been to provide evidence to help determine whether nitrates, nitrites and other nitrogenous air pollutants should be regulated separately from oxides of nitrogen. The title, "In Vivo Fate of Nitrogenous Air Pollutant Derivatives", accurately reflects the scientific objective of the contracts summarized herein.
However, almost nothing was known initially about the organ distribution of any inspired nitrogenous pollutants or their biochemical reactions. Consequently, this research has addressed the distribution and biochemical fate in animals of the simply oxy-anions, nitrate and nitrite. The indirect evidence and theoretical predictions noted in the introduction strongly indicate that understanding the physiological chemistry of these anions is a crucial link in using existing animal toxicology data for the prediction of human health hazards. Answers to the following general questions have been intermediate stepping-stones.

(1) What is the organ distribution in animals of nitrate and nitrite after induction into the lungs, and for comparison to ingestion toxicology studies,

the stomach and the blood?

(2) Do the anions react in the lungs or elsewhere to form new and possible hazardous compounds?

(3) Are they metabolically eliminated and by what route?

(4) Do they behave physiologically like some well-studied chemical species?

(5) What are the time scales for possible excretion or reaction to form innocuous or hazardous metabolites?

(6) With a knowledge of excretory and biochemical dynamics, can ingestion studies of nitrate and nitrite toxicity be related to inhalation hazards of nitrogenous compounds?

(7) Can non-invasive methods for relating the physiological behavior of these anions in animals to humans be found?

Experimental Methodology. The execution of experiments designed to answer the preceding biological questions required advances in radiochemical engineering application of rapid high pressure (to 6000 pounds per square inch) liquid chromatography, a reliable computerized data acquisition and analysis system, and a cyclotron capable of producing at least one-half Curie of tracer N-13 ($t_{1/2}$ = 10 min). The detection limits for a single N-13 labeled compound by radioliquid chromatography have been reduced to one-billionth of a Curie; thus complex experiments requiring several biochemical manipulations can be performed up to a maximum of two hours. Fortunately, many important biochemical reactions of nitrogen compounds take place with time-scales measured in seconds and minutes.

The biological experiments with living animals (in vivo) were done with mice and rabbits. A minimum of 10- to 100-billionth of a gram/kilogram body weight of radioactively labeled nitrate and nitrite were introduced into the lungs, stomach, or blood or test animals; this dosage is far below the level of any known drug effect. The maximum dosage used was about one million times higher than the minimum. Test-tube experiments (in vitro) were done with blood in parallel to experiments in which the agent was given originally to the animal. Blood was then analyzed in detail for extracellular: intracellular distributions and chemical identity of N-13 because it communicates with all other organs and tissues. The origin and chemical identity of labeled compounds appearing in the blood but not formed in blood was found by sampling different organs which showed 99.99% of the compounds detected to be naturally

formed nitrogen compounds produced by intestinal bacteria within 1- to 2-hours of tracer instillation. The formation of other compounds, hazardous or benign, were not detected if they incorporated less than Ø.Ø1% of instilled tracer.

Summary of Results and Conclusions.

Related work. In a pioneering California Air Resources Board research program (CARB-1116) conducted a few years ago, we demonstrated that radioactive N-13 labeled nitrogen dioxide could be made in sufficient quantities at the University of California, Davis cyclotron for inhalation experiments with rhesus monkeys. Non-human primates were exposed to Ø.6 parts-per-million NO_2 which contained radioactive tracer, $^{13}NO_2$. This level was an upper limit, but within the range of ambient levels at the time.

We found that a large fraction of the N-13 tracer atoms left the lungs and were distributed throughout the body. This result contradicted contemporary thinking which held that nitrogen dioxide should react with lipid molecules in lung tissue and trap the N-13 atoms in the lung. No rapid chemical analysis technology was available during the early inhalation experiments so the chemical form of the N-13 in the lung or leaving the lung by the blood was unknown. New basic chemistry results indicate that nitrogen dioxide at ambient air concentrations will form nitrous acid (a nitrite precursor) in the lungs. We have shown in the present work that the N-13 label from nitrite in not trapped in the lungs either. This is the logical keystone relating nitrogen dioxide, nitrite, and ultimately, nitrate metabolic fates.

More recently, other ARB sponsored research (CARB A9-Ø76-31) has shown that chronic inhalation of NO_2 at concentrations near those encountered in polluted urban air cause spleen enlargement, and more importantly, cause a higher level of lung metastases to form from blood-borne cancer cells. We believe that our work shows this to be most likely the result of direct insult to the lung tissue. More complex organic nitrogen compounds may be degraded to hazardous compounds in other tissues.

Conclusion. In this conclusion we link what we believe to be the elementary in vivo chemical reactions or formation of molecular lesions to either a physiological or pathological response. First, we should note that our exploratory experiments with breeds of mice other than the Balb/C, with

which most of our work was done, gave similar results. We give the model for nitrogen dioxide, nitrite, and nitrate because these chemical species are intimately linked in both atmospheric chemistry and in vivo biochemistry.

Nitrogen Dioxide. For NO_2, the first reaction appears to be abstraction of a hydrogen atom from pulmonary lipids to form HNO_2 which is neutralized to form NO_2^- ion at physiological pH in the well-buffered pulmonary fluid. The chronic formation of chemically induced lesions in the cell membranes of pulmonary tissues results in an immune response which can explain part of the splenic enlargement observed by toxicologists in animals. The remainder of the splenic enlargement may be derived from splenic congestion due to increased formation of red-blood cells as a compensating mechanism for decreased oxygen-carrying capacity of the blood. Decreased oxygen capacity is caused by the reaction of NO_2^- ion with hemoglobin to form methemoglobin which does not carry oxygen. We have shown that NO_2^- rapidly leaves the lung and enters the blood where it is oxidized along with hemoglobin. However, in a chronic NO_2 exposure situation, nitrite ion is being continuously formed in the lung and topically applied to the pulmonary tissue. This topical application of nitrite or its precursor probably induces vascular leakage of the post capillary venules by damaging the endothelial cells and perturbing the intracellular junctions. Hence, the reported greater migration of blood-borne cancer cells into the lungs and the subsequent formation of metatases in animals exposed to NO_2 may derive from an increased permeability of the venular endothelium.

Nitrite Ion. For inhaled NO_2^- ion, the first interaction is expected to be the physiological effects of topical application to pulmonary tissue as discussed in the preceding. Nitrite has been shown by us to be oxidized to nitrate in the red blood cells of living animals. This oxidation is accompanied by oxidation of hemoglobin and reduction of oxygen carrying capacity which may induce splenic congestion as a consequence of increased red blood cell populations in cases of chronic exposure. The long-term consequences of such splenic compensation are unknown. In our experiments, nitrite in the blood was 50% converted to nitrate in 5-10 min and simultaneously transported throughout the body. Inside the small intestine, nitrite oxidation was retarded and the possiblity of formation of potentially

carcinogenic nitrosamines is enhanced although none were identified in our animal experiments. The fate in blood of nitrite formed by metabolic degradation of complex organic nitrogenous air pollutants would parallel that of inorganic nitrite after it exited the lung. Nitrite which entered the large intestine (5-15%) was 50% converted to ammonia, glutamate, and other naturally occurring amines within 5-10 min. No nitrosamines were identified in large intestine extracts from exposed mice. Our detection level was 0.01% of the intratacheally instilled nitrite.

Nitrate Ion. Nitrate ion, like nitrite ion, is derived from emmissions of nitrogen oxides and exists in the atmosphere as a component of particles which form the ambient aerosol. It is transported out of the lung unchanged. It does not undergo chemical modification in the blood and appears to be cleared by the kidneys to urine with a half life of about 12 hours. Nitrate does cross the intestinal wall and is reduced in the large intestine to ammonia, glutamate, and other naturally occurring amines. It seems unlikely that inhaled nitrate will contribute an excess health hazard over that which might accrue from the much larger quantities which are ingested. It should be noted that the N-13 label from nitrate does cross the placental barrier in pregnant animals. The health hazard potential arising from the continuous inhalation of nitrate precursors or nitrate directly by pregnant human females is not well known.

RECOMMENDATIONS

1. The large body of existing toxicological data for exposure to nitrogen dioxide appears to be relevant to suspended nitrate health concerns. Since nitrates are derived from emissions of nitrogen oxides (NO_x), a general recommendation is that maintenance of atmospheric NO_2 concentrations below levels at which adverse health effects are known to occur may adequately protect the population against inorganic nitrate. No recommendation is made at this time regarding organic nitrates.

2. It is recommended that the metabolic fate of pollutant organic nitrates be studied and determined.

3. With the 100-fold greater amounts of N-13 and chemical identification capability now available, extension of the earlier $^{13}NO_2$ studies with rhesus monkeys (CARB-1116) to a rodent species in which organ distributions and biochemical reactions can be cost-effectively determined is now warranted. This should be combined with a modest chronic exposure program using stable ^{15}N labeled NO_2. The enhancement of lung metastasis by chronic exposure is not understood.

4. In vitro studies to determine whether NO_2 reduces pulmonary immunity by interfering with the production and removal of superoxide ion by pulmonary macrophages are recommended.

5. It is recommended that synthetic methods for candidate ^{13}N labeled pollutant organic nitrates be developed and their metabolic fate be studied and determined. There is no information at all in this area.