UCD-CNL 225

FINAL REPORT
to the
CALIFORNIA AIR RESOURCES BOARD
ARB Contract # AO-Ø31-31
Feb 2Ø 1982

PROJECT TITLE:
IN VIVO FATE OF NITROGENOUS
AIR POLLUTANT DERIVATIVES
Report 3

PRINCIPAL INVESTIGATORS:

Norris J. Parks
Laboratory for Energy-Related Health Research
School of Veterinary Medicine

Kenneth A. Krohn
Division of Nuclear Medicine
School of Medicine

SUBMITTING INSTITUTION:
Crocker Nuclear Laboratory
University of California
Davis, CA 95616

TABLE OF CONTENTS

		Page
	List of Figures and Illustrations	3
	List of Tables	4
	Abstract	5
	Acknowledgements	7
	Disclaimer	8
Section I:	Introduction	9
Section II:	Project Objectives	12
Section III:	Conclusions	16
Section IV:	Recommendations	23
Section V:	Animal and Radiochemical Experimental Methods	24
Section VI:	Results	44
Section VII:	Discussion	70
Section VIII:	Publications	77
Section IX:	References	78

LIST OF FIGURES

Figure	Title	Page
Figure 1.	Time dependency of organ distributions	45
Figure 2.	Radiochromatograms illustrating the oxidation of $^{13}NO_2^-$ in animals.	57
Figure 3.	Comparison of oxidation rates for $^{13}NO_2^-$ to $^{13}NO_3^-$ in humans and rabbits.	59
Figure 4.	Liquid chromatogram of mouse small intestine contents after IT $^{13}NO_2^-$.	65
Figure 5.	Thin layer electrophoretoram of mouse small intestine contents after IT $^{13}NO_2^-$. Activity shown to the right of the arrow is times 100.	66
Figure 6.	Liquid chromatogram of mouse large intestine contents after IT $^{13}NO_2^-$.	67
Figure 7.	Thin layer electrophoretogram of mouse large intestine contents after IT $^{13}NO_2^-$.	68

LIST OF TABLES

Table	Title	Page
Table 1	Blood fractionating scheme	30
Table 2	Summary of results	31
Table 3	^{13}N distribution data ANOVA for per organ	47
Table 4	^{13}N distribution data ANOVA for per gram	48
Table 5	^{13}N Distribution Data: Mean uptake in organs percent injection dose per organ -+ std. dev. (adjusted mean from ANOVA with time covariate)	49
Table 6	^{13}N Distribution Data: Mean uptake per gram percent injected dose per organ -+ std. dev. (adjusted mean from ANOVA with time covariate)	50
Table 7	^{13}N Distribution Data: Three-Way ANOVA	51
Table 8	Bolus instillation of carrier nitrite: organ distribution	54
Table 9	Fractional distribution of ^{13}N in blood at 10 min after administration of $^{13}NO_2^-$ pr $^{13}NO_3^-$	55
Table 10	Summary of N-labeled product distribution in various tissues after intratracheal instillation of $^{13}NO^-$ in mice.	62

ABSTRACT

We have investigated the previously unknown metabolic fate of nitrate (NO_3^-) and nitrite (NO_2^-) after introduction into the respiratory tract because they exist in the ambient California urban aerosol or can be derived from known nitrogenous air pollutants such as nitrogen dioxide (NO_2), nitric acid (HNO_3), or nitrous acid (HNO_2). This study deals exclusively with chemically neutral sources of NO_3^- and NO_2^-. Nitrate and nitrite distribution and metabolic chemistry measurements in blood fractions and pertinent organs of both mice or rabbits were made possible by our development of new radiochemical methods. Radioactive nitrogen-13 ($t_{1/2}$ =10 min) has been used to synthesize $^{13}NO_3^-$ solutions with radioactivity concentrations of 230 mCi/mL and $^{13}NO_2^-$ solutions with radioactivity concentrations of 150 mCi/mL (Section 5). These concentrations are 10-100 million times our minimum detectable quantity and permit longer, more complex biochemical tracer experiments than previously possible. The radioactive label allowed us to account for 100% of the administered nitrogen throughout the period required for metabolism of the original species.

We have found that $^{13}NO_2^-$ and $^{13}NO_3^-$ are cleared rapidly from the lungs in the presence and absence of added carrier. Both anions penetrate the red cell membrane and NO_2^- is converted to NO_3^- which diffuses out. The N-13 label from both anions was loosely bound to plasma and cellular proteins but did not form a covalent bond (Section 6, 7). Nitrate does not undergo detectable reactions in blood. Nitrite is oxidized to nitrate in blood cells and not in plasma. In mice, over 70% was oxidized within 10 min, and in rabbits, only 46% was oxidized within 10 min. Comparable in vivo and in vitro oxidation rates for mice and rabbits suggest that in vivo oxidation of nitrite to nitrate can be estimated for humans from in vitro blood studies and provide a basis for interspecies extrapolation of NO_2 gas toxicity studies. A preliminary model of nitrate and nitrite metabolism after introduction into the respiratory tract is proposed (Section 7). We previously observed an unidentified non-anionic compound or mixture of labeled compounds in blood and now have found it to be derived from metabolic reactions in the gastrointestinal tract where the possibility of forming carcinogenic nitrosamines exists. The compounds formed in the intestines by bacteria were found to be naturally occurring ammonia,

glutamate, glutamine and small amounts of urea and neutral amino acids. A selective search for simple nitrosamines did not reveal their presence under our experimental conditions.

ACKNOWLEDGMENTS AND PREFACE

The third year of work on this project has been exceptionally rewarding in that several important questions which arose from our initial research have been answered. We have successfully extended our radiobiochemical methods to meet the difficult problems in enviromental toxicology of nitrogen compounds. Our work has been well-received by the scientific community and has resulted in several publications in the open literature. We wish to acknowledge our colleague Neal Peek who was co-investigator with us for years 1 and 2, our collaborating undergraduate, graduate and post-graduate researchers, Joseph Chasko, Kenneth Geiger, Marsha Gregor, Laura Swartz and Chester Mathis, Ph.D.; our colleagues from the N-13 research group, John C. Meeks, Ph.D. and James R. Thayer, Ph.D.; the staff of Crocker Nuclear Laboratory and the Laboratory for Energy-Related Health Research. We also acknowledge the Research Division of the California Air Resources Board for their support.

Norris J. Parks

Kenneth A. Krohn

February, 1982

Disclaimer: "The statements and conclusion in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein in not to be construed as either an actual or implied endorsement of such products."

Section I

INTRODUCTION AND SUMMARY OF PREVIOUS CONTRACTS

The existence of atmospheric pollutants that can form nitrite (NO_2^-) or nitrate (NO_3^-) upon inhalation is well documented. The health hazard potential associated with their inhalation is still poorly resolved. In this report, we elucidate the in vivo metabolic fate of pulmonary anionic nitrogen; a subject about which past ignorance has been a notable impediment to our understanding, and assessment, of the potential health risks associated with nitrogenous air pollutants. This is the final report of California Air Resources Board (CARB) contract A0-031-31. This is the third contract-year report of our investigations of the in vivo fate of nitrogenous air pollutants which included a radiochemical engineering effort to develope a nuclear probe, radioactive ^{13}N ($t_{1/2}$=10 min), that permitted these otherwise unobtainable biological measurements to be made.

In the first contract-year (CARB A7-190-30) (1), we proceeded along parallel lines to initiate measurement of N-13 organ distribution and to develope high specific-activity (1-7 Curies/micromole) reagents which, together with rapid high pressure liquid radiochromatography (HPLC), would allow metabolite analysis after several half-lives of the label had elapsed. We found that the organ distribution of the N-13 label from nitrite and nitrate entering the lungs after intratracheal (IT) administration was the same after 30 minutes as that following intravenous (IV) administration. The distribution of N-13 following intraesophageal (IE) administration was qualitatively similiar to that found after IT or IV, but there were detectable temporal variations in the distribution pattern. This raised the question of whether significant fractions of nitroxy (NO_x) ions entering the respiratory tract might end up as carcinogenic nitrosamines which had been reported (37) to form from ingested nitrite (NO_2^-). Additional concern was raised by our finding that the N-13 label crossed the placental barrier in pregnant rats (1). The label was also found to freely cross red cell membranes. Bonding to plasma or cellular constituents was deemed to be electrostatic in nature. These experiments were performed under no-carrier-added (NCA) tracer conditions so that pharmacological perturbation of the experimental animals was avoided. The essence of the NCA distribution studies was that N-13 tracer

entered predominantly into organs highly perfused with blood and that the biological "target" appeared to be a ubiquitously distributed biochemical pool rather than a specific organ or organs. This work was extended in subsequent contract-years to include a study of how graded dosages of carrier nitrite or nitrate (NO_3^-) might perturb organ distribution of the label. The final analysis of all the organ distribution studies are included in this report.

In the second contract-year (CARB A8-121-31) (2), our first priority was to establish the chemical identity of the N-13 label that was systemically distributed by the blood. However, this required continuing effort to increase the specific concentration (radioactive disintegrations per unit volume) of the labeled pollutant analogues in order to extend the time course of the measurements. Concurrently, we determined that IT dosages of NO_2^- up to 30 mg/kg body weight of mice did not alter the organ distribution of N-13 tracer from that observed with NCA concentrations although met-hemoglobin was produced. The similiar organ distribution of N-13 from acute high dosage and low dosage experiments indicated that formation of met-hemoglobin (which cannot carry oxygen) does not markedly retard clearance of nitrite from the lung by blood. The N-13 label from both nitrate and nitrite distributed fairly evenly across the red cell membrane and was not controlled like chloride ion which has an extracelluar:intracellular ratio of 25:1. We demonstrated by thin-layer electrophoresis that the N-13 activity from both anions which precipitated with plasma and cellular proteins was not covalently bound.

Our first priority question in the second year, identification of metabolic products in blood and their rate of formation, was addressed with N-13 reagent concentrations that permitted chemical identification of labeled compounds up to two hours after administration in some cases. We found that nitrate does not undergo detectable reactions in blood cells or plasma. Nitrite, on the other hand, is largely oxidized to nitrate inside blood cells of mice and rabbits in 10-20 minutes. There are species differences in oxidation rates of nitrite to nitrate in blood, but in vivo rates for a given species are similiar to in vitro rates which indicated that in vivo rates for humans could be estimated from in vitro studies. Finally, a labeled non-anionic (NA) compound or mixture of compounds was found to constitute about 3% of the blood activity within 10 minutes of IT administration of nitrate or nitrite in both mice and rabbits. We hypothesized that the NA component originated from the 20% of the N-13 label that was transported

from the lung to the gastrointestinal (GI) tract, and that a fraction of it might be a carcinogenic nitroso-compound. The answer to questions about the identity of NA has been a major focus of the third contract-year.

In this final report of the third contract-year (CARB AØ-Ø31-31) and the final report of this project, we address new experimental areas which include identification of NA, the influence of bolus administration of nitroxy ion carrier on distribution and metabolism, the influence of chronic carrier administration on the distribution and metabolism of subsequently administered N-13 labeled ions, and the invitro comparison of nitrite oxidation rates in human and animal blood. The project objectives given in the next section represent the objectives for years 1, 2, and 3. These are the important questions which have distilled out of the initial goal to document the heretofore unknown fate of nitrate and nitrite which might be inhaled directly or be formed as metabolites of other nitrogenous air pollutants such as nitrogen dioxide or nitro-aromatics. In the discussion and conclusion sections, we present a tentative model relating our pharmacological results to toxicological results with a nitrite precursor.

Section II

PROJECT OBJECTIVES

The purpose of these projects was to develop and apply the radioactive nitrogen-13 based radiochemical technology to address the following biological objectives:

(1) Determine the time dependence of nitrite oxidation.

In the second contract year, we showed the rapid oxidation of labelled nitrite ($^{13}NO_2^-$) to nitrate ($^{13}NO_3^-$) in the mouse model. Further studies were needed to obtain accurate estimate of the in vivo and in vitro mechanisms of oxidation. The determination of these rates was required in order to make interspecies comparisons of in vitro experiments and extrapolate these to the expected in vivo results.

(2) Determine the chemical form of the ^{13}N inside rabbit and mouse red blood cells.

We have shown that $^{13}NO_3^-$ oxidizes to $^{13}NO_3^-$ in the blood, and the red blood cells are a likely site for this process. Determining the chemical form of the ^{13}N label within the erythrocyte would indicate whether this is an intra- or extra-cellular process.

(3) Examine the nature of protein bound activity.

After intratracheal administration, both $^{13}NO_2^-$ and $^{13}NO_3^-$ yield a small percentage of the label which precipitates with proteins. A covalently bound label would indicate the irreversible reaction of the labeled nitrogen with a protein residue. Such a reaction, if it were a nitrosation process, would be an important consideration in the production of human health risks from inhalation of nitrogen oxides.

(4) Compare the in vitro oxidation kinetics between animal models and human.

Once we have determined the time dependence of both the in vivo and in vitro chemistry of nitrite in the mouse model, we can use this to estimate the in vivo fate of nitrite in humans using in vitro experiments.

(5) Determination of whether the intestine is a site of ^{13}N-labeled non-anionic (NA) compound production.

Studies relating bacterial activity to nitrosamine synthesis suggest that the intestinal microglora may contribute to the body burden of potential nitrosation products.

(6) Determine whether NO_3^- is reduced to NO_2^- in the intestine.

Nitrate occurs in mammals fed nitrogen free diets. Some of this NO_3^- may be derived from inhaled sources. Thus, the synthesis of NO_2^- in the intestine could contribute to chemical production of nitrosamines where NO_3^- alone would not.

(7) Compare the effect of addition of a "bolus" of carrier NO_2^- or NO_3^- on the biodistribution of 13-N.

Under no-carrier-added (NCA) conditions we have demonstrated that 13-N from NO_3^- and NO_2^- is distributed uniformly on the basis of weight. If an organ-specific accumulation of NO_3^- or NO_2^- metabolites occurs at higher administered NO_3^- or NO_2^- concentrations, target organs for potential health risk might be identified.

(8) Examine the effect of addition of a bolus of carrier NO_2^- or NO_3^- on the level of NO_2^- and NO_3^- in the blood (plasma) and intestine.

If NO-2 is acting as a precursor to the chemical synthesis of nitrosamines in the body, the addition of carrier to the N-13 additions could increase the rate of nitrosamine formation

(9) Determine the metabolic products of pulmonary NO_2^- instillation. Are they nitrosamines, NO_2^- protein conjugates or innocuous products of bacterial nitrogen assimilation in the intestine?

(1Ø) Determine if NO_2^- can be produced by oxidative pathways in mammals. Nitrate is synthesized by mammals fed a diet containing no nitrogen. Can nitrite by produced in vivo from reduced forms of nitrogen?

We have found that $^{13}NO_2^-$ is either oxidized to $^{13}NO_3^-$ or reduced to $^{13}NH_4^+$ or neutral products. The oxidation pathway from NH_4^+ to NO_2^- must be considered as a possible source of NO_2^-.

(11) Examine an intratracheal (IT) bolus of added carrier effects the metabolism of $^{13}NO_2^-$?

Nitrogen-13 fro no-carrier-added (NCA) $^{13}NO_2^-$ distributes rapidly and uniformly throughout the biological fluids of the mouse, mot o the $^{13}NO_2^-$ is rapidly oxidized to $^{13}NO_3^-$, and a small percentage is chemically reduced to other products. Previous experiments showed little effect on these processes by the addition of carrier NO_2^-. Both in vivo and in vitro experiments were conducted to verify these results.

(12) Determine how $^{13}NO_2$ metabolism is affected by previous chronic administration of carrier NO_2^-?

The bolus experiments mentioned above provide valuable insight into the pathways of NO_2 metabolism relating to the ingestion of a large single dose of NO_2^- (as is possible in foods). However, these do not adequately simulate chronic exposure to low levels of NO_2^- or NO_2^- producing air pollutants. Such chronic exposure could burden the biochemical pathway and decrease the ability to metabolize NO_2, or could alternatively sensitize the system to NO_2^-, resulting in more rapid metabolism. Chronic exposure simulation with a new technique utilizing subcutaneous implantation of miniature osmotic pumps ws used. These pumps can deliver a wide range of concentrations of NO_2^- solutions at a uniform rate for a period of one to two

weeks. This eliminates uncertainties in total dose and dose rate associated with dosing through food or water. The IV route of administration also more closely simulates exposure through the lungs.

(13) Do in vivo results relate to in vitro results, (how can the results in mice be related to humans?)

We have extensively studied ^{13}NO-2 metabolism in the mouse model. If in vitro and in vivo resluts are directly comparable, as appears to be the case, then the results in mice can be extrapolated to humans by comparison of in vitro results.

(14) Examine the chemical identity of the reduced species product from $^{13}NO_2^-$. Are nitrosamines produced?

We have shown that a non-anionic (NA) produced species produced from $^{13}NO_2^-$ by reduction in the intestine. The presence of nitrosation products would have important implications in the consideration of health impact. Further studies utilizing high conentrations of radioactivity and expanded HPLC analyses were needed to determin the chemical forms of the reduction products.

Section III

CONCLUSIONS

In this section, we first give our specific conclusions that are matched to the individual biological objectives listed in the preceding section.

(1) Time dependence of nitrite oxidation. In the second contract year, we showed the in vivo oxidation of labelled nitrite ($^{13}NO_2^-$) to nitrate ($^{13}NO_3^-$) in mouse blood to have a half life of about 5-7 min. Further studies were needed to correlate the in vivo and in vitro rates of oxidation. The determination of these rates was required in order to make interspecies comparisons of in vitro experiments and extrapolate these to the expected in vivo results. We conclude (a) that in vivo and in vitro oxidation rates for mice and rabbits are similar; therefore in vitro rate measurements with human blood are expected to predict in vivo rates, and (b) the New Zealand rabbit gave nitrite oxidation half lives on the order of 10-12 min and appeared to be a less satisfactory animal for assessing human metabolic rates.

(2) The chemical form of the ^{13}N inside rabbit and mouse red blood cells. We have previously shown that $^{13}NO_2^-$ oxidizes to $^{13}NO_3^-$ in the blood, and that red blood cells were a likely site for this process. The chemical form of the ^{13}N label within the erythrocytes of animals exposed to nitrite was nearly all nitrate. In plasma, a mixture of nitrite and nitrate was found, indicating transport of nitrate out of the cells after it is formed. Nitrite introduced into separated plasma was not oxidized, indicating that the formation of nitrate is clearly an intra-cellular process.

(3) The nature of protein bound activity. After intratracheal administration, both $^{13}NO_2^-$ and $^{13}NO_3^-$ yield a small percentage of the label which precipitates with proteins. A covalently bound label would indicate the

irreversible reaction of the labeled nitrogen with a protein residue. Such a reaction, if it were a nitrosation process, would be an important consideration in the production of human health risks from inhalation of nitrogen oxides. We found that repeated washing removed the ^{13}N label, indicating no covalent binding.

(4) Compare the in vitro oxidation kinetics between animal models and human. Human in vitro oxidation measurements gave a half-life of 5-7 min which were comparable to mouse values and indicated the mouse to be a good model for assessing human metabolism of simple nitroxy compounds.

(5) Determination of whether the intestine is a site of ^{13}N-labelled non-anionic (NA) compound production. Studies relating bacterial activity to nitrosamine synthesis suggested that the intestinal microflora may contribute to the body burden of potential nitrosation products. Primarily the large intestine is the site of ^{13}N labelled NA production. No nitrosamines were identified in our experiments.

(6) Determination of whether NO_3^- is reduced to NO_2^- in the intestine. Nitrate occurs in mammals fed nitrogen free diets. Some of this NO_3^- may be derived from inhaled sources. Thus, the synthesis of NO_2^- in the intestine could contribute to chemical production of nitrosamines where NO_3^- alone would not. We did not observe synthesis of NO_2^- in the intestine.

(7) Compare the effect of addition of a "bolus" of carrier NO_2^- or NO_3^- on the biodistribution of 13N. Under no-carrier-added (NCA) conditions we have demonstrated that 13N from NO_3^- and NO_2^- is distributed uniformly on the basis of weight. If an organ-specific accumulation of NO_3^- or NO_2^- metabolites occurs at higher administered NO_3^- or NO_2^- concentrations, target organs for potential health risk might be identified. Carrier dosages as high as 60 mg/kg did not reveal a statistically significant ($P=0.997$) difference in organ distribution from that observed under NCA conditions.

(8) The effect of addition of a "bolus" of carrier NO_2^- or NO_3^- on the level of NO_2^- and NO_3^- in the blood (plasma) and intestine. If NO_2^- is acting as a precursor to the chemical synthesis of nitrosamines in the body, the

addition of carrier to the 13N additions could increase the rate of nitrosamine formation. Carrier NO_2^- and NO_3^- increased urinary excretion of NO_3^- as measured optical spectroscopy, but did not appreciably change the metabolite distribution from the NCA case. No nitrosamine formation was detected during our observation period.

(9) The metabolic products of pulmonary NO_2^- instillation. Are they nitrosamines, NO_2^- -protein conjugates or innocuous products of bacterial nitrogen assimilation in the intestine? The products are about 80% nitrate formed in the blood, and about 20% non-anionic (NA) compounds which are formed by bacteria in the intestine. The NA fraction was 50-67% glutamate, 28-44% ammonia, 2-6% glutamine, and about 2% urea and neutral amino acids.

(10) Is NO_2^- produced by oxidative pathways in mammals. Nitrate is synthesized by mammals fed a diet containing no nitrogen. Can nitrite be produced in vivo from reduced forms of nitrogen?

We have found that $^{13}NO_2^-$ is either oxidized to $^{13}NO_3^-$ or reduced to $^{13}NH_4^+$ or neutral products. The oxidation pathway from NH_4^+ to NO_2^- was considered as a possible source of NO_2^-; however, no evidence for oxidation of $^{13}NH_4^+$ to $^{13}NO_2^-$ or NO_3^- was observed.

(11) Does an intratracheal (IT) bolus of added carrier effect the rate of metabolism of $^{13}NO_2^-$? Nitrogen-13 from no-carrier-added (NCA) $^{13}NO_2^-$ distributes uniformly throughout the biological fluids of the mouse within minutes, most of the $^{13}NO_2^-$ is rapidly oxidized to $^{13}NO_3^-$, and a fraction is chemically reduced to other products. Previous experiments showed little effect on these processes by the addition of carrier NO_2^-. Both in vivo and in vitro experiments were conducted to verify these results. With carrier levels as high as 60mg/kg, the half life for nitrite oxidation increased by no more than a factor of two and was on the order of 7-10 min.

(12) Is $^{13}NO_2^-$ metabolism affected by previous chronic administration of carrier NO_2^-? The bolus results given previously provide valuable insight into the pathways of NO_2 metabolism relating to the ingestion of a large single dose of NO_2^- (as is possible in foods). However, these do not adequately simulate chronic exposure to low levels of NO_2^- or NO_2^- producing air

pollutants. Such chronic exposure could burden the biochemical pathway and decrease the ability to metabolize NO_2^-, or could alternatively sensitize the system to NO_2^-, resulting in more rapid metabolism. Chronic exposure simulation with a new technique utilizing subcutaneous implantation of miniature osmotic pumps was used. These pumps can deliver a wide range of concentrations of NO_2^- solutions at a uniform rate for a period of one to two weeks. This eliminates uncertainties in total dose and dose rate associated with dosing through food or water. The IV route of administration also more closely simulates exposure through the lungs.

(13) Do in vivo results relate to in vitro results, (how can the results in mice be related to humans?). We have extensively studied $^{13}NO_2$ metabolism in the mouse model. If in vitro and in vivo results are directly comparable, as appears to be the case, then the results in mice can be extrapolated to humans by comparison of in vitro results.

(14) Are nitrosamines produced from intratracheal NO_2^-? We have shown that a non-anionic (NA) produce is formed from $^{13}NO_2^-$ by reduction in the intestine. The presence of nitrosation products would have important implications in the consideration of health impact. Studies utilizing high concentrations of radioactivity and expanded HPLC analyses were used to determine the chemical forms of the reduction products. We looked specifically for di-methyl, di-propyl, and di-phenyl nitrosamine formation with a detection limit of 0.01% of the installed radioactivity and did not find them or other compounds expected to have similar chromatographic retention. This does not mean that this hazardous class of compounds is not formed either transiently or permanently, but rather that the specific representatives we looked for were not formed in detectable quantities in our animal model within a time frame of 30 min to 1 hour.

(15) Elucidate the effect of added carrier NO_2^- on the formation of NA. The effect of added carrier up to 60 mg/kg increased the half life for reductive processes in the intestine about 30-40%. These levels did not appear to markedly interfere with the normal functioning of the animal.

This brings us to an important question with respect to CARB sponsored toxicology work by Richters, Kuraitis, and Sherwin (CARB A9-Ø76-31). They found in their main study that a 12 week exposure of C57 BL/6J male mice to NO_2 at Ø.8ppm resulted in greater metastasis to the lungs of blood borne cancer cells. They also observed increased spleen weights in about 2ØØ Swiss/Webster mice exposed for six weeks to Ø.35ppm NO_2 as compared to a like number of controls. The question is,"Does the observed pharmacology form a consistent picture with the observed toxicology?" We believe that it does and give our conclusions in the following summmary model.

General Conclusion: Model of Pharmacology and Toxicology. In this model, 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 (4). 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 innocuous amines within 5-10 min. No nitrosamines were identified in large intestine extracts from exposed mice.

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.

Section IV
RECOMMENDATIONS

1. The large body of existing toxicological data for exposure to nitrogen dioxide appears to be relevant to suspended nirate 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 (ARB-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.

Section V

ANIMAL AND RADIOCHEMICAL EXPERIMENTAL METHODS AND SUMMARY OF EXPERIMENTS PERFORMED

The complete details of cyclotron-based radiochemical methods for routinely producing up to Ø.5 Ci of radioactive N-13 (T 1/2 = 1Ø min) labeled air pollutant analogues, including NO_3^- and NO_2^-, by proton bombardment of a recirculating water target have been previously reported (3-7). A complete description of animal exposure methods and the high performance liquid radiochromatography system for chemical analysis is described in the final report of earlier contract years (1,2). In this report, we describe some newly developed or newly adapted methods in animal experimentation and in radiochemical syntheses and analyses that have dramatically increased the amount of detailed biochemical information that can be obtained with the mouse.

Animal Methods and Experiments. The experimental animals were Balb/C mice and New Zealand white rabbits fed commercial laboratory chows and water and libitum. Intratracheal (IT) instillation of 15ul tracer into mice was done by the Sedgwick and Jahn technique (8). Intravenous (IV) injections were made into a tail vein of mice or ear vein of rabbits. Intraesophageal (IE) instillations into mice were done with a small stomach tube. Organ distribution studies of $^{13}NO_3^-$ and $^{13}NO_2^-$ in mice were completed with 1Ø-12 mice for each combination of anion and route of administration. Measurements were made at time intervals from 5 to 3Ø minutes between injection and sacrifice. Organs were excised within 1Ø min after sacrifice and weighed. The tracer concentrations in the lungs, heart, kidneys, liver, stomach, small intestine, large intestine, bladder, and carcass were determined by gamma ray counting.

In order to extend blood fraction analysis to mice so that detailed chemical evaluation of metabolites would be available for this cost-effective animal in which we had distribution data, we adapted a cardiac-puncture method of blood sampling that had been developed for rats by M. Al-Bayati, D.V.M. of

the Laboratory for Energy-Related Health Research, University of California, Davis. Each mouse was anethesized by an intraperitoneal injection of 0.04 mL sodium pentothal. Anesthesia occured within 10 to 15 minutes, as which time the NO_2^- or NO_3^- was administered intratracheally for the in vivo studies. At a predetermined time after instillation, blood was taken (for in vitro studies blood can be taken at this time).

Because our mice typically weighed only 20-25 grams, small animal surgical procedures were required to access the heart for cardiac puncture. A small hole was made with scissors in the diaphragm, which caused the lungs to collapse away from the diaphragm. This enabled one to use small scissors to cut the diaphragm laterally, thus exposing the heart and lungs. A heparinized syringe with needle was then inserted obliquely into the appropriate chamber of the heart (right ventricle). The plunger was gently pulled back until maximum blood was obtained (0.6 - 1.0 ml). The needle was then removed from the syringe and blood was gently extruded into a centrifuge tube for separation of the plasma and cellular fractions.

Tables 1 and 2 at the end of this section summarize the experiments performed. The flowchart for separation and analysis of blood fractions is given in Table 1. Analysis of homogenized tissue samples followed a similar scheme. Nineteen cyclotron runs were executed during this third contract period for the exposure to synthesized N-13 labeled air pollutants or related compounds. The animal experiments performed during these runs are summarized in Table 2., "Summary of Experiments and Results" on page 31 .

Our activities were divided approximately into four general categories. The first quarter was spent measuring in vivo and in vitro oxidation rates for nitrite in mouse and rabbit blood. The second quarter was occupied with biochemical studies with mice and exploration of methods improvement that would permit us to find out where reduced ^{13}N compounds were produced and what their identity was. The third quarter was devoted to range-finding experiments with mouse tissues, comparison of carrier-added (CA) and no-carrier-added (NCA) effects on metabolic rates, the ratio of oxidized to reduced forms, and optimization of the thin layer electrophoresis biological analysis procedures for mouse studies. The fourth quarter was used to investigate in more detail the rates of metabolic conversion of nitrate and nitrite with mice chronically exposed to nitrite with implanted osmotic pumps, or acutely exposed by bolus instillations (IT) up to 60 mg/kg.

The first step in these experiments is to measure the time-dependent course of distribution for the radiotracer in body tissues. These experiments can be done with radiotracers in a way that is quantitative and accounts for 100% of the administered material. The second step is to measure the chemical form of the radiotracer in all tissues where it achieves an appreciable concentration. This can be done by high performance liquid chromatography using radiation detectors to quantitate the amount of tracer in each chromatographic peak as it is eluted. The biodistribution kinetics and radiochemical analysis experiments together will lead to a complete picture of the in vivo fate of the labeled substance.

The first phase of these experiments involved measurement of the time-dependent tissue distribution of high specific activity $^{13}NO_3^-$ and $^{13}NO_2^-$ in mice. Under these conditions the mass of nitrogenous anion administered to the animal is insignificant compared to its normal in vivo contentration. The result is a pulse-labelling of the existent in vivo pool. Other experiments will compare differences in tissue distribution and radiochemical form of the radiotracers when their specific activity has been diluted with increasing amounts of nonradioactive nitrite or nitrate. The experiments with added carriers step logarithmatically from the normal in vivo concentration of nitrogeous anions up to the very large levels that have been reported to cause cancer. By plotting various biological reponse variables (% ID/gram tissue at various times, radiochemical form in each tissue type, etc.) versus the dose of administered anion, one would expect to see inflection points where the biological handling of the administered substance changes from a low dose regime where it is not destructive of mammalian biochemistry to a high dose regime characterized by potential toxic effects. These series of experiments could be repeated under various conditions of exposure; for example, intravenous, intratracheal, and intraesophageal routes of administration could be compared or administration with added chemicals such as vitamin C. (potential protectors) or secondary amines (potential promoters of nitrosamine formation).

The baseline measurement for all of these comparisons is the pharmacokinetic study of the basic radiotracer anion administered at very high specific activity. In this paper we report our measurements of the

distribution of $^{13}NO_2^-$ and $^{13}NO_3^-$ administered by three routes and studied at multiple times after administration. A large number of animals were tested and careful statistical analysis of the results has been applied to arrive at the best possible data set for further comparison.

Radiochemical Experimental Methods. Our previous methods (1,7) for synthesizing labeled nitrate ($^{13}NO_3^-$) and nitrite ($^{13}NO_2^-$), the principal reagents used in this study, generate about 500 mCi of N-13 in 50 mL of aqueous solution. However, this volume and the 10 minute half-life of N-13 restrict the complexity and duration of tracer biochemical experiments because the total activity that can be introduced into a small 20-30 gram mouse is inadequate. J.H. Chasko and J.R.Thayer of our research group at Crocker Nuclear Laboratory University of California, Davis, have developed a radioactivity concentration system (5) that eliminates the small animal restriction and also permits improved biochemical evaluations with larger 2-3 kilogram animals such as rabbits.

Nitrogen-13 is produced by the $^{16}O(p,alpha)^{13}N$ nuclear reaction of a 20-A beam of 20-MeV protons on recirculating water target to yield ~500 mCi of ^{13}N (90-97% NO_3^-, 3-10% NO_2^-, 0.5-2% NH_4^+) in 50 mL of solution as previously described (6). The target solution is pumped from the cyclotron vault through a neutral alumina column for removal of a radioactive contaminant, ^{18}F-flouride ion (6), to 60 mL reservoirs in a lead shielded hood containing the concentration system.

This system produced aqueous $^{13}NO_3^-$ or $^{13}NO_2^-$, as needed for radiotracer experiments. The target solution is concentrated without pretreatment for $^{13}NO_3^-$ production, and contaminating $^{13}NO_2^-$ is removed after concentration by chemical oxidation or by chromatography. For $^{13}NO_2^-$ production, the target $^{13}NO_3^-$ is first reduced to $^{13}NO_2^-$ with a copperized cadmium reduction column (6), which may further reduce the $^{13}NO_2^-$ to $^{13}NH_4^+$, resulting in 1 to 20% $^{13}NH_4^+$ depending on conditions. The reduced solution is then concentrated, rinsed to remove the $^{13}NH_4^+$, eluted, and taken for biological experiments. Times and flow rates in the procedure will vary according to the experiments.

The target or reduced target solution flows from the reservoir through a six-way Teflon solvent selection valve (Rheodyne, Inc.), to high pressure pump (Simplex 3967-57 Milton Roy Mini-pump, Laboratory Data Control) by gravity and inlet suction. The solution is then pumped through a pair of cartridge

mini-columns (4.6 mm x 3.0 cm MPLC, Brownlee Labs) packed with a microporous, silica based, high performance anion exchanger (Partisil-10 SAX, Whatman, Inc.), which retains the radioanions. The MPLC Cartridge System consists of a cartridge holder in which two 3-cm guard columns are butt-joined together with a cartridge union fitting. This permits facile disassembly for column replacement without disturbing the high pressure, low volume, end fittings. The effluent from the column is routed to a fraction collector (Model FC ^{80}H, Gilson Medical Electronics) with a drain to a waste resevoir. The residual cationic and neutral compounds are rinsed from the columns with water. Direct collection of the concentrate is done by switching the selection valve to a high ionic strength eluant for elution of the radioactivity. The pump speed is reduced to prevent spillage of activity between collected fractions. As the concentrated radioanions appear at the detector, the fraction collected is advanced to collect 0.6 mL fractions. Concentrated $^{13}NO_2^-$ is used without further purification. Concentrated $^{13}NO_3^-$ target solution is treated with H_2O_2 (3% final concentration) at pH 1.8 for 2 minutes to oxidize trace $^{13}NO_2^-$ to $^{13}NO_3^-$ (7). The solution is neutralized with NaOH containing phosphate buffer; peroxide is destroyed with catalase (Boehringer Manheim GmbH analytical grade, crystalline.)

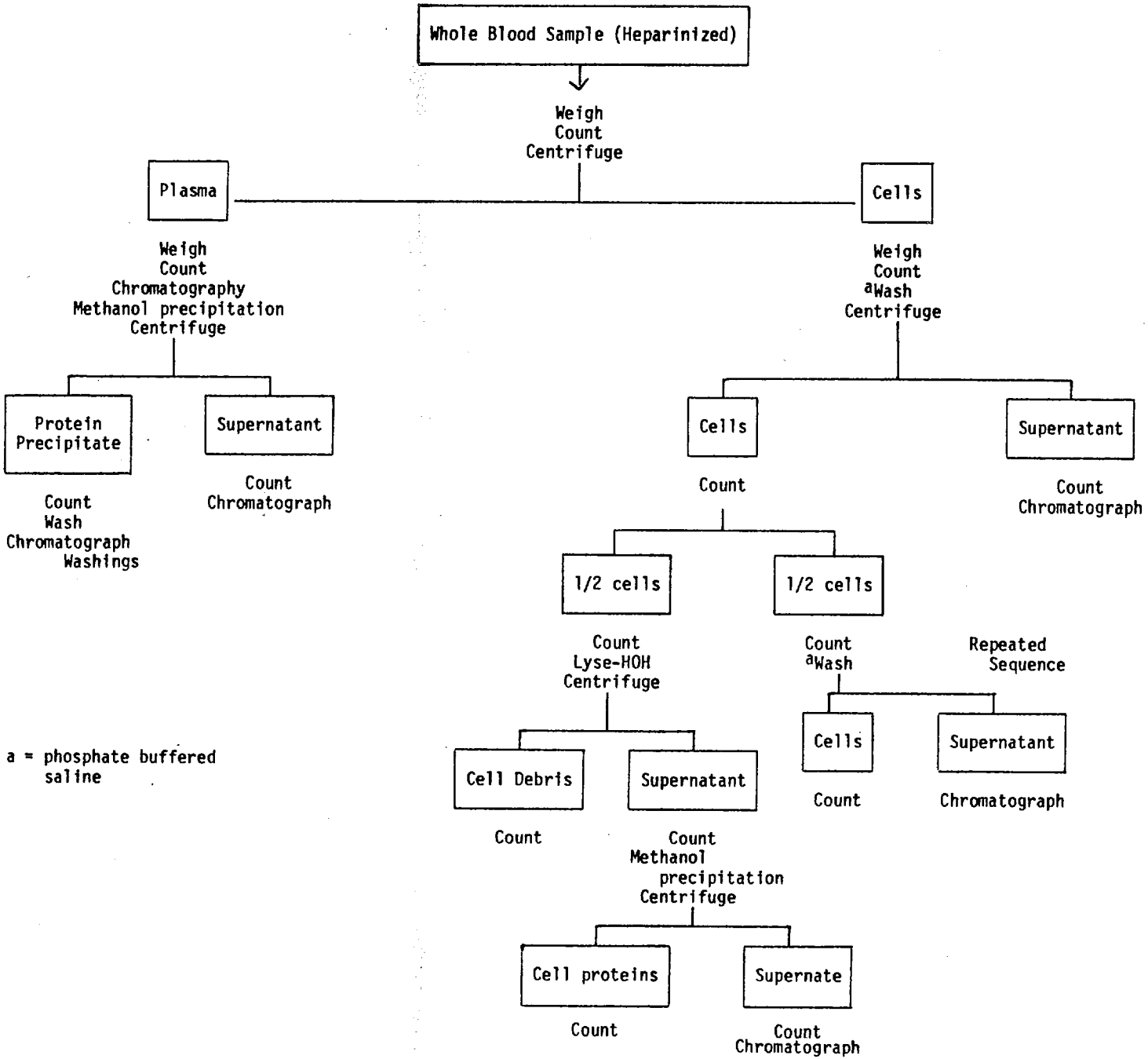
For animal experiments, normal saline (0.9% NaCl) is used to remove the ^{13}N-labelled anions from the mini-columns. The mini-columns are prepared for reuse by rinsing with distilled water to remove residual saline. Saline is used to elute the radioanions from the mini-columns; typically 140 mCi of $^{13}NO_3^-$ or 90 mCi of $^{13}NO_2^-$ are delivered in a volume of 0.6 mL within 10 to 12 minutes after end of bombardment. This corresponds to radioactivity concentrations of 230 mCi/mL $^{13}NO_3^-$ and 150 mCi of $^{13}NO_2^-$. Concentration of target solutions followed by collection of the most active 0.6 mL fraction have resulted in 20-fold net increases in concentration of $^{13}NO_3^-$ (or 40-fold if decay corrected).

The radiochemical purity of the products if usually 99% or more. The procedure effectively removes $^{13}NH_4^+$; the 20% $^{13}NH_4^+$ sometimes present in reduced target solutions is decreased to 0.2 to 1.0% of the radioactivity. The radiochemical purity of $^{13}NO_3^-$ from target solution is increased from 90 to 99% by removal of $^{13}NH_4^+$ and peroxide oxidation of $^{13}NO_2^-$.

With the instillation of optical detectors on the liquid chromatography system that span the wavelength region from 190 to 700 nanometers, we have

achieved the important goal of measuring non-radioactive nitrate and nitrite concentrations in some biological fluids (10). In aqueous media we can measure nanogram quantities of these anions, thus permitting us to measure the ubiquitous background concentration of nitrate and nitrite that reduces the specific activty of our labeled material from true "carrier-free" to about $10^3 - 10^4$ Ci per millimole for nitrate and about $10^4 - 10^5$ Ci per millimole for nitrite. Nitrate (NO_3^-) can be measured optically in blood serum with present separations. Nitrite can be measured also but the precision of the measurements is much more strongly affected by interferences in biological samples than is nitrate. The capability of making measurements of nonradioactive nitrate and nitrite concentrations in necessary in chemical toxicology experiments such as the present one in order to insure that the physiological system under study is not perturbed, or is pharmacologically perturbed in a controlled way.

Table 1. Blood Fractionating Scheme



a = phosphate buffered saline

TABLE 2

SUMMARY OF EXPERIMENTS AND RESULTS

First Fiscal Quarter -1980

Date	Type of Experiment	Results
14 Aug 1980		
	Continued In Vitro pilot studies of mouse blood fraction chromatography completed and extended to IT experiments with $^{13}NO_2^-$ (12 mice)	In Vitro: 10 min incubations with $^{13}NO_2^-$ and whole blood oxidation to $NO^{13}is_3^-$total in blood cell fluid. Protein associated activity measured. In vivo: Oxidation of $^{13}NO_2^-$ in blood parallels in vitro. Confirmed formation of small fraction of ^{13}N (< 10%) as non-anionic species in vivo.
21 Aug 1980		
	Completion of activity distribution from $^{13}NO_2^-$ given IT; activity distribution from $^{13}NO_3^-$ given IT; explore nature of	N from both $^{13}NO_2^-$ and NO_3^- precipitate with various protein fractions. Activity removed by repeated washing. Label introduced (IT) as $^{13}NO_2^-$

Date	Type of Experiment	Results
	binding to proteins in different fractions. (12 mice)	less easily washed from blood cells than $^{13}NO_3^-$, but both are removed by aqueous washes.
12 Sept 1980		
	Kinetics study of in vivo mouse oxidation of $^{13}NO_2^-$ for comparison with in vitro kinetics. IT $^{13}NO_2^-$; 6 mice, 6 - 25 min incubation times.	In vivo oxidation proceeds at the same rates as in vitro.
	Comparison of in vitro oxidation kinetics in plasma and blood cell fluid between mouse and rabbit. Rabbit: 6 samples, 5 - 21 min. Mouse: 6 samples, 5 - 21 min.	Cell fluid in both mouse and rabbit shows almost total conversion to $^{13}NO_3^-$ (85-100%). Plasma oxidation of $^{13}NO_2^-$ in vitro is slower in the rabbit blood than in the mouse.
26 Sept 80		
	Comparison of in vitro blood oxidation kinetics of $^{13}NO_2^-$	The oxidation is slower in

Date	Type of Experiment	Results
	between human and mouse. Mouse: 7 samples, 5 - 30 min. Human: 8 samples, 5 - 30 min.	in the human blood than in the mouse.
10 Oct 80	Repetition of previous experiment comparing mouse and human. Human: 10 samples, 2 - 30 min. Mouse: 6 samples, 1 - 15 min.	
18 Nov 80	Comparison of in vitro blood oxidation kinetics between human and rabbit. Sampling method changed from discrete samples for each time point to serial samples taken from a single pool of blood to which $^{13}NO_2^-$ has been added.	Rabbit blood oxidizes NO_2^- slower than human. The serial sampling technique yields smoother kinetics plots than the discrete method.

TABLE 2: Summary of experiments performed during the second quarter (Dec 1 1980 - Mar 31 1981) of the third contract year.

Date	Type of Experiment	Results/Comments
9 Dec 1981	1) To determine if intestinal microflora are metabolic source of non-anionic (NA) ^{13}N-labeled NO_2^- metabolites.	a) penicillin treated (transiently abolishes microflora) showed variable (0-2%) N.A. (2 mice). b) normal mice showed 4-6% of plasma activity as NA, and >99% of intestinal activity as NA. (5 mice). c) Exenterated (intestines removed) mice showed no plasma NA (2 mice). d) In vivo intestinal ^{13}N included NH_4^+ and other "neutral" components
	2) To determine some of the in vitro/in vivo products of $^{13}NO_2^-$ administration using anion and cation exchange HPLC.	e) in vitro products of $^{13}NO_2^-$ in excised mouse intestines:

TIME exposed	NH_4^+	NEUT	NO_2^-	NO_3^-
added (0)	<.02	<.01	94%	6%
30 sec	36%	ND	60%	4%
60sec	25%	ND	66%	1%
120 sec	84%	ND	15%	1.4%
5 min.	98%	1.7%	ND	ND

15 Dec 1980 to 1 Feb 1981 Cyclotron Maintainance: We used this period to assemble a remote control $^{13}NO_3^-/^{13}NO_2^-$ concentration system.

17-Feb-81	A) To compare conventional and germ-free mice for production of ^{13}N-NA from $^{13}NO_2^-$. B) to further characterize products of $^{13}NO_2^-$ metabolism.	A) No NA products formed in any tissue from GF mice, only NO_2^- and NO_3^- detected. Control mice showed 2-4% plasma NA, 72-93% intestinal NA, and 3.3-7% urine NA. B) Plasma ^{13}N-NA was mainly neutral components, up to 15% glutamate (GLU). Intestinal NA included a mixture of $^{13}NH_4^+$ and neutral components.

DATE	TYPE OF EXPERIMENT	RESULTS/COMMENTS
26-Feb-81	1) Determine if $^{13}NO_2^-$ is made in the intestine from $^{13}NO_3^-$. 2) Does addition of carrier to $^{13}NO_2^-$ or $^{13}NO_3^-$ affect the biodistribution or metabolism of pulmonary ^{13}N.	A) up to 0.4 mg added carrier NO_2^-. No change in activity per gm was detected for any organ except in blood, where an increase in carrier resulted in an increase in blood ^{13}N activity. B) No intestinal NO_2^- was detected in mice instilled with $^{13}NO_3^-$ at any added carrier from 0 to 0.6 mg (ie. 0 to 26.5 mg/kg body wt.)
13-Mar-81	To determine effect of addition of a bolus of added carrier on: A) Biodistribution of $^{13}NO_3^-$ B) Chemistry of product ^{13}N from $^{13}NO_3^-$. C) Biodistribution of $^{13}NO_2^-$. D) Chemistry of product ^{13}N from $^{13}NO_2^-$.	A) On a per gram basis, the $^{13}NO_3^-$ remaining in the lungs increases with increasing carrier. B) Scatter of values as NH_4^+ NA, NO_2^- and NO_3^- in tissues. No clear correlation of chemical form with increasing carrier NO_3^- (6 mice). C) On a per gram basis distribution of ^{13}N from $^{13}NO_2^-$ shows little carrier effects. Activity in the heart, and lung may decrease with increasing time or carrier NO_2^-. D) Addition of carrier NO_2^- apears to slow $^{13}NO_2^-$ destruction in blood, stomach, liver, heart and intestine.

SUMMARY OF EXPERIMENTS PERFORMED DURING THE

THIRD QUARTER OF THE THIRD CONTRACT YEAR

Date - Type of Experiment	Results
13-April-81	
A) In vivo test for oxidation products of $^{13}NH_4^+$ in mice. Intratracheal (IT) administration of no-carrier-added (NCA) $^{13}NH_4^+$ to 2 mice; incubation periods of 20 min and 38 min.	A) No $^{13}NO_2^-$ or $^{13}NO_3^-$ was detected in plasma, intestine, liver, or heart. Most of the $^{13}NH_4^+$ was converted to organic non-anionic products.
B) Attempt to synthesize ^{13}N labelled dimethyl-nitrosamine by reaction of carrier-added (CA) $^{13}HNO_2$ and dimethylamine.	B) No labelled nitrosamine was produced.

Date - Type of Experiment	Results
C) Determine if intra-esophegeal (IE) administration of NCA $^{13}NO_2^-$ forms nitrosation products in the gastro-intestinal tract of the mouse; 2 mice, incubations of 20 and 40 min.	C) No cationic nitrosation products were detected at the 0.1% level.
D) Confirm the observation that the addition of carrier NO_2^- to IT administered $^{13}NO_2^-$ results in an increased residence time of $^{13}NO_2^-$ in the intestine of the mouse; 3 mice with NCA and 2 with 10mg/kg added carrier. Incubation times 20 min.	D) A bolus of carrier decreases the rate of reduction of $^{13}NO_2^-$ in the intestine resulting in a longer residence time.
29-May-81	
A) Determine the effect of a bolus of CA IT $^{13}NO_2^-$ (60mg/kg) on the organ distribution of ^{13}N and on the chemical identity; 6 mice CA, 3 mice NCA.	A) Added carrier increases the rate of ^{13}N transport out of the lung. Distribution in other organs is unaffected. The rate of oxidation of $^{13}NO_2^-$ to $^{13}NO_3^-$ may be decreased.

Date - Type of Experiment	Results
B) Use anion, cation exchange HPLC and high-voltage electrophoresis to identity the chemical forms of the non-anionic (NA) ^{13}N species produced in mice after IT administration of CA and NCA $^{13}NO_2^-$ by IT administration; 2 mice CA, 2 mice NCA.	B) The NA component consists of several compounds; primarily $^{13}NH_4^+$ and amino acids or urea. No cationic labelled species appear in the plasma. Added carrier has no significant effect on the relative amounts of the various NA products.
19-June-1981 and 25-June-1981	
A) Inter-species comparison of $^{13}NO_2^-$ oxidation to $^{13}NO_3^-$ in mouse, rat, and human blood in vitro. Blood samples were incubated with CA or NCA $^{13}NO_2^-$ for 30 min.	A) Mouse, rat, and human blood produce very similar results. The rate of $^{13}NO_2^-$ oxidation is not very different between the 3 species, and the chemical species formed are much the same. Rat blood appeared to show a greater decrease in oxidation rate with added carrier NO_2^-.

Date - Type of Experiment	Results
B) Samples were withdrawn at regular intervals and separated into plasma, cell material, and cell fluid to determine activity distribution and chemical form.	B) Small amounts (1.5-3%) of $^{13}NO_2^-$ were found in cell fluid, contrary to previous experiments using lower levels of radioactivity. Non-anionic species appear to be present in cell fluid but not plasma.
15-July-1981	
A) Continuation of bolus added carrier study in mice. Labelled NO_2^- was administered IT at levels of 0, 2 and 10mg/kg of added carrier (2 mice at each level). Incubations were 15-30 min followed by organ distibution and anion and cation exchange chromatography of plasma, liver, stomach, and intestine.	A) Preliminary analysis indicates verification of above carrier added results.

Date - Type of Experiment	Results
30-July-81	
A) Continuation of carrier study in mice. This experiment initiated a new method utilizing subcutaneous miniature osmotic pumps to deliver sub-acute to acute levels of NO_2^- for a period of one to two weeks.	A) The mice were not affected by the lower levels of nitrite administration. The mice at the highest level suffered weight loss and yellow discoloration of hair. Cold measurements of urine showed increased nitrate levels. Most of the administered nitrite was excreted as nitrate in urine.
B) No-carrier-added $^{13}NO_2^-$ was then administered to see if these levels affect the organ distribution or chemical distribuition of the tracer. Nitrite was delivered to 16 mice at levels from 0-200 mg/kg/day for six days. The mice were then given NCA $^{13}NO_2^-$ by IT administration and sacrificed after 20-30 min. The organ distribution of ^{13}N was measured and anion, cation exchange HPLC done on plasma and intestine.	B) The organ distribution of ^{13}N was not significantly affected by the chronic nitrate administration. The chemical form of the ^{13}N in plasma was unchanged, while in the intestine the reduced fraction was apparently different. However, no significant trend was observed with increasing chronic dose levels. Results not conclusive.

SUMMARY OF EXPERIMENTS PERFORMED IN THE
FOURTH QUARTER OF THE THIRD CONTRACT YEAR

DATE	PURPOSE	RESULTS
Aug 5-12	Administration of low medium and high dose of $NaNO_2$ to mice for 1 week by implanted osmotic pumps. Determine effect of chronic carrier on biodistribution and chemistry in plasma & intestine. Vit C effect at high dose [16 mice, 3 died early].	Biodistribution: higher %/gm in blood, lung, kidney, heart and liver. % RD most in carcass 11-15% in intestine, etc. Chemistry: Plasma; no cationic ^{13}N in saline, 3.5 mg, or 350 mg & Vitc mice. 0.2-0.3% of ^{13}N cationic in 35 and 350 mg NO_2^- mice. NO_2^- clearance increases from sal to 35 mg NO_2^-/ml in pump decreaes again at 350 mg/ml. Intestine: Cationic fraction increases when Vit C is present. Neutral fraction decreases. Conversion of NO_2^- to NA + NO_3^- decreases with increasing NO_2^- in pump.
Aug 19	A) Determine distribution of intestinal N-13 in small and lg intestinal tissue & contents and if Chemistry or distribution shift between 15 & 30 min.	A) The fraction of intestinal N-13 in the small intestinal content varied from 8-12% (@ 30') to 16-17% (at 15 min.); that in the small intestinal tissue decreased from 45-54% (15 min) to 36-39% (30 min); that in the large intestinal contenst shifted from 14-17% at 15 min to 20-39% (at 30 min); that in the large intestinal tissue varied from 16-23%

DATE	PURPOSE	RESULTS
	B) Determine chemical form of N-13 in these tissues by HPLC and in cecum by HPLC and TLE. C) Can we condense $^{13}NO_2^-$ with aminopyrine in acid to synthesize dimethyl nitrosamine? D) does addition of carrier N in the cecum (lg intest) "trap" the ^{13}N compounds metabolized there? (or, do these components exist in an exchangable pool in the intestine?) (8 mice).	with no apparent time dependence. Tissue had 16% NH_4^+ ~12% neut and time dependent NO_2^- (17-39%) and NO_3^- (35-52%) small contents had ~8% NH_4^+, ~4% neutral and higher, but still time dependent NO_2^- (73-43%) and NO_3^- (16-44%) large contents had 50% NH_4^+, 50% neut and essentially zero NO_2^- and NO_3^-. B) What is chemical form of anionic component in large intestinal contents (cecum)? 41.8%, NH_4^+, 4.4% GLN, 2.1% urea, 51.1% GL4, 0.2% NO_2^- and 0.3% NO_3^-. (no NH_4^+ is observed in plasma of these mice). C) Several cationic N-13 labeled deriviatives were formed. Up to 44% of the label was found in one peak which corresponded to the elution positions of standard nitrosamines, purification and yield variable. D) Addition of carrier NO_3^- to the mouse cecum traps $^{13}NO_3^-$ there (88% vs 37% for NCA) carrier NH_4^+ tends to trap $^{13}NH_4^+$. Therefore, these are likely free or exchangeable metabolites in the mouse cecum.

DATE	PURPOSE	RESULTS
Aug 26	$^{13}NO_3^-$ and $^{13}NO_2^-$ metabolic product determination (HPLC +/- urease, with TLE of selected organs) intestinal compartments analyzed separately.	(A) $^{13}NO_3^-$ Plasma: 1-2% urea, 7% NO_2^-, 92% NO_3^- Liver: 7% ARG, 5% urea, rest NO_3^- Lg Intest: 14% NH_4^+, 3-5% GLN, urea, 77% GLU Sm Intest: Ø-2% NH_4^+, 1-3% urea/neut. AA's 7-16% NO_2^-, rest as NO_3^- Whole Int. Tissue: 13% urea/neut. AA's, 4% NO_2^-, 83%NO_3^-. (B) NO_2^- Plasma: Ø-3% NH_4^+, 1% neut. AA/urea, 17% NO_2^-, 79% NO_3^-. Sm Intest: 12% NH_4^+, urea/neut. AA's, ~29% NO_2^-, ~59% NO_3^-
Sept 21	Final experiment of project. Complete Aug 26 expt. and measure metabolite distribution in animals chronically exposed with osmotic pumps.	Previous results confirmed. No new products. Some changes of conversion rates and product distribution.

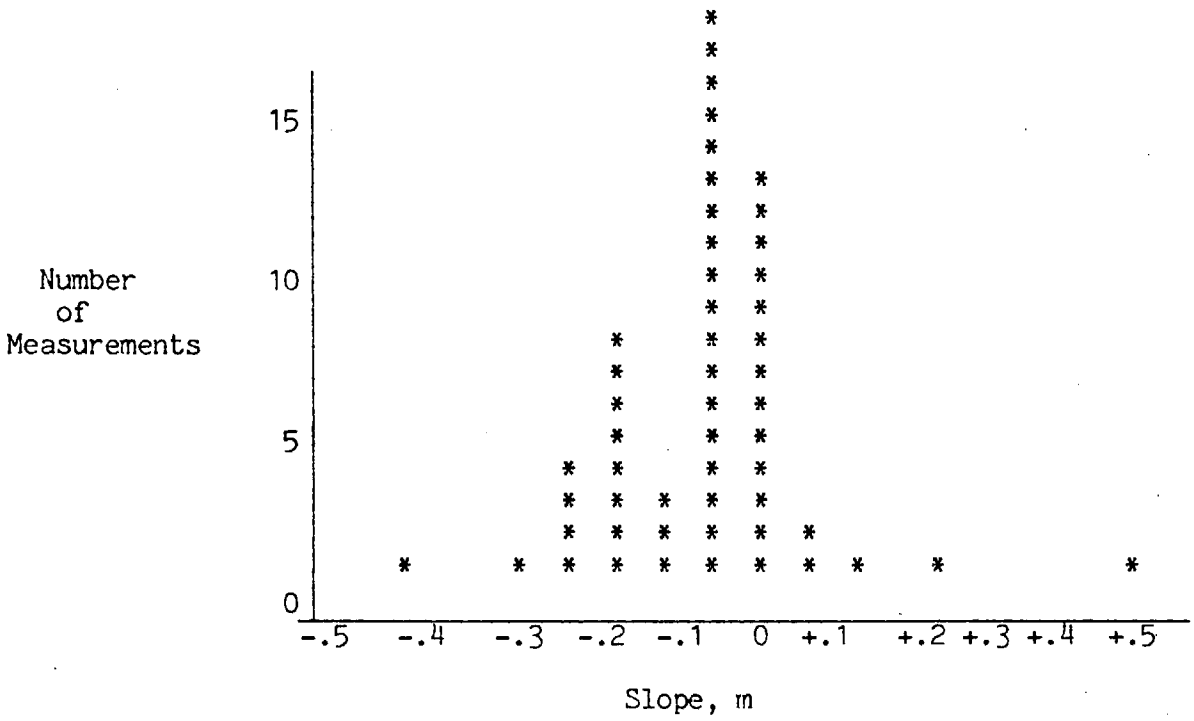
Section VI
RESULTS

The results section includes an analysis of all the organ distribution studies performed during the last three contract years with tracer nitrogen introduced by different routes and in different chemical forms. We also include the results from a similiar matrix of experiments for tracer distribution among the cells and fluids of blood. The results of in vitro NO_2^- oxidation rate measurements with human and rabbit blood are given. The chemical product spectrum represented by metabolites of nitrite and nitrate and their rates of formation or disappearance are given. The last results presented describe the influence of chronic carrier exposure from implanted osmotic pumps on distribution and metabolism.

Organ Distribution. We first analyzed all the organ distribution data taken at different times by the simple linear least squares regression model of % ID(t) = mt + b where % ID(t) is the percent of dose in each organ at time t, and m and b are the calculated slope and intercept, respectively. The slopes for the combinations of organ, tracer ion and route of administration are shown in the histogram (Figure 1). The distribution centers around zero and demonstrates that there were no significant variations with time in the radioctivity distributions from 5 to 30 minutes after carrier-free $^{13}NO_3^-$ or $^{13}NO_2^-$ was administered intratracheally (IT), intravenously (IV) or intraesophageally (IE). The exceptions, not included in Figure 1, were the lung and stomach/intestines when they represented the initial organ of entry for the labeled tracer. For example, following intraesophageal gavage, the stomach showed some tendency toward clearance (m = -0.56) with a complimentary increase to intestinal activity (m = +0.52).

Clearly there must be some time dependency in the distribution kinetics because initially all of the tracer is isolated at the site of administration and none is in the other organs. However, the time-dependent flux of tracer achieves dynamic equilibrium very rapidly when administered by any of the three routes. It is slightly slower from the lung, but in all instances these half-times are of the order of a few minutes or less and cannot be determined

Figure 1. Time dependency of organ distributions. Each data set was fitted to a Y (time) = mX + b function using a linear least squares regression program. The calculated slopes, m, were closely centered around zero and showed that from 5 to 30 min after injection our measured distributions did not vary with time.



any more precisely by our data which begins at 5 minutes after injection.

Because linear regression analysis of the time-dependence of the distributions showed slopes of essentially zero, the data could be combined at all times to test for the normality of distributions. When measurements at different times and routes were Z-transformed and combined, each data set included 30-45 measurements and was tested for normality. Only measurements of % ID/organ were included in this analysis. The third moments were all positive, right skewed, but were all a $\leq$ 0.7 except for the lungs where a values of 1.11 (NO_2^-) and 1.57 (NO_3^-) were dominated by the IT data sets. The fourth moments ranged from 1.46 to 4.67 with a mean of 3.05$\pm$0.98 and Geary's statistic ranges from 0.725 to 0.919 with a mean of 0.798 $\pm$ 0.054. The normal means for these measures of kurtosis are 4.0 for a_4 and 0.889 for g with 95% confidence intervals for n=35 of 2.06 and 4.18, respectively. Thus, the distributions are slightly right skewed and platykurtic, but are sufficiently close to gaussian to be subjected to statistical comparison.

The analysis of variance results are summarized in tables 3-7. The first two give the detailed summary of 2-way ANOVA for each organ and each organ reported on a per-gram basis. The third and fourth tables give the measured mean values and standard deviations when data for all times are combined. Included in karats is the adjusted mean when time covariance is included. This number is the best measure of central tendency because there is a small time factor and there were some differences in the number of data sets per time point in our measurements. The numbers in these two tables will be the reference for our comparisons in later manuscripts.

The last table summarizes the 3-way ANOVA and also gives the probability associated with each F-ratio. The per organ analysis was done with and without including the remaining carcass because its large mass may totally dominate the analysis.

BIOLOGICAL INDICATIONS FROM STATISTICAL ANALYSIS

What pharmacokinetic differences exist as identified by analysis of variance? A review of the NO_3^-/NO_2^- comparisons shows that seven of the 2-way conparisons were significant at the 10% level but nine were not. Of these ogans where difference was suggested by P<10%, the differences did not hold up

Table 3.

^{13}N Distribution Data: ANOVA for Per Organ

ORGAN	Model R^2	Adjusted Means			F Ratios a.(P Values)				F Ratios for Route Contrast			
		IV	IE	IT	Cov	b. NO_3^-/NO_2^-	c. Route	d. Interaction				
Carcass n=88	33.0	61.6	43.9	60.1	.02	.40 (.53)	15.4 (10^{-6})	2.08 (.027)	IV/rest IE/rest IT/rest	9.27 30.8 6.44	IV/IE IE/IT IT/IV	23.2 23.4 .20
Lungs n=88	26.2	1.49	1.45	4.48	1.26	.91 (.34)	12.1 (10^{-5})	.27 (.992)	IV/rest IE/rest IT/rest	4.97 5.27 24.2	IV/IT IE/IT IV/IE	17.0 17.2 .002
Kidneys n=84	8.53	3.28	2.73	3.21	.72	3.2 (0.077)	1.26 (.29)	.08 (.999)	IV/rest IE/rest IT/rest	.92 2.52 .46	IV/IE IE/IT IT/IV	2.10 1.78 .035
Liver n=86	25.1	8.69	6.45	9.70	5.89	.74 (.39)	6.72 (.002)	.43 (.947)	IV/rest IE/rest IT/rest	.59 11.5 7.94	IV/IE IE/IT IT/IV	5.57 13.2 1.28
Stomach n=84	63.4	3.25	31.4	3.69	.58	3.61 (.061)	63.1 (10^{-17})	6.15 (10^{-7})	IV/rest IE/rest IT/rest	3.50 126 36.5	IV/IE IE/IT IT/IV	93.9 103.1 .03
Sm. Int. n=79	2.76	7.52	8.92	8.41	.02	.64 (.43)	.57 (.57)	.21 (.997)	IV/rest IE/rest IT/rest	.24 .38 .03	IV/IE IE/IT IT/IV	1.11 .17 .05
Lg. Int. n=74	21.4	5.70	3.57	7.66	.23	.96 (.33)	8.37 (10^{-4})	1.03 (.43)	IV/rest IE/rest IT/rest	.01 13.2 12.2	IV/IE IE/IT IT/IV	4.77 16.7 3.94
Total Int. n=85	4.43	13.2	12.5	15.0	.22	.44 (0.51)	1.14 (.32)	.10 (.999)	IV/rest IE/rest IT/rest	.13 1.03 2.14	IV/IE IE/IT IT/IV	.14 2.06 1.09
Gut n=81	54.4	16.4	43.7	18.4	.13	3.93 (.051)	41.4 (10^{-13})	4.97 (10^{-6})	IV/rest IE/rest IT/rest	27.3 82.8 18.9	IV/IE IE/IT IT/IV	66.5 62.1 .41

a = Probability, b = Chemical form, c = IT,IE,IV, d = Interaction of form and route

Table 4.

^{13}N Distribution Data: ANOVA for Per Gram

ORGAN	Model R^2	Adjusted Means			F Ratios [a] P.Values				F Ratios for Route Contrast			
		IV	IE	IT	Cov	[b] NO_3^-/NO_2^-	[c] Route	[d] Interaction				
Col 4 Carcass n=84	22.6	4.15	2.94	8.50	.29	4.11 (.046)	6.54 (0.0023)	2.50 (.0074)	IV/rest IE/rest IT/rest	10.18 10.43 .03	IV/IE IE/IT IT/IV	13.07 3.51 4.12
Col 5 Lungs n=86	30.6	9.51	8.65	23.4	4.11	6.31 (.014)	8.95 (.0003)	.96 (.49)	IV/rest IE/rest IT/rest	2.83 4.55 17.66	IE/IT IE/IT IT/IV	.04 13.97 10.91
Col 6 Kidneys n=82	13.7	9.05	8.12	7.94	.47	9.44 (.003)	.58 (.56)	.83 (.62)	IV/rest IE/rest IT/rest	1.11 .16 .54	IV/IE IE/IT IT/IV	.68 .03 1.08
Col 7 Liver n=84	17.5	6.48	5.04	7.55	1.03	.004 (.95)	6.2 (.0031)	.51 (.90)	IV/rest IE/IT IT/rest	.08 9.12 8.30	IV/IE IE/IT IT/IV	3.29 12.3 1.96
Col 8 Stomach n=85	54.1	10.27	65.0	9.78	1.65	.31 (.58)	40.4 (10^{-13})	.30 (.99)	IV/rest IE/rest IT/rest	17.6 79.0 23.8	IV/IE IE/IT IT/IV	52.8 68.7 .005
Col 9 Sm. Int. n=77	3.54	5.22	5.64	5.23	.65	.34 (.56)	.18 (.84)	.62 (.82)	IV/rest IE/rest IT/rest	.09 .36 .08	IV/IE IE/IT IT/IV	.29 .27 .0002
Col 10 Lg. Int. n=74	20.6	8.18	4.37	6.58	.10	3.65 (.060)	7.32 (.0013)	.10 (.999)	IV/rest IE/rest IT/rest	9.34 12.39 .12	IV/IE IE/IT IT/IV	14.38 5.01 2.44

a = Probability, b - Chemical form, c = IT,IE,IV, d = Interaction of form and route

Table 5

^{13}N Distribution Data: Mean Uptake in Organs
Percent injected dose per organ (std. dev.)
<adjusted mean from ANOVA with time covariate>

ORGAN	IV		IE		IT	
	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-
Carcass	64.5(7.9) <64.4>	58.9(7.1) <58.8>	39.4(15.7) <39.5>	48.3(16.0) <48.4>	59.0(11.2) <59.0>	61.1(11.5) <61.1>
Lungs	1.55(1.14) <1.42>	1.57(0.56) <1.55>	1.18(0.44) <1.24>	1.54(0.78) <1.67>	3.88(3.92) <3.90>	5.12(4.58) <5.06>
Kidneys	3.10(0.77) <3.05>	3.51(1.93) <3.51>	2.47(0.91) <2.50>	2.90(1.34) <2.95>	2.83(1.08) <2.86>	3.60(1.71) <3.56>
Liver	9.79(3.23) <9.45>	7.96(3.49) <7.93>	6.55(2.56) <6.70>	5.84(1.89) <6.19>	9.91(3.79) <9.63>	9.89(4.62 <9.76>
Stomach	2.14(1.09) <2.46>	4.02(2.15) <4.04>	39.3(14.5) <39.3>	23.8(19.8) <23.5>	3.08(1.84) <2.99>	4.33(1.95) <4.40>
Sm Int	8.06(3.29) <8.04>	7.00(2.90) <7.00>	9.73(8.42) <9.74>	8.06(4.64) <8.10>	8.40(1.77) <8.38>	8.46(4.37) <8.45>
Lg Int	5.64(2.89) <5.58>	5.83(2.70) <5.83>	3.64(2.01) <3.67>	3.39(1.89) <3.47>	8.90(6.77) <8.86>	6.48(3.63 <6.46>

TABLE 6

^{13}N Distribution Data: Mean Uptake per Gram

Percent Injected dose per organ (std. dev.)
<adjusted mean from ANOVA with time covariate>

	IV		IE		IT	
ORGAN	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
Carcass	4.13(0.80) <4.11>	4.21(0.92) <4.20>	2.28(1.17) <2.28>	3.58(1.73) <3.61>	3.43(0.72) <3.43>	3.59(1.00) <3.58>
Lungs	6.81(1.36) <5.56>	13.97(12.36) <13.47>	6.60(3.71) <6.90>	8.99(4.09) <10.40>	15.99(17.87) <16.25>	31.03(27.08) <30.60>
Kidneys	7.91(1.99) <7.82>	10.31(4.80) <10.28>	6.08(2.37) <6.12>	10.00(4.92) <10.13>	7.20(2.29) <7.23>	8.73(4.87) <8.65>
Liver	7.06(1.89) <6.95>	6.06(2.33) <6.01>	4.86(1.85) <4.89>	5.06(1.89) <5.19>	7.26(2.07) <7.28>	7.85(4.38) <7.81>
Stomach	5.70(2.34) <7.00>	13.04(9.10) <13.53>	66.7(43.4) <66.5>	65.0(45.3) <63.5>	7.04(2.05) <6.73>	12.40(9.67) <12.83>
Sm Int.	5.17(1.28) <5.25>	5.17(2.25) <5.19>	6.34(5.16) <6.31>	5.08(2.24) <4.97>	5.06(21.29) <5.10>	5.34(2.34) <5.37>
Lg Int.	7.09(3.76) <7.14>	9.20(4.05) <9.22>	3.73(1.68) <3.71>	5.10(2.33) <5.03>	5.92(2.91) <5.95>	7.21(4.5) <7.22>

TABLE 7

^{13}N Distribution Data: Three Way ANOVA

Source of Variation	df	F-Ratio	P
A. Percent per organ data (Error df = 535)			
NO_3^- or NO_2^-	1	2.17	.14
Organ	6	637.6	(10^{-90}
Injection Route	2	1.058	.35
Interaction	--not considered--		
B. Percent per organ data excluding carcass (Error df = 461)			
NO_3^- or NO_2^-	1	5.07	.025
Organ	5	89.09	10^{-11}
Injection Route	2	50.92	10^{-20}
Interaction	12	112.32	$>10^{-90}$
C. Percent per gram data (Error df - 528)			
NO_3^- or NO_2^-	1	.00071	.979
Organ	6	18.93	10^{-20}
Injection Route	2	3.35	.036
Interaction	12	23.05	10^{-41}

for both per organ and per gram measurements, with the possible exception of the kidneys. Neither of the full 3-way tests were significant at 10%. We conclude that there is no difference between tracer $^{13}NO_3^-$ and tracer $^{13}NO_2^-$ pharmacokinetics in the mouse organs studied.

The P-values for interaction were even more clear in establishing the total absence of interaction between either NO_3^- or NO_2^- and the route of injection. Many of the probabilities approached 1, with differences for only the carcass and the stomach as whole organs. The stomach difference disappeared when ratioed to the organ's weight. This was not surprising because stomach weights were highly variable, especially when the tracer was introduced via that organ. Table 7 includes organ and route data as separate layers in the 3-way analysis of variance and in that case interaction bcomes very important (P~0). One may conclude, however, from comparison of the 2- and 3-way analyses that the important interaction is between route and organ and not between tracer anion and route. The possible interaction between the tracer and the organ was not treated.

The P-values for organ distributions as a function of the route of administration show large differences. Ten of fourteen 2-way comparisons had P-values of 0.003 or less and the 3-way analysis resulted in even lower probabilities. There can be no doubt that the route of injection is a major factor in the distribution of these two N-13-labeled anions.

A more complete understanding of the differences associated with the route of injection comes from the far right hand section of Tables 3 and 4. In this section individual routes are contrasted and each route is contrasted with the average of the remaining two routes. In this table P-ratios of greater than 4 are required for strong evidence of difference ($P<0.05$). Thus the important differences are in IT versus the other routes to the lungs, and in IE versus the other routes in stomach, large intestines and liver. These contrasts are not surprising as they represent the initial organs of result by intratrachial and intraesophageal administration, respectively.

The contrast in the liver is primarily between IE and IT administration and is not as easily explained.

The question of differences between organs seems trivial; it was assumed and not tested by 2-way ANOVA> In the 3-way analyses F-ratios for organs ranges from 19 to 600 with probabilities for this occuring by chance that were zero to ten or more significant figures. The differences between

organs remained when analyzed on a per gram basis, indicating that uptakes were more than a consistent fraction of each organ's weight.

In table 5, we have given the mean and standard error for nitrite and nitrate distribution after IE, IV, or IT administration. These data were taken over a period of 5-30 minutes. In table 8, we have given the organ distribution of nitrite with carrier added material in concentrations up to 60 mg/kg. These animals were sacrificed at 30 minutes. Our analysis of variance showed no significant difference (P=0.997) between the various dose groups which included no-carrier-added. The main finding here is that high levels of carrier do not reveal a dominant target organ that was not seen under NCA conditions.

Blood Fraction Distributions. Blood samples (0.5 - 1.0 mL) were taken by cardiac puncture from anesthetized mice and rabbits 10 min after IT instillation of $^{13}NO_3^-$ or $^{13}NO_2^-$. The samples were separated into cells and plasma by centrifugation. The plasma was fractionated further by precipitation of the plasma proteins (PP) with 50% methanol (MeOH) and the cells were lysed with distilled water and recentrifuged to remove cellular debris (CD). The lysate was treated with an equal volume of MeOH and centrifuged to remove the intracellular protein precipitates (CP). There were slight differences in the blood fraction distributions of ^{13}N from $^{13}NO_3^-$ and $^{13}NO_2^-$. Three washes of precipitates PP and CD in phosphate buffered saline solubilized ~95% of the ^{13}N and indicated minimal incorporation of ^{13}N into high molecular weight products. High voltage electrophoresis of the protein fraction showed a similar small fraction of the activity incorporated into protein. The results upon which the following discussion is based are given in Table 9.

The rabbit was chosen as a second animal species because previous experiments (10) showed increased blood levels of NO_3^- and NO_2^- following exposure to NO_2 gas, and gross organ distribution in rabbits were conveniently obtained with our Anger scintillation camera. With intravenous $^{13}NO_2^-$ and $^{13}NO_3^-$, a rapid homogeneous distribution of the radioactivity throughout the rabbit was observed as previously seen in mice. Equilibrium between the intravascular and extravascular compartments was reached within 5 minutes after injection of either radiochemical. The activity was distributed evenly throughout the soft-tissue organs. Only 2-3% of the ^{13}N appeared in the urinary bladder during the first 30-45 minutes after injection. The blood

Table 8.

Bolus Instillation of Carrier Nitrite: Organ Distribution

	Group I	Group II	Group III
Intestine	15.356 ± 4.3989	8.08 ± 3.78	7.02 ± 0.92
Stomach	2.74 ± 1.08	1.32 ± 0.35	1.88 ± 0.13
Blood	6.53 ± 1.82	5.96 ± 1.05	3.78 ± 1.46
Liver	8.12 ± 2.53	6.27 ± 1.096	14.69 ±7.43
Heart	1.03 ± 0.23	0.784± 0.106	0.71 ±0.02
Spleen	0.63 ± 0.3255	0.468± 0.106	0.45 ±0.04
Lung	3.62 ± 1.54	1.56 ± 0.65	1.31 ±0.11
Kidney	3.17 ± 0.953	2.17 ± 1.31	2.08 ±5.32
Carcass	58.24 ±12.46	71.03 ± 8.09	64.32 ±5.32
Bladder	0.37 ± 0.18	0.129±0.048	0.110±0.007
	99.80	97.77	96.31

Table 9. Fractional distribution of ^{13}N in blood at 10 min after administration of $^{13}NO_2^-$ or $^{13}NO_3^-$

	Mice[a]		Rabbits[c]	
	NO_3^-	NO_2^-	NO_3^-	NO_2^-
Plasma	0.58 (0.05)[b]	0.49 (0.04)	0.62 (0.10)	0.64 (0.08)
MeOH ppt. (PP)	0.12 (0.03)	0.11 (0.03)	0.18 (0.12)	0.10 (0.04
Supernatant	0.46 (0.03)	0.38 (0.03)	0.44 (0.15)	0.54 (0.06)
Cells	0.42 (0.05)	0.51 (0.04)	0.38 (0.10)	0.36 (0.08)
Cell Debris (CD)	0.16 (0.05)	0.28 (0.05)	0.17 (0.07)	0.13 (0.03)
HOH Lysate	0.26 (0.06)	0.23 (0.05)	0.21 (0.09)	0.23 (0.05)
MeOH ppt. (CP)	0.07 (0.03)	0.05 (0.03)	0.06 (0.03)	0.05 (0.02)
Supernatant	0.19 (0.04)	0.18 (0.04)	0.15 (0.03)	0.18 (0.07)

[a]. 5 mice were used for each anion; intratracheal instillation

[b]. Standard deviations are given in parentheses

[c]. 5 rabbits were used for NO_3^- and 4 rabbits used for the NO_2^- date; **IV injection**

fraction data (Table 9) are very similar for both anions in the rabit and are similar to the mouse data.

The hematocrit for mice was about 0.55 and for rabbits about 0.40. These were approximately the fractions of tracer found in the cells, suggesting that the ^{13}N from NO_3^- or NO_2^- was distributed uniformly on a per volume basis throughout the blood. However, it was observed that the phosphate-buffered saline washes of cells from animals exposed to nitrate gave higher removal values per wash than cells from animals exposed to nitrite. Comparison of first wash values for cells from mice given the anions by IT administration 10 minutes previously were 64.9% ± 5.3 (SD)% of the cell activity for $^{13}NO_3^-$ and 41.6 ± 10.9 (SD)% of the cell activity for $^{13}NO_2^-$.

Chemical Identification. On the basis of the several range-finding experiments for the determination of chemical conversion rates, where applicable, an in vivo or in vitro incubation period of 10 minutes was determind to be suitable for comparison. Aliquots of each supernatant from the blood distribution experiments were also analyzed by rapid high pressure liquid chromatography (HPLC) (Fig. 2). The chromatographic results obtained from mouse plasma taken 10 minutes after IT instillation of $^{13}NO_2^-$ revealed that 70 ± 5% (SD) of the ^{13}N was converted to $^{13}NO_3^-$ and 27 ± 2% remained as $^{13}NO_2^-$. Nonanionic (NA) compounds and their chemical identification has been the subject of an extensive separate investigation wchich is discussed under a subsequent heading. One site of NO_2^- conversion to NO_3^-. By comparison, the product distribution in rabbit plasma 10 minutes after $^{13}NO_2^-$ injection was 46 ± 3% $^{13}NO_2^-$, 51 ± 3% $^{13}NO_3^-$ and 3 ± 1% non-anionic, indicating a slower conversion rate than in mice.

Ten minutes after IT instillation of $^{13}NO_3^-$ into mice, 100% of the ^{13}N label was found as $^{13}NO_3^-$ in all blood fractions. The $^{13}NO_3^-$ IV injection data in rabbits revealed no differences from the mouse IT data. Thus, any $^{13}NO_2^-$ formed by reduction of $^{13}NO_3^-$ by the intestinal or oral microflora was not detected in blood of either species.

The possible formation of labeled nitrosyl (-NO) compounds or exhalable gases such as nitric oxide (NO), dinitrogen (N_2), nitrogen oxide (N_2O) in the blood of animals given $^{13}NO_3^-$ or $^{13}NO_2^-$ was examined in two experiments. In first, $^{13}NO_2^-$ and $^{13}NO_3^-$ were added under NCA conditions, in vitro, to whole blood and incubated to 37 C for varying periods (5-30 min). Nitrite was found

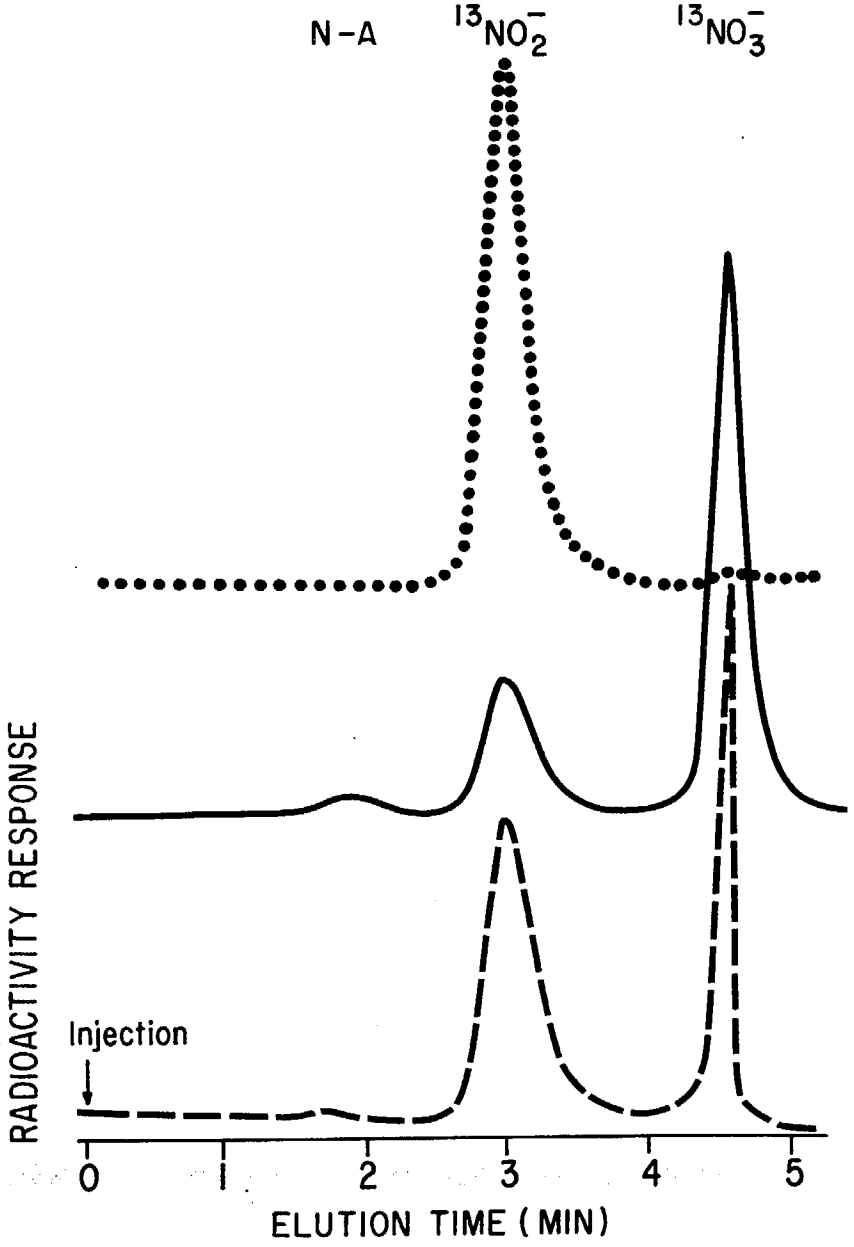


Figure 2. Radiochromatograms illustrating the oxidation of $^{13}NO_2^-$ in animals. Nitrogen-13 labeled anions were separated on a high performance liquid chromatograph fitted with a 4.6 mm ID x 250 mm Partisil-10 SAX (Whatman, Inc.) strong anion exchange column. The eluent was 30 mM phosphate buffer, pH 3.1; the flow rate was 3.0 mL/min. The 511 keV positron annihilation photons from ^{13}N were detected with NaI (Tl) crystals. The elution order on this column is non-anionic (N-A) labelled compounds, nitrite ($^{13}NO_2^-$), and nitrate ($^{13}NO_2^-$). The decay-corrected percentages, given as N-A, $^{13}NO_2^-$, $^{13}NO_3^-$, respectively, for introduced material are 0.2, 99.3, 0.5; for 10 min mouse plasma are 3, 27, 70; for 10 min rabbit plasma are 3, 46, 51.

Figure Legend: .....$^{13}NO_2^-$ solution introduced into animals.
———mouse plasma from blood drawn 10 min after instillation.
-----rabbit plasma from blood drawn 10 min after injection.

to react and be converted to nitrate at a rate similar to those observed in vivo. Nitrate did not undergo any detectable reactions.

The results of in vitro oxidation rate studies with $^{13}NO_2^-$ in human blood and in rabbit blood are compared in Figure 3. What we found was an oxidation rate for humans that corresponded to a half-life of 5 to 7 minutes for a variety of but not exhaustive set of conditions. We learned that excess NO_2 inhalation from smoking by a male does not give dramatically different results from those observed in an adult female non-smoker. Additional experiments with three other adult males and one adult female produced similar results when NCA nitrite was added to blood samples. An additional series of measurements were made with added carrier (2mg/mL) with blood from the male donor. We found that even with this substantial loading of the oxidative mechanism that the rate of oxidation to nitrate was only slightly depressed. Because the rate of in vitro oxidation of nitrite in human blood is essentially identical to both in in vivo and in vitro rates for our primary animal model, the Balb/C mouse, we believe that the in vitro measurements with human blood are predictive of the in vivo situation. In such a case, the rapid destruction of nitrite is expected and the extensive formation of hazardous nitrosyl-compounds in blood is unlikely. By comparison, the much slower oxidation rates observed for the rabbit indicate this species may be a less reduced and formed nitrosyl-hemoglobin or other nitrosyl compounds was probed with an exchange labeling technique. The $^{13}NO_2^-$ spiked blood samples were purged of free oxygen and then slowly purged with nitric oxide (NO) gas in anticipation that N-13 labeled nitrosyl (NO-) ligands bound to heme iron would exchange to form ^{13}NO gas which could be identified by gaschromatography. Labeled NO gas was observed but it could not be shown at the 5% significance level that the concentrations were greater than blank runs with buffered saline. The second experiment tested for exhalation of N-13 compounds by radioactivity counting of exposed animals. At the 5% significance level, we did not find that these animals' total radioactivity content declined faster than could be accounted for by physical decay.

Nonanionic compounds. In these metabolic studies, we used $^{13}NO_2^-$ to minimize any pharmacological action of the tracer (3,4). Labeled NO_2^- was prepared by reduction of cyclotron-produced $^{13}NO_3^-$ (6,7) and concentrated into saline solution prior to instillation (5). All the experiments were done under

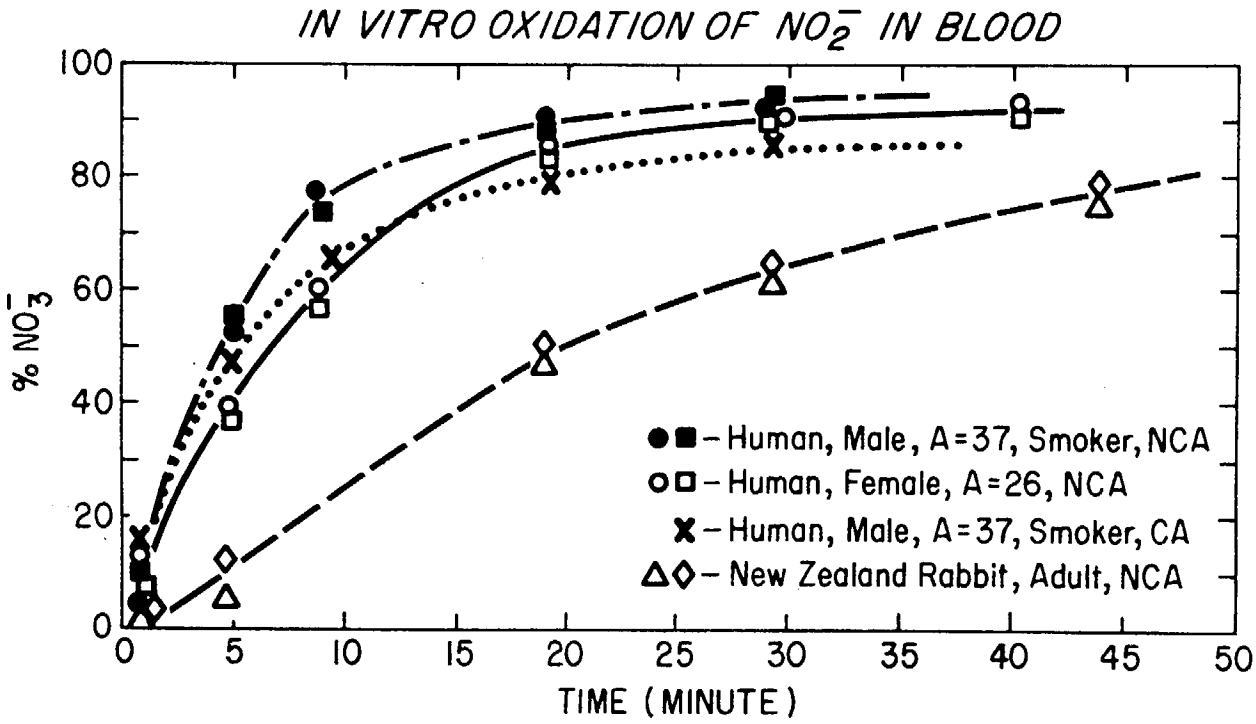


Figure 3. Comparison of oxidation rates for $^{13}NO_2^-$ to $^{13}NO_2^-$ in humans and rabbits.

"no-carrier-added" (NCA) conditions, with specific activities of to 1-7 Ci/umole of NO_2^-. Consequently, total NO_2^- dosages ranged between 10 and 100 ng per kilogram of body weight. The composition of the labeled components was determined by high pressure liquid chromatography (HPLC), together with thin layer electrophoresis (TLE) (9).

Conventional (CV) balb/c mice were fed commercial feed and water without restriction. The effect of the microflora on NO_2^- metabolism was assessed by comparing CV mice with germ-free (GF) mice and specific-pathogen-free (SPF) mice. Conventional Balb/c mice (20-23 gm) were obtained from Simonsons, Morgan Hill, Ca.. These mice were housed in a controlled environment by the U. C. Davis Animal Resource Services and fed Purina rodent lab chow #5001 and water without restriction until use. Germ-Free [CD-1(ICR)GN] and Specific-Pathogen-Free [CD-1(ICR)BR] mice derived from the same line were obtained from Charles River Labs and kept in the vendor's isolation chambers until used (<24 hours). Further comparisons employed exenterated (i.e. small and large intestines removed) or penicillin treated CV mice (29).

Table 10 shows the distribution of intratracheally administered $^{13}NO_2^-$ in the various mouse tissues and indicates the relative amounts of radioactivity in the NO_3^-, NO_2^-, and non-anionic (NA) fractions. The chemical determinations were done by anion exchange chromatography (26,27,28). Cation exchange HPLC utilized Altex and Whatman Partisil cation exchange media in 25cm by 4.6mm ID columns. Cations were eluted with sodium phosphate buffers at a pH of 3.7 to 6 and concentrations from 7 to 25 mM with respect to sodium. The flow rates for all columns was between 2.5 and 3.5 ml/minute. When anion peaks eluting at the void volume of cation exchange columns (e.g., $^{13}NO_2^-$, $^{13}NO_3^-$) masked the presence of neutral components, a 3cm "stripper" column containing AG1-X8 anion exchange resin (Bio-Rad) was used ahead of the analytical column to remove the interfering anions. The NA components include all nitrogenous compounds not retained on the anion exchange column.

Mean values and asymmetric 68% confidence intervals about the means were determined using the logit transformation. The mean estimate for the fractions (f) bounded by the fixed margins of 0 and 1 are transformed by $L=\ln[f/1-f]$. The logit mean $\bar{f} = 1/[1+\exp(-\bar{L})]$ where $\bar{L}$ is the arithmetic mean of the L values. The standard error limits about $\bar{L}$ are transformed to error limits about $\bar{f}$ (lower to upper limit) as $1/[1+\exp -(\bar{L}+SE_{\bar{L}})]$ to

$1/[1+\exp -(\bar{L}-SE_{\bar{L}})]$. This transformation gives asymmetric error limits when transformed back to a linear scale. This technique adequately estimates the central tendency of fractional values which are bounded by fixed margins (eg. 0 and 1) (20.)

The fractions of whole body activity (WBA) in the various tissues of conventional (CV) mice shown in Table 10 are consistent with a uniform distribution of ^{13}N on the basis of weight (3,4.) These values show that 10-20% of the ^{13}N introduced into the lungs as NO_2^- ends up in the gut. The chemical distribution of intestinal radioactivity in CV mice is dramatically different from that in the blood, acidic stomach contents, and other body fluids. This indicates that oxidation of $^{13}NO_2^-$ to $^{13}NO_3^-$ in blood is primarily responsible for the products observed in non-intestinal fluids and tissues. Table 1 also shows that the chemical distribution of ^{13}N in tissues of the SPF controls for GF mice is minimally different from that in CV mice.

The data for GF mice show that 1) The intestinal microflora are solely responsible for the production of ^{13}N-NA, 2) NO_3^- and NO_2^- in the blood move into the intestine, and 3) in the examined tissues, oxidation of $^{13}NO_2^-$ to $^{13}NO_3^-$ occurs most rapidly in the liver and blood and least rapidly in the intestine. Additional evidence for the first observation was obtained from two exenterated CV mice and one treated with penicillin for 24 hours prior to administering $^{13}NO_2^-$. Table 10 shows that the results were the same.

Because the intestinal microflora are responsible for ^{13}N-NA production we determined the identity of these labeled components in intestinal tissues from CV mice. The NA was separated into neutral and cationic components by cation exchange chromatography with anion stripping to remove $^{13}NO_3^-$ and $^{13}NO_2^-$ and further characterized by TLE (17). Eight CV mice intratracheally instilled with $^{13}NO_2^-$ were sacrificed as before except that their intestines were extracted with 100% methanol to stop bacterial metabolism prior to chemical analysis. Of the neutral ^{13}N-NA components only glutamate (GLU) and urea or neutral amino acids were detected. Of the cationic components, only NH_4^+ and glutamine (GLN) were found. The presence of urea in the intestinal extracts was confirmed by $^{13}NH_4^+$ production upon incubation of the extracts with urease. Twenty-five to thirty minutes after $^{13}NO_2^-$ administration, the intestinal ^{13}N-NA were: 59% (50-67%) GLU, 36% (28-44%) NH_4^+, 3.8% (2.3-5.8%) GLN, and 1.8% (1.2-2.1%) urea and neutral amino acids.

This metabolic process took place primarily in the large intestine as

Table 10

SUMMARY OF ^{13}N-LABELED PRODUCT DISTRIBUTION IN VARIOUS TISSUES AFTER INTRATRACHEAL INSTILLATION OF $^{13}NO_2^-$ IN MICE [a]

Tissue	Fraction of WBA[b]	Percent of tissue ^{13}N activity as:[c] NA[d]	NO_2^-	NO_3^-
Conventional (CV)[e] (4 mice: 12-31 min)				
Plasma	.044 (.032-.059)	3.9 (3.5-4.2)	6.8 (5.2-8.8)	89 (87-91)
Intestine	.122 (.111-.134)	84 (78-88)	.18 (.08-.40)	16 (11-22)
Liver	.070 (.064-.076)	33 (30-36)	ND[f]	67 (64-70)
Stomach	.020 (.019-.021)	6.8 (5.1-9.0)	3.2 (1.7-6.0)	90 (85-93)
Urine	<.03	7.8 (7.5-8.1)	1.4 (1.0-2.0)	90 (87-93)
Others[g]	.736 (.702-.767)			
Exenterated[h] (2 mice: 6,25 min)				
Plasma		ND	67	33
Plasma		ND	27	72
Penicillin treated[j] (25 min)				
Plasma		ND	13	86
Germ Free (GF) (3 mice: 12-34 min)				
Plasma		ND	14 (10-18)	86 (82-90)
Intestine		ND	28 (23-34)	72 (66-77)
Liver		ND	2.2 (1.0-4.8)	98 (94-99)
Specific Pathogen Free (SPF) (3 mice: 12-29 min)				
Plasma		3.0 (2.6-3.5)	20 (15-25)	77 (71-82)
Intestine		85 (72-92)	8 (7-10)	5 (1-18)
Liver		14 (9-23)	11 (7-17)	72 (70-74)
Urine		5 (4-7)	4 (3-5)	90 (86-93)

FOOTNOTES

a) Fifteen uL of ^{13}N labeled nitrite was intratracheally instilled without added carrier. The mice were sacrificed as before (3) 12-31 min after instillation and selected tissues were then homogenized in saline, centrifuged for 60 sec. @ 12,000 x G, and the supernatant filtered through 0.5 um disposable filters prior to chromatographic analysis.

b) WBA = whole body activity, given as: mean (asymetric error interval).

c) Percent of recovered label in the indicated form (NA, NO_2^-, NO_3^-) in the extracted tissue given as: mean (68% confidence interval about the mean). The measure of central tendency and dispersion for the chromatographically determined product activity fractions which are bounded by the fixed margins of 0 and 100%, was made on the basis of the logit logarithmic transformation (20).

d) NA = non-anionic

e) CV = conventional Balb/C mice obtained from Simonsons.
GF = germ free mice Charles River [CD1-(ICR)-GN].
SPF = specific pathogen free control mice of the same of the same strain as the GF mice [CD1-(ICR)-BR].

f) ND, not detected.

g) The fraction of total body activity in the organs not listed of CV mice was; lung .017 (.014-.019) heart .008 (.007-.009) kidney .023 (.019- .027) bladder .002 (.001-.003) spleen .0045 (.0042-.0048) and carcass 0.63 (.58-.67).

h) These mice were exenterated prior to instillation of ^{13}N nitrite. Control mice that were surgically invaded and whose intestines were teased out intact and replaced yielded results identical to those of untreated balb-c mice.

j) The drinking water of this penicillin treated mouse was amended with 0.3 g/liter of buffered penicillin G (pfitzerpen)·24 hours prior to instillation of ^{13}N nitrite.

can be seen in Figures 4 through 7. In Fig. 4 and 5, the HPLC and TLE data are shown, respectively, for a representative mouse small intestine contents. The survival of nitrite and nitrate as the dominant chemical species indicates a low level of bacterial conversion. Peaks to the right of the arrow in the TLE plot are on a 100 times more sensitive scale. Urea is expected from degradation of amino acids in the liver. Conversely, the representative HPLC and TLE data in Fig. 6 and 7, respectively, from mouse large intestine contents show no survival of nitrite and nitrate and exclusively show ammonia or amino acids.

Under our chromatographic conditions, dimethyl-, dipropyl-, and diphenyl- nitrosamines have elution times different from the ^{13}N species found in plasma and intestines. Our limit of detection is about 0.1% of the total chromatographed radioactivity. This corresponds to approximately 0.005% of the total instilled activity. No ^{13}N-labeled components were detected at the elution positions of these simple nitrosamines. Thus, these potential nitrosation products are not synthesized at rates comparable to assimilatory products in vivo under our NCA conditions. However, we cannot rule out minor formation of these or other potential nitrosation products.

In summary, $^{13}NO_2^-$ introduced into the respiratory tract at dosages below the level of pharmacological perturbation, enters the intestines as both NO_2^- and NO_3^-. Within 30 minutes, the intestinal fraction of activity accounts for 0.1-0.2 of WBA in CV mice. About 80% of this intestinal activity in both CV and SPF mice is found in reduced forms as NH_4^+, amino acids and urea. Simple ^{13}N-nitrosamines were not detected by HPLC in animals fed a normal diet. In contrast, GF and exenterated CV mice produced no ^{13}N labeled NA components in any of the examined tissues. The metabolism of $^{13}NO_2^-$ and $^{13}NO_3^-$ in CV and SPF mouse intestines is thus rapid and chemically reductive. These observations do not support arguments for nitrification, which is oxidative, in the intestines of mammals (21).

Chronic exposure experiments. The purpose of these experiments was to determine the effect of chronic subcutaneous administration of $NaNO_2$ on the metabolism of intratracheally instilled $^{13}NO_2^-$ in mice. These experiments were accomplished with the use of miniture osmotic pumps implanted under the skin for one week. These pumps delivered 0.5 uL/hr of solutions containing 0, 35, and 350 mg/mL of NO_2^-.

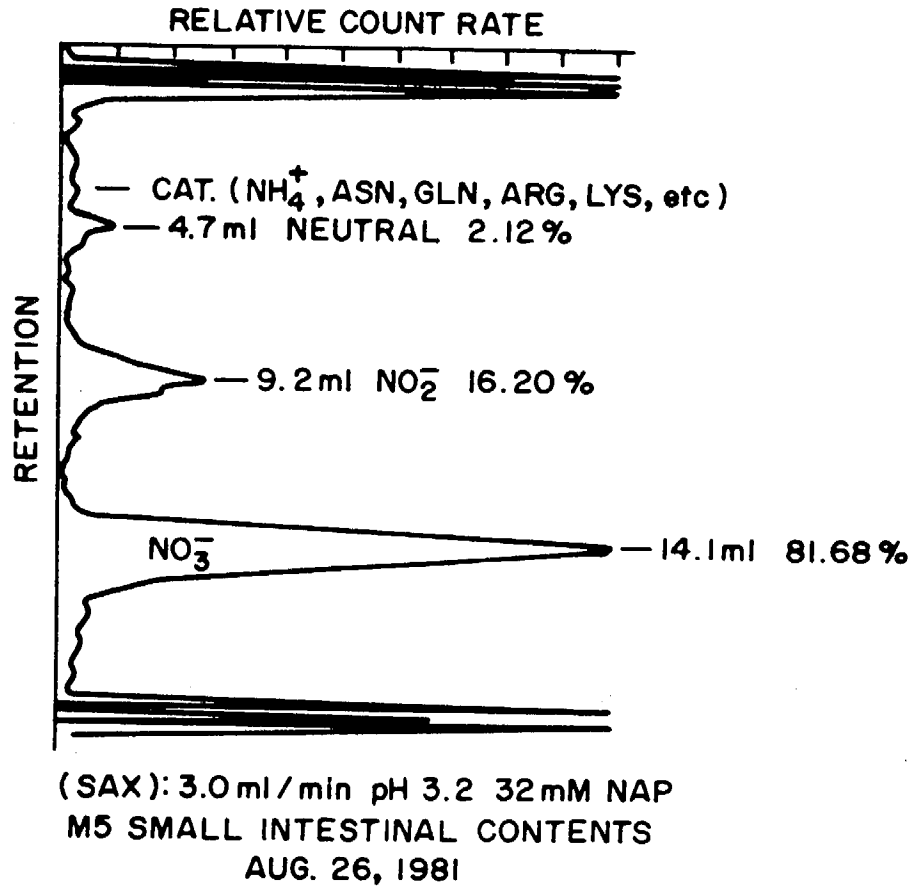


Figure 4. Liquid chromatogram of mouse small intestine contents after IT $^{13}NO_2^-$.

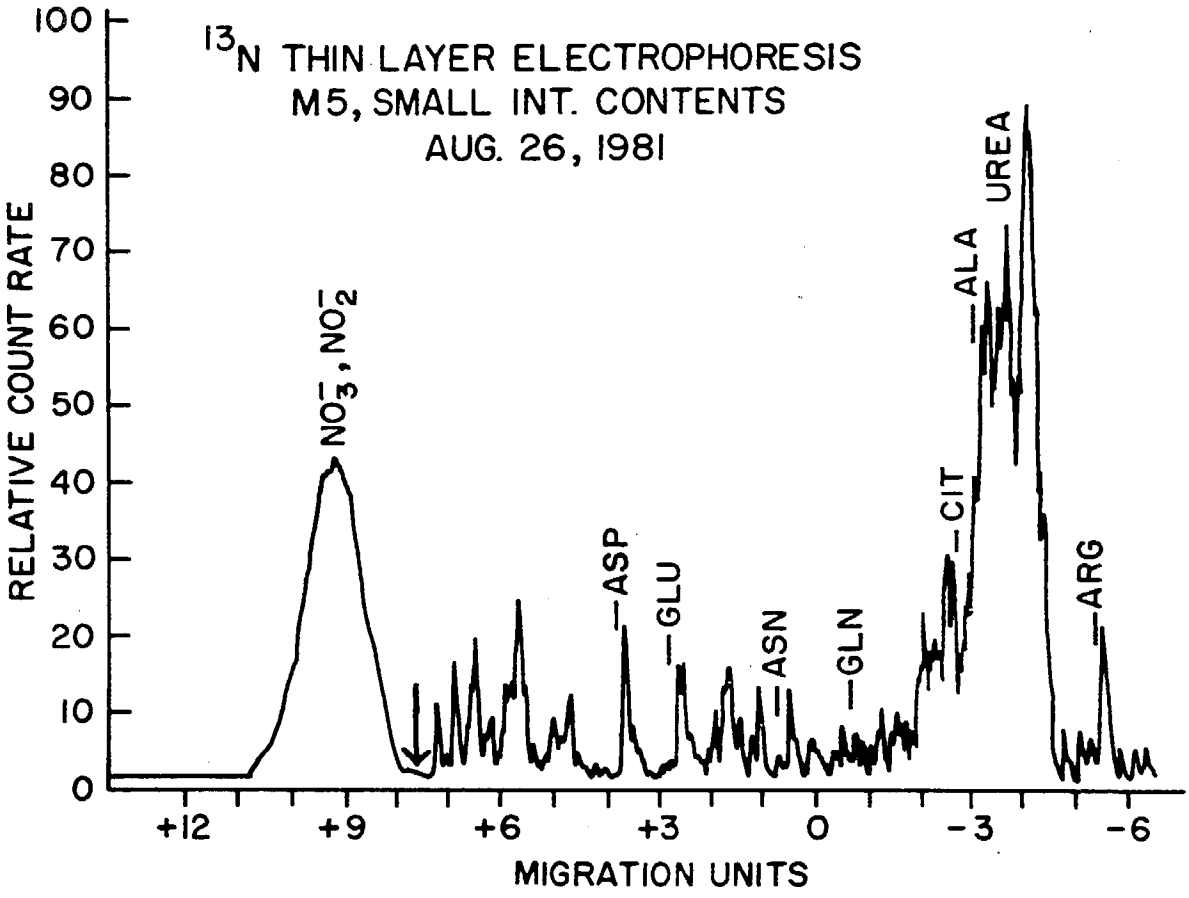


Figure 5. Thin layer electrophoretogram of mouse small intestine contents after IT $^{13}NO_2^-$. Activity shown to the right of the arrow is times 100.

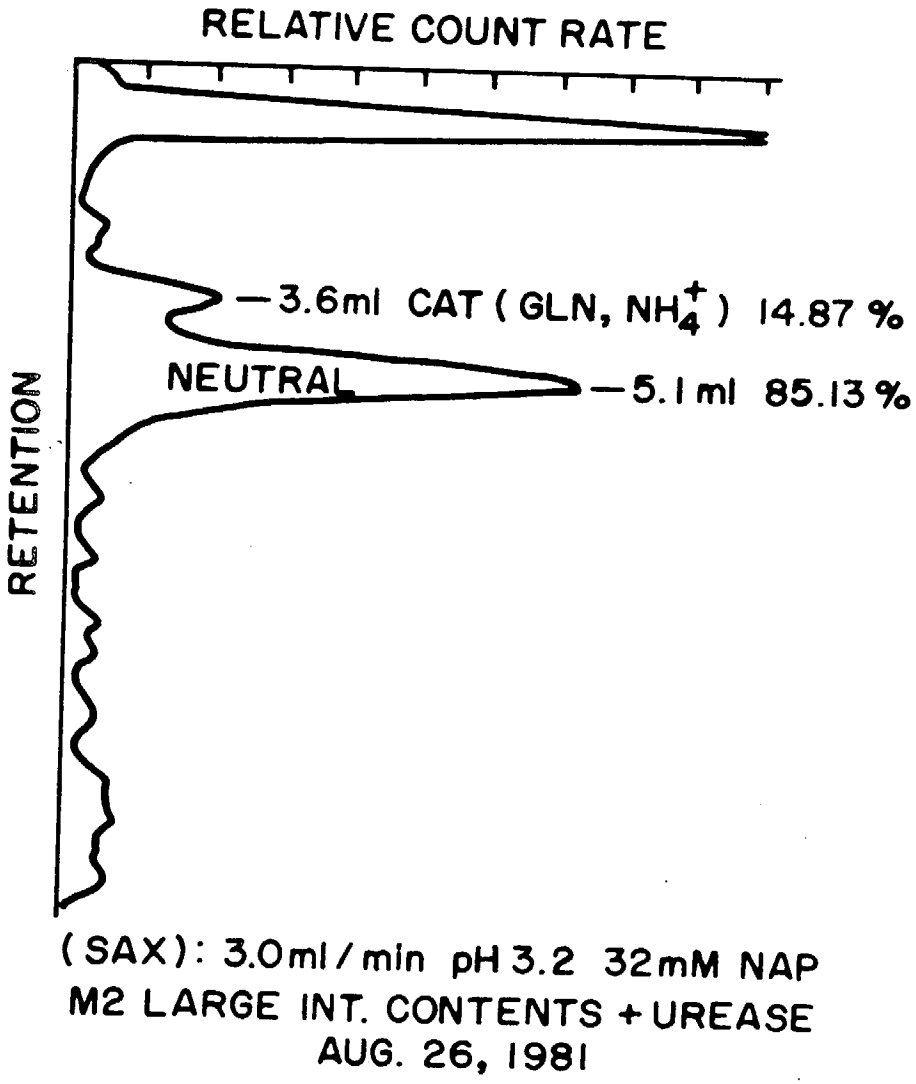


Figure 6. Liquid chromatogram of mouse large intestine contents after IT $^{13}NO_2^-$.

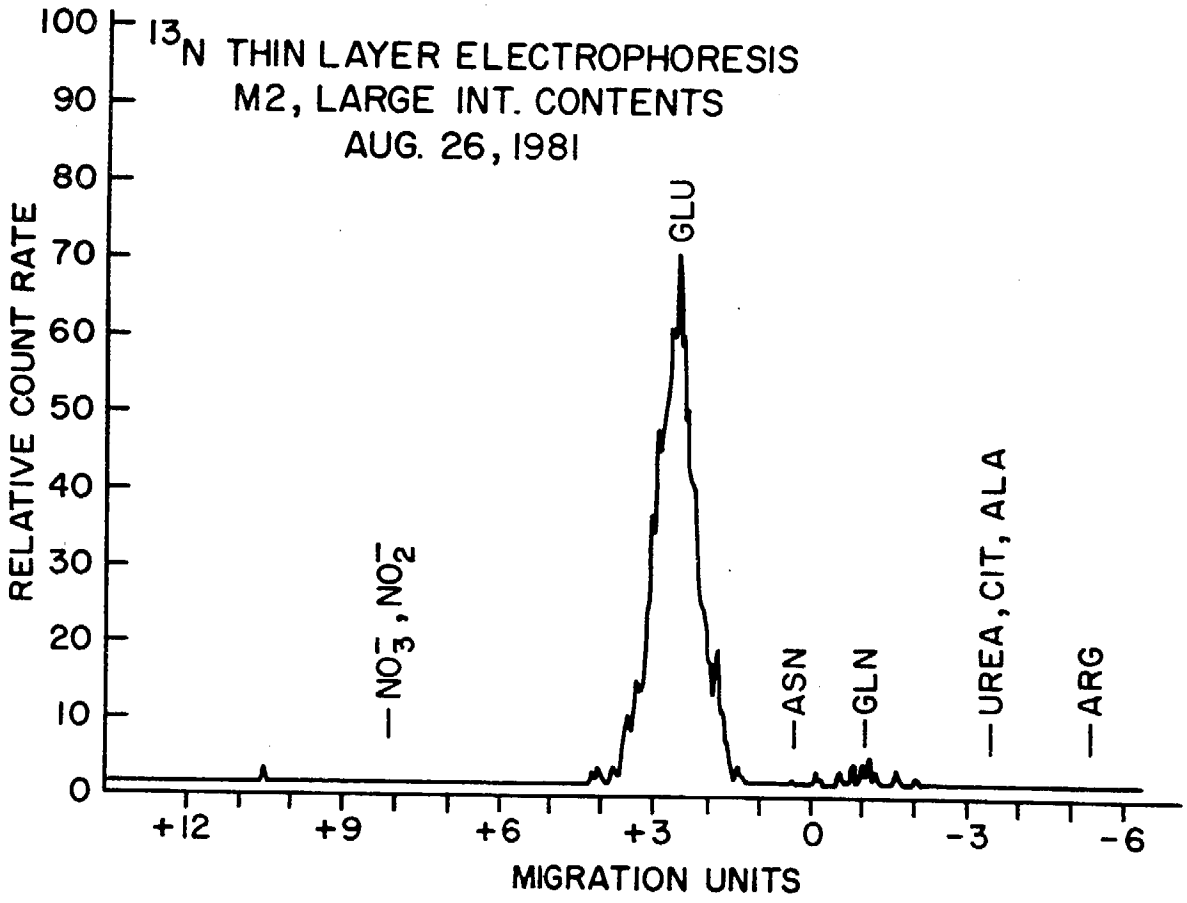


Figure 7. Thin layer electrophoretogram of mouse large intestine contents after IT $^{13}NO_2^-$.

Our conclusions were the following: (1) Excess $^{14}NO_3^-$ excretion occurred only at high to acute NO_2^- pump doses; (2) No "loading" of the plasma with $^{14}NO_2^-$ or $^{14}NO_3^-$ was observed for any mice even up to 350 mg/mL in the pumps; (3) Increasing NO_2^- in the pumps seemed to increase the fraction of administered ^{13}N remaining in the lungs (on a per-gram basis); (4) Inclusion of vitamin C in the drinking water of a mouse during chronic exposure to 350 mg/mL NO_2^- resulted in a lung clearance rate of IT administered $^{13}NO_2^-$ comparable to that in mice whose pumps contained saline; (5) The fraction of plasma ^{13}N as NO_2^- was less in mice whose pumps contained up to 35 mg NO_2^-/mL, and appeared to increase above that concentration; (6) The inclusion of vitamin C in the drinking water of a mouse whose pump contained 350 mg NO_2^-/mL resulted in a lower plasma NO_2^-/NO_3^- ratio in comparison to animals not receiving the vitamin.

In the intestines (whole) of chronically dosed mice, increasing the carrier concentration from 0 to 350 mg NO_2^-/mL had the following results: (a) increased the fraction of neutral (amino acid) products up to 35 mg NO_2^-/mL, then decreased slightly at 350 mg/mL in the pump; (b) did not alter the fraction of ^{13}N as NO_2^- except at 350 mg NO_2^-/mL, where it increased; (c) decreased the fraction of ^{13}N as NO_3^- in this organ group. The inclusion of vitamin C in the drinking water of a mouse exposed to 350 mg NO_2^-/mL in the pump did not significantly alter the fraction of NO_3^- or NO_2^- in the intestines, but did reduce the fraction of neutral ^{13}N while increasing the fraction of cationic ^{13}N in that organ group. The fraction of total ^{13}N administered as NO_2^- that was found as cationic or neutral compounds in the intestines did not appear to change upon increasing pump NO_2^- concentrations. However, the fraction of total ^{13}N appearing as NO_2^- increased while that appearing as NO_3^- decreased upon increasing the pumped dosage. The inclusion of vitamin C in the drinking water of a mouse with a 350 mg NO_2^-/mL pump altered only the fraction of total body activity in the intestine which was cationic.

Section VII

DISCUSSION

The presence of nitrogen dioxide (NO_2) and various nitrate (NO_3^-) compounds in indoor and outdoor air is well established (33-35). These chemical species are either known or suspected to form nitrite (NO_2^-) in vivo. Nitrite is known to form carcinogenic N-nitroso compounds in vivo by reaction with amines in the stomach of experimental animals (38-41) or in vitro under the mediating influence of intestinal microflora (42). The formation of nitrosamines has been reported in rodents gavaged with a precursor amine and exposed to NO_2 gas by inhalation (43). Nitrous acid was postulated as an intermediate although the mechanism or site of nitrosation was not determined. Pathogenesis attributed to NO_2^- alone has been reported but this conclusion is still a source of debate (44,47).

Evaluation of the potential health hazard of inhaled or ingested NO_2, NO_2^- or NO_3^- at concentrations likely to be encountered under realistic conditions has been complicated by Tannenbaum's discovery of NO_3^- synthesis in humans. This was originally attributed to formation of NO_3^- by intestinal microflora,(36) but more recent studies have revealed this to be a mammalian process. Saul et. al. found that human fecal material destroys both NO_2^- and NO_3^- in vitro, but did not identify any reaction product (37). Witter et al. subsequently reported that the presence of intestinal microflora decreased urinary NO_3^- excretion in rats; NO_2^- was speculated to be a metabolite (16).

We previously reported that radioactive ^{13}N ($t_{1/2}$ = 9.96 min) as $^{13}NO_2^-$ or $^{13}NO_3^-$ (carrier dosage less than 100ng per kilogram of body weight) cleared from the lungs of mice to the blood and distributed into the gastrointestinal (GI) tract and other organs within 30 minutes (3), (CARB) Reports,A7-190-30 and A8-121-31). At this low concentration of added NO_2^- (2-3 nM in body fluids) we found $^{13}NO_2^-$ was 70% oxidized to $^{13}NO_3^-$ in blood within 10 minutes. Consequently, NO_2^- derived from nitrogenous air pollutants is partially converted to NO_3^- before reaching the GI tract. However, mammals synthesize NO_3^- and their oral and possibly intestinal microflora reduce it to

NO_2^-. This presented an enigma with respect to evaluation of health risk from either inhaled or ingested oxidized nitrogen compounds.

The uncertainties regarding the metabolism of oxidized N compounds have persisted in part because analysis of trace NO_2^-, NO_3^-, or their metabolites in biological samples is difficult. Mass-spectrometric detection of stable ^{15}N allows tracer chemistry, but requires dosages of ^{15}N compounds which may perturb normal biochemical processes. In our metabolic studies, we used $^{13}NO_2^-$ to minimize any pharmacological action of the tracer. Radioactive $^{13}NO_2^-$ was prepared by reduction of cyclotron-produced $^{13}NO_3^-$ concentrated into saline solution prior to instillation. For experiments done under "no-carrier-added" (NCA) conditions, we achieved specific activities of to 1-7 Ci/umole of NO_2^-. Consequently, total NO_2^- dosages ranged between 10 and 100 ng per kilogram of body weight when no carrier was added. The composition of the labeled components was determined by high pressure liquid chromatography (HPLC), together with thin layer electrophoresis (TLE). We have now determined the sites of most rapid metabolism of $^{13}NO_2^-$ in mice to be the blood and intestines (3,4). In the following, we discuss our findings and present a pharmacologic model which places this research in the context of other relevant work.

Tissue Distribution of Tracer ^{13}N from Nitrite and Nitrate. The very similar organ distributions of ^{13}N labeled tracer NO_2^- and NO_3^- introduced intratracheally into mice or intravenously into rabbits can largely be explained by the conversion of $^{13}NO_2^-$ to $^{13}NO_3^-$ in blood. We directly demonstrated that NO_2^- was rapidly oxidized to NO_3^- at NCA concentrations in mice and rabbits, but $^{13}NO_3^-$ was not reduced to $^{13}NO_2^-$ within the 10 minute period of observation. Measurable species differences were observed in the oxidation rates of NO_2^- to NO_3^-.

Comparison with literature. There is only a limited literature on the biodistribution of NO_3^- and NO_2^-. One potentially important study of the pharmacokinetics of nitrite and nitrate in dogs, sheep and ponies failed to identify the site of the major pool for the injected anions (12). A classic study by Greene and Hiatt involved administration of large quantities of sodium nitrate to dogs (13). They measured plasma nitrate concentrations by an indirect colorimetric method and found volumes of distribution from

analysis of plasma clearance that averages 28% of the dog's total weight. This was 1.16 to 1.20 times the extracellular fluid (i.e. CL^-) volume. When doses as high as 35 g/kg intravenous of 1.7 g/kg organ were given, 92% of the administered dose was recovered in the urine, mostly within the first day after administration. At the smallest dose given (0.04 g/kg IV) urinary excretion decreased to only 30%. This was the first evidence that the biorouting of NO_3^- is dose dependent.

We are aware of two studies involving colorimetric analyses of administered nitrite; one involved mice (14), the other rats (15). Friedman et al. (14) found that 10 min after oral administration of 150 ug of $NaNO_2$ to mice, only 15% remained in the stomach. Of the remainder, a little more than half was absorbed and the balance was oxidized to NO_3^-. This finding was not altered by ligating the gastroduodenal junction and led the authors to conclude that nitrite is absorbed directly from the stomach into the bloodstream. Nitrous acid is essentialy totally dissociated at neutral pH but is at least 50% HNO_2 in the acid environment of the stomach.

Mirvish and colleagues fed $NaNO_2$ in food to starved rats to look for intragastric nitrosation(15). They found a clearance half-time from the stomach of 1.4 hours. At 10 min after IE nitrite, we found 37.8 $\pm$12.2% of the N-13 activity remaining in the stomach. Our clearance rate is intermediate between these two studies but the differences in biological models are too great to warrant further reconciliation of these results.

Witter, et al. used radioisotopes to measure the distribution of $^{13}NO_3^-$ and $^{13}NO_2^-$ in rats (16). The tracer was administered by gavage and a small number of conventional flora rats wre studied with nitrate at 15-30, 45 and 60 minutes and with nitrite at 30 and 45 min. These results are qualitatively similar to ours and even agree quantitatively in stomach, intestines and carcass. The activity in liver and kidney was significantly less in their rats. From their data we estimate a slope for clearance from the stomach of -1.56/min and for accumulation in the intestines of +0.58/min for nitrate data only or +0.42/min for all data. These numbers are very close to our results.

A review of the existent nitrite and nitrate literature shows inconsistencies with our results, but the present study represents the most thorough study of their pharmacokinetics at very low concentrations. But do NO_2^- and NO_3^- behave pharmacokinetically like other anions or are they special

in some way? A similarity was assumed by Greene and Hiatt when they used nitrate as a generic marker to study the mechanism of electrolyte excretion because, they argued, of its similarity in size and valency with the chloride ion (13). None of our measurements were taken at the early times required to see if these ions are freely-diffusable tracers that distribute according to blood flow by the Kety model (17) but this would represent very unusual biological behaviour for anions. We can however, comment on how closely they mimic halide, sulfate and pertechnetate anions. Tracer distribution studies of each of these anions have been reported and they represent a spectrum of pharmacokinetic properties for comparison.

Much of the research with tracer anions has been motivated by the search for a marker for determination of extracellular fluid volume. All of these anions penetrate cells to a significant extent, however, and may suffer from the disadvantage of rapid urinary excretion (18). In addition, several of the anions accumulate in target tissues -- for example I^- in the thyroid, F^- in the hydroxyapatite aspect of bone and $SO_4^=$ in chondrotin. Perhaps the most data exists for the pertechnetate anion because of its important role in clinical nuclear medicine (19). Its distribution in the body is dominated by early accumulation in the salivary and thyroid glands, gastric mucosa and the choroid plexur. It has a plasma clearance half time of about 5 minutes for 70-80% of injected activity and is excreted primarily via the GI tract and kidney, but 85% of the filtered TcO_4^- is reabsorbed by the tubules.

Measurements with most anions fall short of the detail in the present N-13 studies but there is sufficient data to show that NO_3^- and NO_2^- are distinctly unlike the other anions. Their plasma clearance rates are faster than those for I^-, F^-, $SO_4^=$ or TcO_4^-. There is no apparent accumulation of the nitrogen anions in thyroid, bone or cartilage. Both nitrate and nitrite have the same pharmacokinetics under our experimental conditions but they are not mimicked by other anions.

Risk Assessment from Pharmacokinetic Results. These results relate to the prediction and assessment of human health risk associated with the inhalation of nitrogenous compounds that can form NO_2^- in vivo. In an evaluation of biological response data, Dawson and Schenker showed that a one hour inhalation of NO_2 gas at 0.1 part-per-million (ppm) significantly decreases the threshold for asthmatic provocation in predisposed humans (22). An NO_2

concentration of 0.1ppm, which appears to be near the threshold for adverse physiological response, can result in an inspiration of approximately 40ng/kg body weight/minute in humans (assuming a 60 kg man, inspiring 15 L/min, and retaining 90% of the NO_2). Concurrently with this work, Pryor et. al. (23) found that such concentrations of NO_2 gas may form nitrous acid (HNO_2, NO_2^- at physiological pH) by hydrogen abstraction from pulmonary lipids rather than nitrite and nitrate by disproportionation (24). Subsequent to our in vivo work showing that NO_2^- is oxidized the NO_3^- inside the erythrocyte, an in vitro study with perfused rat lungs exposed to NO_2 gas showed that NO_2^- was the only product in the absence of erythrocytes (25). This is consistent with our prior observations that the concentration of ^{13}N in the blood of monkeys who inhaled $^{13}NO_2$ gas rises in proportion to the amount inhaled (11) and that ^{13}N from $^{13}NO_2^-$ rapidly leaves the lung as the ion (3).

Our measurements in mice indicate an exponential destruction of NO_2^- by the combined mechanisms of reduction and oxidation, with a biological half-period of about 7 minutes. If these metabolic studies in mice are applicable to humans, continuous inhalation of air with 0.1 ppm NO_2 gas, or concentrations of other nitrogenous compounds giving rise to NO_2^- at equivalent levels will lead to a concentration of NO_2^- in body fluids and tissues of about 400ng/kg body weight (about 10nM) (4). Consequently, we suggest that continuous exposure to atmospheric levels of NO_2 or other NO_2^- precursors below 0.1ppm are unlikely to produce an extrapulmonary health risk by interfering with normal metabolic mechanisms. By comparison, a continuous exposure to more than 100ppm NO_2 gas would be required to produce gastric concentrations of NO_2^- comparable to the transient micromolar values associated with ingestion of a meal containing 2-3mg of NO_2^-.

Our mice were found to have similar rates of nitrite oxidation as humans in our in vitro tests. In vivo, we found that NO_2^- concentrations of 60 mg/kg administered to live animals did not appreciably alter the results in comparison to those observed under NCA conditions. This high concentration of nitrite is about one-million times higher than that which might be achieved by a 0.1ppm concentration of NO_2. Our chronic exposure studies also showed comparable, although not identical results. The similarity of the in vitro and the in vivo results in animals lead us to believe that humans can successfully metabolize much higher levels, on a short term basis, of nitrite or nitrate derived from NO_2 inhalation or directly inhaled than they are

likely to encounter in polluted air. However, the effect of lifelong inhalation of nitrogenous precursors that form NO_2^- may well be the gradual build-up of hazardous nitrosation products that could not be detected in our short term experiments.

This brings us to an important question with respect to CARB sponsored work by Richters, Kuraitis, and Sherwin (30,31,32). They found in their main study that a 12 week exposure of C57BL/6J male mice to NO_2 at 0.8ppm resulted in greater metastasis to the lungs of blood borne cancer cells. They also observed increased spleen weights in about 200 Swiss/Webster mice exposed for six weeks to 0.35ppm NO_2 as compared to a like number of controls. The question is,"Does the observed pharmacology form a consistent picture with the observed toxicology?" We believe that it does and give our conclusions in the following summmary model.

Summary Model of Pharmacology and Toxicology. In this model, 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 (48). 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 innocuous amines within 5-10 min. Under our experimental conditions, no metabolic products were identified in large intestine extracts in quantities which exceeded our detection limit of 0.01% of the instilled tracer and corresponded to the chromatographic retention times of simple dialkyl-nitrosamines.

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.

Section VIII

PUBLICATIONS

(Contracts:CARB A7-190-30, A8-121-31,and A0-031-31)

1. Thayer, J.R., Chasko, J.H., Swartz, L.A. and Parks, N.J., Gut Reactions of Radioactive Nitrite after Intratracheal Administration. Science, 217:151 (1982).

2. Parks, N.J., Krohn, K.A., Mathis, C.A., Chasko, J.H., Geiger, K.A., Gregor, M.E., Peek, N.F., Nitrogen-13 labeled nitrite and nitrate: Distribution and metabolism after intratracheal administration. Science, April 1981, pg. 58-61.

3. Parks, N.J., Use of nitrogen-13 in environmental studies. Symposium on Practical Applications of Nuclear Radiochemistry, Second Chemical Congress of the North American Continent, joint with the 180th American Chemical Society Meeting, Las Vegas, Nevada, August 25-29, 1980, Eds. N. Morcas and R.M. Lambrecht (Pergamon Press, New York), 1982, in press.

4. Krohn,K.A. and C.A. Mathis, The Use of Isotopic Nitrogen as a tracer in Chemistry and Biology: in Short-Lived Radionuclides in Chemistry and Biology. Adv. Chem. Ser. 197, (ACS, Washington, D.C., 1981), p.233.

5. Chasko, J.H., Thayer, J.R., Rapid concentration and purification of 13N-labeled anions on a high performance anion exchanger. Int. J. appl. Radat. Isotopes, 32, 645-649 (1981).

6. McElfresh, M.W., Meeks, J.C., and Parks, N.J., Radioanalyt. Chem. 53, 337(1979).

Section IX

REFERENCES

1. Parks, N.J., Krohn, K.A., and Peek, N.F. In Vivo Fate of Nitrogenous Air Pollutant Derivatives. Final Report: California Air Resources Board (Contract CARB A7-190-30), University of California, (1979).

2. Parks, N.J., Krohn, K.A., and Peek, N.F. In Vivo Fate of Nitrogenous Air Pollutant Derivatives: Report No. 2 Final Report: California Air Resources Board (Contract CARB A8-121-31), University of California, (1980).

3. Parks, N.J., Krohn, K.A., Mathis C.A., Chasko, J.J., Geiger, K.R., Gregor, M.E., and Peek, N.F. Science 212:58 (1981).

4. Thayer, J.R., Chasko, J.H., Swartz, L.A., and Parks, N.J., Science, 1982, 217; 151 (1982).

5. Chasko, J.H. & Thayer J.R., Int. J. appl. Radiat. Isotopes. 32:645(1981).

6. McElfresh, M.W., Meeks, J.C. & Parks, N.J., Radioanalyt. Chem. 53:337 (1979).

7. Parks, N.J., Krohn K.A. Int. J. appl. Radiat. Isotopes. 29:754 (1978).

8. Sedgwick, C. and Jahn, S. California Vet. 34:27 (1980).

9. Wolk, C.P., Thomas J., Shaffer, P.W., Austin S.M., & Galonsky, A., J. Biol. Chem. 251:5027 (1976).

10. Thayer, J.R., and Huffaker, R.C. Anal. Biochem. 102:110, (1980).

11. Goldstein E., Peek, N.J., Parks, N.J., Hines, H.H., Steffy E.P., & Tarkington, B. Am. Rev. Respir. Dis. 115:403 (1977).

12. Schneider, N.R., Yeary, R.A. Am. J. Vet. Res. 36:941-947 (1975).

13. Green, I., Hiatt, R.P. Am. J. Physiol, 176:463-476 (1953).

14. Friedman, M.A., Greene, E.J., Epstein, S.S. J. Pharmaceutical Sciences 61:1492-1494 (1972).

15. Mirvish, S.S., Patil, K., Ghadirin, P., Kommieni, V.R.C. J. Natl. Cancer Inst. 54:869-875 (1975).

16. Witter, Balish, Gatley. CV rats. Appl. Env. Microbiol. 38:870-878 (1979).

17. Kety, S.S. Methods Med. Res. 8:228-236 (1960).

18. Walser, M., Seldin, D.W., Grollman, A. J. Air Invest. 32:299-311 (1953).

19. Alderson, P.O., Krohn, K.A., Welch, M.J. Radiopharmaceuticals. In: Diagnostic Nuclear Medicine, Gottschalk, A. & Potchen, E.J. eds. The Williams & Wilkins Company Baltimore, publishers p. 26-50 (1976).

20. McNeil, D.R. Interactive Data Analysis, John Wiley & Sons NY (1977).

21. Kurzer, M.S. & Calloway, D.H. Am. J. Clin. Nutrition 34:1305 (1981).

22. Dawson, S.V. & Schenker, M.B. Am. Rev. Resp. Dis. 120:281 (1979).

23. Pryor, W.A., & Lightsey, J.W. Science 214:435 (1981).

24. Lee, Y.N. & Schwartz, S.E. J. Phys. Chem. 85:840 (1981).

25. Postelthwait, R.M. & Mustafa, M.G. J. Toxicol & Env. Health 7:861 (1981).

26. Thayer, J.R. & Huffaker, R.C. Anal. Biochem. 102:110 (1980).

27. Thayer, H.R. Altex Chromatogram 3:2 (1979).

28. Cooper, A.J.L., Macdonald, J.M. Gelbard, A.S., Gledhill, R.F. & Duffy, T.E. J. Biol. Chem. 254:4982 (1979).

29. Savage D.C. In: Defining the Laboratoy Animal. IV symposium, International Committee on Laboratoy Animals; (National Acadamy of Sciences Washington, D.C. p. 60 (1971).

30. Richters, A. Facilitation of Cancer Metastases by an Air Pollutant, Journal of Surgical Oncology 17:159-162 (1981).

31. Kuraitis, K.V., Richters, A., Sherwin, R.P. Spleen Changes in Animals Inhaling Ambient Levels of Nitrogen Dioxide, Journal of Toxicology and Environmental Health 7:851-859 (1981).

32. Richters, A., Kuraitis, K.V. Inhalation of NO and Blood Borne Cancer Cell Spread to the Lungs, Archives of Environmental Health, Vol 36, No. 1:36-39 (1981).

33. Appel, B.R., Tokiwa, Y., & Haik, M. Atmospheric Environment 15:283 (1981).

34. Spengler, J.D., Ferris, B.G. Jr., Dockery, D.W., Speizer, F.E. Env. Sci. & Tech. 13:1276 (1979).

35. Pitts, J.N. Jr., In: Nitrogen Oxides and Their Effects on Health , Lee, S.D., Ed. (Ann Arbor Science, Ann Arbor, Michigan, p. 77 (1980).

36. Tannenbaum, S.R., Fett, D., Young, V.R., Land, P.D. & Bruce, W.R. Science 200:1487 (1978).

37. Saul, R.L., Kabir, S.H., Cohen, Z., Bruce, W.R. & Archer, M.C. Cancer Res. 41:2280 (1981).

38. Lijinsky, W, & Greenblatt, M. Nature (London) New Biol. 236:177 (1972).

39. Lijinsky W., & Epstein, S.S. Nature (London) 225:21 (1970).

40. Mirvish, S.S., Toxicol. Appl. Pharmachol. 31:325 (1975).

41. Sen, N.P., Smith, D.C., Schwinghamer, L. Food Cosmet. Toxical. 7:301 (1969).

42. Klubes, P., & Jondorf, W.R. Res. Commun. Chem. Pathol. Pharmacol. 2:24 (1971).

43. Iqbal, Z.M., Kahl, K., Epstein, S.S. Science 207:1475 (1980).

44. Newberne, P. Science 203:1079 (1979).

45. Mirvish, S.S. Bulay, O., Runge, R.G., Patil, K., J. Natl. Cancer Inst. 64:1435 (1980).

46. Aoyagi, M., Matsukura, N., Uchida, E., Kawachi, T., Sugimura, T., Takayama, S., Matsui, M., J. Natl. Cancer Inst. 65:411 (1980).

47. Interagency Working Group, "Evaluation of the MIT nitrite feeding study to rats" (Report to the Food and Drug Administration, Washington, D.C., August 1980)

48. Ryan, G.B. & Majno, G. Am. J. Pathology, 86:185 (1977).