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ABSTRACT

The previously unknown metabolic fate of nitrate and nitrite introduced into the respiratory tract has been studied because they exist in the ambient California urban aerosol or can be derived from known nitrogenous air pollutants. The metabolic fate of NO_X ions in the digestive tract also has been studied for comparative purposes because these ions are known to form cancer-causing nitrosamines therein under superambient exposure conditions. Radiochemical syntheses (Section 3.2) for 0.5 Ci quantities of radioactive nitrogen-13 (half-life = 10 min) labeled NO_X tracer ions have been developed to address the objectives of this study. This has permitted the fate of these ions to be studied at pharmacologically non-perturbing concentrations which may more accurately reflect their biological fate under realistic ambient exposure conditions. The development and evaluation of first-generation radiochromatographic methods for chemical analysis of N-13 labeled compounds in biological fluids are described (Section 3.2).

The organ distribution of N-13 has been measured following intratracheal, gavage, and intravenous administration to adult mice and rabbits and to pregnant mice. The tracer NO_X ions were in the chemical form of nitrate and nitrite (no added carrier) (Section 3.3). The N-13 label from both nitrate and nitrite was rapidly cleared from the organ of introduction and distributed throughout the body. An equilibrium distribution was reached in about 5 minutes after injection and did not change over the next 30 minutes. Less than 3% of the tracer was excreted rapidly into the urine. The label from both nitrate and nitrite crossed the placenta to the fetus. Both nitrate and nitrite were found to react in vivo, but the conversion of nitrite was much more extensive than for nitrate. These pharmacokinetic results suggested a need for additional work.

The overall results suggest that the toxicological data bases for inhaled nitrogen dioxide gas, a precursor to nitrate and nitrite, and ingested inorganic nitrates and nitrites are useful in evaluating the health hazard associated with air pollutant inorganic nitrates. A biochemical mechanism is postulated for pathogenic effects associated with exposure to compounds containing nitrogen in its higher oxidation states.

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Disclaimer: "The statements and conclusions 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 is not to be construed as either an actual or implied endorsement of such products."

ACKNOWLEDGEMENTS AND PREFACE

"In completing one discovery, we never fail to get an imperfect knowledge of others of which we could have no idea before, so that we cannot solve one doubt without creating several new ones."

-Joseph Priestly, 1733-1804

As Californians and scientists at the University of California, the investigators have considered this research into air pollution and health effects relationships a privilege and a duty. This obligation derives from the unique availability at Davis of the novel cyclotron based nuclear technology and scientific expertise necessary to address certain environmental health issues pertaining to nitrogenous pollutants. This report presents independently useful information, but in a larger view, it represents the logical extension of previous California Air Resources Board sponsored research into the fate of inhaled nitrogen dioxide.

This work has proven to be scientifically exciting, particularly because of the new and unresolved questions which have arisen. It is a cooperative effort drawing on the resources of the cyclotron laboratory as well as the School of Veterinary Medicine and the School of Medicine.

We wish to acknowledge: the clinical assistance of Gayle Vial, D.V.M., and Evelyn Profita who volunteered their special expertise in difficult animal preparations; the spartan dedication of the cyclotron crew whose technical prowess made it possible for them to faithfully deliver the 100,000 trillion protons per second required for the nuclear chemical syntheses; the staff of Crocker Laboratory, the Laboratory for Energy-related Health Research, and the California Air Resources Board who have helped resolve various logistics problems in a collegial spirit; collaborating undergraduate, graduate, and post-graduate scholars, Michael McElfresh, Kenneth Geiger, Marsha Gregor, Scott Steffen, Joseph Chasko, and Chester Mathis, Ph.D.; our colleagues from the N-13 Research Group, Donald Dohan, Ph.D., John C. Meeks, Ph.D., and James Thayer, Ph.D., for their innovative suggestions; the U. S. Department of Energy and the National Science Foundation who supported the design and fabrication of the N-13 target system.

PROJECT OBJECTIVES

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

(1) to compare and quantitate the in vivo distributional fate of intratracheally administered, ingested or gavaged, and intravenously injected labeled nitrate and nitrite when administered in realistic dosages that were not pharmacologically perturbing,

(2) to evaluate, on the basis of the comparative studies, how toxicology research on these compounds from outside the realm of air pollution effects studies might contribute to the presently very limited data base about the health effects of inhaling these compounds, and

(3) to advance the present understanding of nitrogen dioxide effects studies since most evidence points to the conversion of NO_2 to nitrites and nitrates in the respiratory tract.

Section 1: Conclusions

1.1 Introduction

The presence of inorganic and organic nitrates in the urban aerosols of California is well documented, but the health impact of chronic inhalation of these compounds is almost completely unknown. However, extensive toxicological studies with inhaled nitrogen dioxide gas and limited studies with super-ambient amounts of ingested nitrites and nitrates are useful in evaluating the health risk associated with inhaled nitrates. These classical high-dose toxicologic studies and our tracer approach are complementary.

Our metabolic tracer studies with <0.1 picomoles of nitrite and nitrate labeled with radioactive nitrogen-13 (half-life = 10 min) are consistent with previous work (ARB-1116) concerning the metabolic fate of inhaled radioactive nitrogen dioxide. The N-13 label from inhaled nitrogen dioxide was rapidly taken up by the blood and distributed systemically, probably following reaction of nitrogen dioxide with aqueous lung mucous to form nitrous and nitric acids which dissociate to ions in vivo.

In the current study, N-13 from radioactive nitrite and nitrate was introduced into the lungs, stomach, and blood of experimental animals at subambient concentrations. Again, the tracer was rapidly taken up by the blood and systemically distributed.

1.2 Summary and Conclusions

The conclusions reached are the following:

(1) The organ distribution of N-13 nitrite and nitrate entering the lungs was the same after 30 minutes as that following intravenous injection.

(2) The distribution of N-13 following gavage with both ions had the same qualitative distribution as after lung administration.

(3) There were small quantitative temporal variations of N-13 organ distribution following different entry routes.

(4) The N-13 radioactivity concentration from both ions was about 2-3 times higher in vital organs as compared to carcass.

(5) The N-13 from both ions was uniformly distributed among blood fractions.

(6) The N-13 label from both ions introduced into the stomach of pregnant rats appeared in the fetuses.

(7) We believe that the primary "biological target" of inhaled inorganic nitrates or organic nitrates degraded in vivo to produce nitrite is not a specific organ, but a ubiquitously distributed biochemical pool that is also supplied by ingested nitrates and nitrites.

(8) We found from limited analytical experiments that nitrite is largely and nitrate is slightly metabolized to other compounds in vivo.

(9) Our synthesis of literature information suggests that among the important and detrimental potential metabolites are methemoglobin and nitrosyl compounds. The latter can influence the physiologic levels of biological molecules that regulate cellular function and growth and are pathogenic if in vivo repair mechanisms are suppressed.

(10) Substantially superambient exposure levels used in some toxicology studies may saturate normal processes by which nitrogenous compounds such as NO_2 are metabolized nonpathogenically. Thus, pathogenic manifestations may be observed following high doses which are extremely unlikely following exposure to ambient concentrations of the same pollutant. The high dose metabolic experiments can be done on a large scale without tracer, but the low dose experiments are uniquely dependent upon tracer methods.

Section 2
RECOMMENDATIONS

2.1 The large body of existing toxicological data for exposure to nitrogen dioxide appears to be relevant to suspended nitrate health concerns. Since nitrates are derived from emissions of nitrogen oxides (NOx), a general recommendation is that maintenance of atmospheric NOx 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.

2.2 The N-13 label from $^{13}NO_2^-$ and $^{13}NO_3^-$ is incorporated into other chemical moieties. It is recommended that these species be studied and identified to test the hypothesis that nitrogenous air pollutants can lead to nitrosyl compounds which perturb normal physiochemical processes.

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

2.4 The metabolic effect of nitrate concentrations greater than the ambient background concentration should be studied using tracer methods in order to better interpret existing toxicologic data bases for nitrogenous pollutants.

2.5 The effects of "protector" free radical scavengers such as vitamins on the metabolic fate of NO_2^- and NO_3^- should be examined by both tracer and classical toxicologic methods.

Section 3

The In Vivo Distribution of N-13 Introduced as Nitrate and Nitrite

This report describes a pioneering effort to bring a novel application of nuclear technology to bear on heretofore unassailable problems in understanding the environmental toxicology of nitrogenous compounds. A preliminary report of this work presented graphical illustrations of key data distributions which are numerically summarized herein. The illustrations represent a useful alternative data presentation and are included in this report as Appendix A. The development of radioactive N-13 as an experimental probe in environmental research has been greatly advanced under the auspices of the California Air Resources Board; the comparative merits of this methodology are included in this report as Appendix B.

3.1 Introduction

The presence of nitrogenous air pollutants in Californian urban aerosols has been well documented although chemical identification and precise quantitation continue to be an area of active investigation (1-5). The primary nitrogenous emissions include nitric oxide (NO) and nitrogen dioxide (NO_2), plus their known derivatives, nitric acid, inorganic particulate nitrates, and organic nitrates such as peroxyacetylnitrate (PAN), which may be accompanied by formation of nitrosamines, nitramines, and amides (6). Many of these compounds have been implicated, either directly or indirectly, as having adverse health effects, including potential for inducing mutagenesis and carcinogenesis (7-14). Our efforts have concentrated on increasing our understanding of the biochemical fate of two of the simplest nitrogen oxide ions; NO_2^- and NO_3^-, labeled with the short-lived positron/gamma emitter, N-13 (T, 1/2, = 10 min). These ions were selected since (1) inhaled nitrogen dioxide is expected to result in their formation through a disproportionation mechanism, (2) they exist in the ambient aerosol, and (3) organic nitrates may be rapidly metabolized to one or both in vivo. This work has further general significance because, in addition

to the continual insult of nitrate and nitrite from atmospheric sources, we ingest them as natural components of some foods and as additives/preservatives of others. Furthermore, the human digestive tract generates some nitrite and nitrate in situ. A review of sources and pathways for nitrogenous environmental pollutants that lead to the endogenous presence of nitrate and nitrite in humans was included in a presentation by the investigators at the 1979 fall meeting of the American Chemical Society and is included as Appendix A (15).

The available evidence suggests that low concentrations of these compounds are well tolerated by human physiology; yet recent toxicologic studies by Newberne (14) produced evidence that high concentrations of NO_2^- are carcinogenic. The mechanism was postulated to involve something more direct than the highly mutagenic and carcinogenic N-nitroso derivatives which can be formed by reaction of NO_2^- with dietary amines. The present concern is the complete lack of information regarding the possible formation of N-nitrosamine compounds after inhalation of candidate precursors such as nitrogen or nitrate because the in vivo biochemical fate of these compounds is unknown.

Another concern is the established connection between high nitrate concentration in drinking water and infant methemoglobinemia (9), a condition in which the blood loses its essential O_2 transport property. The disease is characterized by conversion of hemoglobin (Hb) which contains Fe (II) to methemoglobin (MetHb) containing Fe(III) and is caused by NO_2^- entering erythrocytes directly or secondary to in vivo reduction of nitrates by intestinal bacteria.

Clinical methemoglobinemia apparently appears only in infants, suggesting some unusual susceptibility or absence of a protective or repair mechanism in the immature physiologic state. Nitrites have been administered to lactating rats via their drinking water and no abnormal rise was found in MetHb levels of the suckling rats. By contrast, the mothers showed high MetHb levels and this was interpreted as evidence that nitrites are apparently not transferred via the milk. Gruener and colleagues (21) measured the transfer of nitrites to the fetus in utero and the subsequent production of MetHb by giving sodium nitrite orally or intraperitoneally to pregnant rats. Their results showed that nitrite concentrations rose in fetal blood about 20 min. after injection and the nitrite levels were followed closely by MetHb levels.

Consequently, it is important that we first understand the biochemical fate and, through that, the ultimate health consequences of nitrite and nitrate taken into the body. An additional advantage to this starting point is it provides a basis for relating the toxicological data base pertaining to ingested nitrate (16) to the data-base pertaining to inhaled nitrogen dioxide (17, 18). Thus, the toxicological information available for ingested nitrate can be interpreted in a more efficacious manner with respect to estimating the health impact of inhaled nitrates.

The route of entry of these compounds into the body is complex. Atmospheric nitrogen compounds that are inhaled may enter the mouth, trachea and respiratory tract, disproportionate, and react with those tissues or be absorbed into the vascular and lymphatic systems. If attached to particles, they may traverse the mucociliary escalator and be swallowed. Pharmacologists interested in the application of $NaNO_2$ and organic nitrates as vasodilators have reported order-of-magnitude differences in drug potency, depending upon the route of administration (19). For example, there is considerable GI absorption of nitrates administered orally, but they are degraded by the liver and little intact drug reaches the systemic circulation. In contrast, nitrates absorbed through the sublingual mucosa are maximally effective.

The experiments performed in the course of the present investigation of nitrite and nitrate derived N-13 organ distribution were designed to test for (1) possible metabolic variations dependent on route-of-entry, and (2) possible metabolic variations dependent on the different oxidation states of nitrogen in NO_2^- and NO_3^-. These experiments are unique in that they are the only comparative study of the fate of [N-13]-nitrite and [N-13]-nitrate in mammals. A limited study complementary to this one was reported by a University of Wisconsin group who compared [N-13]-nitrate metabolism in rats and humans (20). In addition, we have examined the transfer of N-13 from both anions across the placenta in pregnant animals.

In our experiments, labeled compounds were administered without added carrier so that the fate of the N-13 in the tracer molecule would be that of a similarly bonded nitrogen in a physiological system unperturbed by macroscopic carrier material. This is a critical point because most toxicology studies (17, 18) have employed super-ambient levels of material which may have saturated normal physiological protective processes.

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3.2 Development of Experimental Methods

Much of our effort this year was directed towards development of methods for efficient synthesis and analysis of high specific activity labeled nitrogen compounds. This section summarizes our progress in cyclotron target and synthetic chemistry and the development of a computer based hardware and software system for rapid analysis of labeled inorganic compounds and biological samples.

We have developed a cyclotron target for production of ^{13}N by the $^{16}O(p,\alpha)$ reaction on recirculating aqueous solutions in a stainless steel target chamber. The total target system includes facilities to produce $^{13}NO_3^-$, $^{13}NO_2^-$, ^{13}NN and $^{13}NH_3$, although only $^{13}NO_3^-$ and $^{13}NO_2^-$ production are relevant to this project and will be covered here. Further details of this target system have been discussed elsewhere (1,2, see also Appendix B).

3.2.1 Target System

The details of the target installed at the University of California, Davis, "193 cm" Crocker Cyclotron Facility are shown in Fig. 1. The irradiation cavity (IC) has a volume of $\sim$2.35 cm^3 and a surface area exposed to the beam of $\sim$6 cm^2. The incident 20 MeV proton beam is defocused in the vertical plane and swept continuously in the horizontal plane, thus irradiating about 2 cm^3 of solution in the IC with protons degraded to about 18 MeV by the foils. Primary cooling with an 80 cm^3s^{-1} flow of water over the rear surface of the target maintains the target solution in the external circuit at $< 75°C$ with beam currents < 35 μA. Continuous deionization of the cooling water minimizes electrolytic processes during irradiation. Circulation ($\sim$60 cm^3s^{-1}) of cooled helium (15°C, 1.2 atm) through the interfoil region of the target block assembly provides a non-oxidizing environment for the front side of the target-foil and auxiliary cooling.

A schematic of the entire target system is shown in Fig. 2. The target is connected to the other system components, 5 meters away and outside the 2-m thick cyclotron shielding walls, by 3.175 mm o.d. x 1.5 mm i.d. Teflon tubing. The flexible Teflon connections provide electrical isolation and permit remote

removal of the target block from the beam-line and storage in a nearby "hot" cell without breaking the system's flow integrity. The target solution (TS) volume is typically 60 cm^3 with approximately ±5 cm^3 variation allowed by the filling level of the 15-cm^3 gas collector. The TS is circulated typically at 2 $cm^{-3}s^{-1}$ by a variable-speed, magnetic drive, centrifugal pump (Micropump Corp., Concord, CA; model 12A-41-316). The four-way valve arrangement (Fig. 2) permits complete replacement of the TS without interrupting the proton bombardment.

The products of interest or their precursors are formed by 18-MeV proton bombardment of water or aqueous solutions, which induces the $^{16}O(p,\alpha)^{13}N$ nuclear reaction (3). The ^{13}N radioactivity yield under our conditions is 29 ± 1 mCi/μA for 20-minute irradiations.

Labeled nitrate comprises > 80% of the ^{13}N species ($^{13}NO_3^-$, $^{13}NO_2^-$, $^{13}NH_4^+$) recovered after recirculated H_2O TS has been irradiated with ∿18 MeV H^+ beams of 1 to 40 μA intensity for from 2 to 30 minutes. Any combination of current intensity and time that results in charge deposition of more than 18,000 μC in the TS produces 96 to 99% $^{13}NO_3^-$ as determined by high performance ion-exchange chromatography.

Irradiation of target solutions with beam currents (40 μA maximum) of 18 MeV protons routinely produced one curie of ^{13}N accompanied by ^{15}O ($T_{1/2}$ = 2 min) and ^{18}F ($T_{1/2}$ = 110 min). At the end of a 20-min irradiation, 0.176 mCi of ^{15}O and 0.0015 mCi of ^{18}F are present per mCi of ^{13}N. After a 10-min period required for the various chemical manipulations or separations, the ^{15}O radioactivity is reduced to about 1% of the ^{13}N radioactivity. Thus, about 0.5 Ci of ^{13}N in the chemical form of $^{13}NO_3^-$, $^{13}NO_2^-$, $^{13}NH_4^+$ is available for subsequent use.

The target water used is distilled in glass, with no further pretreatment. Under the relatively high dose conditions of our 20 μA x 20-min irradiations, the radiochemical composition is typically 0.2% $^{13}NH_3$, 1.5 to 4% $^{13}NO_2^-$, and 96 to 98% $^{13}NO_3^-$. We have also irradiated water with an overpressure of various gases to determine some of the parameters affecting the radiochemical product spectrum. These irradiations were done at lower doses, typically 1 to 5 μA for one minute to facilitate the running of multiple samples. Under

these conditions using air-saturated target water, the yields range around 1% $^{13}NH_3$, 10% $^{13}NO_2^-$, and 90% $^{13}NO_3^-$. Using water purged with oxygen, argon, or nitrogen the yields shifted dramatically towards $^{13}NO_2^-$, with 1% $^{13}NH_3$, 35 to 50% $^{13}NO_2^-$ and the balance $^{13}NO_3^-$. Carbon dioxide increased the $^{13}NH_3$ yield to 10% and decreased the $^{13}NO_2^-$ to 1%. We are continuing to study those factors with the aim of direct production of $^{13}NO_2^-$ in the cyclotron target.

3.2.2 Synthesis of [N-13]-Labeled Nitrite

Labeled nitrite is prepared by direct discharge of 60-cm^3 target solution that has been irradiated under the previously described high ^{13}N-nitrate production conditions through a cadmium-copper reducing column. The reducing column is prepared freshly and operates at a flow rate of 60 cm^3 min^{-1} without preconditioning with carrier nitrate. The description that follows is taken from a more detailed discussion by McElfresh et al. (4).

3.2.3 Preparation of Reduction Column

Preparation of the cadmium reaction column was adapted from that of Wood et al. (5). Packing for a 7 mm inside diameter and 24 cm in length column is prepared by filing a cadmium bar (99.999% purity, Ventron, Alfa Products, Beverly, Mass.) with a half-round rasp. Iron from the rasp is removed by drawing a magnet across the filings. Approximately 20 g of filings are placed in a 250 ml beaker and treated with 2N HCl. Each 20 min treatment is followed by washing with distilled water until the pH is neutral.

The treated filings are transferred to a container designed to minimize contact with air (cf. Ref. 5, Fig. 2). When treated filings are in contact with air (out of solution) they undergo an underdetermined chemical reaction that greatly reduces their capacity to produce $^{13}NO_2^-$. The filings in the container are treated with two changes of 0.08 M $CuSO_4$ solution over a 20 min period. The solution turns grey-blue and a copper precipitate forms. The precipitate is removed by careful flushing with distilled water and gentle agitation. The distilled water is replaced with 0.15 M NH_4Cl (pH 5.2) by repeated decanting and refilling.

Prior to packing, the base end of a column is fitted with a 1 cm copper wool plug, prepared by washing for 5 min in 2N HCl followed by a distilled water rinse. A graduated reservoir is attached to the top of the column and the column then filled with 0.15 M NH_4Cl (pH 5.2). A given amount of the treated cadmium filings is displaced into the column by inverting the treatment container into the reservoir atop the column. The filings are packed by gentle tapping to a final length of 20 cm. A second acid treated copper wool plug of 2.5 cm is fitted at the top of the filings. Prior to use, 300 ml of glass-distilled water is drawn through the column. The column is stored under 0.15 M NH_4Cl (pH 5.2).

3.2.4 Synthesis and Purification of $^{13}NO_2^-$

Forty to sixty milliliters of irradiation solution from the recirculating target system is transferred to the 70 ml calibrated reservoir atop the cadmium reduction column (Fig. 3). A peristaltic pump is used to control the flow rate of $^{13}NO_3^-$ solution and, in some experiments, to recirculate the eluent through the cadmium column at approximately 80 mL/min. Normally after one passage through the column the eluent containing primarily $^{13}NO_2^-$ and $^{13}NH_4^+$ is directed into the input tube of a Buchi Rotovapor-R (Brinkmann Instruments, Inc., Cantiague Road, Westbury, NY). The flask contains 0.15 mL of 0.1 M NaOH, which yields a solution of pH greater than 11 with eluent volumes of 40 to 50 mL. This is sufficient to convert $^{13}NH_4^+$ to $^{13}NH_3$. The rotary evaporation condenser coils are cooled with circulating ice water and the vapor trap is maintained at -196°C with liquid nitrogen. The round bottom flask is immersed in a water bath maintained at 65°C. The evacuated $^{13}NH_3$ is captured in the rotary evaporator vapor trap and in a second liquid nitrogen trap placed prior to the vacuum pump. Sixty milliliters of solution can be reduced to dryness in about 8 min. The dry residue is neutralized with 0.15 ml of 0.1 N HCl and the $^{13}NO_2^-$ dissolved in buffers appropriate to produce the desired volume and ionic strength.

The irradiated target solution, reduction column eluent, and final solution for biological experiments are analyzed by the methods described in the following solution. Routinely $^{13}NO_2^-$ of radiochemical purity greater than 97% is obtained in the eluent of the cadmium column alone. Rotary evaporation of the eluent at pH 11 removes essentially all of the contaminating $^{13}NH_3$, resulting in a final solution of $^{13}NO_2^-$ with radiochemical purity greater than 99.2%. Fig. 4a-c are radiochromatograms of target solution, reduction column eluent (one pass), and final solution, respectively. The reduction of $^{13}NO_3^-$ to $^{13}NO_2^-$ is evident by the shift in peak areas between Fig. 4a and 4b. The removal of $^{13}NH_4^+$ by rotary evaporation is observed at the decrease in the $^{13}NH_4^+$ peak between Fig. 4b and 4c.

3.2.5 Summary of Radiochemicals Produced

The radiochemical parameters are summarized as follows:

	Concentration	Total Activity	Radiochemical Purity	Radionuclidic Purity
$^{13}NO_3^-$	8-10 mCi/mL	~500 mCi	>96%	<10% ^{15}O
$^{13}NO_2^-$	> 30 mCi/mL	~200 mCi	99% >95% required	~ 0% ^{15}O <0.3% ^{18}F

The ^{15}O produced with the $^{13}NO_3^-$ presents no problems in analysis or tracer studies as it is separated by chromatography for analysis and has decayed at a negligible concentration by the time animal organ distributions are measured. The ^{18}F is also separated by chromatography and is corrected for in organ distributions by taking a duplicate count after long times (>1.5 hr) when the ^{13}N has decayed to a small amount. We have attempted removal of the ^{18}F with an alumina column to eliminate the necessity of the second measurements with some success. The ^{18}F was quantitatively removed, but too much of the ^{13}N activity was also adsorbed on the alumina. Further experiments will be done to increase the specificity of the removal of ^{18}F.

3.2.6 Development of Analytical Methods

Analyses of cyclotron target solutions, purified $^{13}NO_2^-$, and $^{13}NO_3^-$, serum fractions and urine samples is done by high performance liquid chromatography (HPLC). The present 3-column system permits characterization of samples by three different chemical mechanisms of separation. Each system consists of a high pressure pump, a 25 cm x 4 mm I.D. micro-particle HPLC column and a NaI (Tl) well-type scintillation detector with associated electronics. A sample is loaded into the variable-volume injector and pumped through the column where the constituents are separated based upon their chemical properties before the effluent passes through the well of the scintillation crystal. The radioactive counts generated as a peak is eluting pass from the photomultiplier-preamp to an amplifier, thence to a single channel analyzer (SCA) which discriminates the gamma rays produced by positron annihilation and feeds the pulses to a ratemeter which generates an analog signal for the recorder trace, and to a high speed, four-channel buffered scaler where the pulses for each detector are integrated and transmitted to the PDP 11/40 at two second intervals (see Figure 5). The scaler can also be outputted directly to a teletype at 6-second intervals. In addition to the radiation detector, we have a 254 nM UV absorbance detector which can monitor the optical density of the effluent from any one column. This permits calibration with cold samples of UV-absorbing compounds such as aromatic molecules and nitrosamines.

The HPLC columns used are Partisil-10 SAX and Partisil-10 SCX ion exchange columns from Whatman, and μ-Bondapak C_{18}, a microparticulate silica column from Waters that has organic functional groups attached to give partitioning effects. Partisil-10 SAX is a strong anion exchanger which separates ions by their degree of negative charger at the pH of the eluting buffer. Neutral molecules and cations have no attraction to the column and elute with the breakthrough or void volume. Anions are retained according to their relative size and charge; NO_3^- has the longest retention time of about three column volumes, NO_2^- and nucleic acids elute sooner depending on the properties of the eluting buffer. This column is used to determine the radiochemical composition of the cyclotron target solution and the radiochemical purity of ^{13}N labeled $^{13}NO_2^-$ and $^{13}NO_3^-$ (see sample chromatogram, Figure 6). It is also used to determine $^{13}NO_2^-$ and $^{13}NO_3^-$ in blood fractions, urine and other biological samples.

Partisil-10 SCX is a strong cation exchanger which retains positively charged species. Anionic $^{13}NO_2^-$, $^{13}NO_3^-$ and uncharged molecules elute with the void volume, $^{13}NH_4^+$ is retained about two column volumes, and less basic amino acids elute at intermediate volumes, again depending on the properties of the eluting buffer. This serves as a crosscheck on the radiochemical purity of $^{13}NO_2^-$ and $^{13}NO_3^-$ and biological samples are analyzed for biosynthetically labeled cationic species (see sample chromatogram, Figure 7).

The μ-Bondapak C_{18} column consists of silica particles with octadecyl hydrocarbon chairs chemically bonded to the surface, presenting a very non-polar partitioning interaction. Non-polar molecules such as benzene have long retention times, whereas species of mixed character, such as nitrosamines are eluted earlier, and very polar molecules elute with column breakthrough. This column is used to analyze blood fractions and urine samples for molecules in which ^{13}N has been incorporated into organic molecules, particularly nitrosamines (see sample chromatogram, Figure 8).

The samples of radiochemicals produced are injected directly into the chromatograph for routine quality controls. Cold experiments have demonstrated that there are no anomalies in the measurement of nitrite and nitrate by this method (6). Biological samples, however, require special precautions to avoid damaging the HPLC columns. All biological samples are centrifuged and filtered through a 0.45 micron disposable filter before injection to remove gross amounts of solid material. In addition, a guard column is placed between the sample injector and the analytical column. The guard column contains a pellicular packing of the same type as the particulate packing of the analytical column. This removes large molecules or other compounds which would bind irreversibly to the analytical column and shorten its useful life. The guard column is repacked with fresh material as necessary. The use of guard columns can greatly extend the lifetime of the more expensive analytical columns.

We expect that some of the unidentified labelled compounds in biological samples are high molecular weight protein molecules. To separate such compounds we require a model of separation that will differentiate molecules on the basis of size rather than charge or polarity. This can be accomplished

with high performance gel permeation chromatography (HPGPC). Recent advances in HPGPC have produced gel permeation columns of high efficiency and speed which can be used on our present equipment.

In addition to liquid chromatography we have obtained a gas chromatograph (Bendix 2600) for analysis of dissolved labelled gases in biological samples. This is used to separate ^{13}NN, ^{13}NO, and $^{13}N_2O$ and is interfaced to a scintillation crystal, electronics and the computer in the same way that our liquid chromatographs are set up.

3.2.7 Development of Data Processing Capabilities

The purpose of the PDP 11/40 computer system is to provide automatic data acquisition and on-line analysis of chromatographic and organ distribution data. This required hardware development, the interfacing of radiation detectors and an electronic balance to the PDP 11/40, and extensive software development. The software system will provide (1) continuous acquisition of data from the various radiation detectors and from the balance, (2) console communication, and (3) automatic or interactive on-line data analysis with video display of results.

The hardware interface of the detectors to the computer is completed and is in routine use; additional work is required to interface the electronic balance. The data acquisition system reads a buffered four-channel scaler at two second intervals along with the clock within the computer and stores the data on an RK05 disk. This procedure is now accomplished on a routine basis and the data saved for analysis after completion of the cyclotron run. Console communication serves two tasks, program control and insertion of data. Operator program control is used to pen data files, initiate and control acquisition of data, request analyses of previous data, and terminate acquisition. The data to be inputted from the console includes chromatographic and experimental conditions, sample identification, and a signal to indicate injection of each HPLC sample.

The object of the data analysis is to detect chromatographic peaks, background and decay-correct the data, integrate the peaks, and provide a composite analysis promptly at the end of each chromatogram. These capabilities have been incorporated into the software system. At the time of the cyclotron irradiation each HPLC sample identifier and column number are entered at

the console and stored with the injection time in a file on the RK05 disk. The chromatogram can later be automatically retrieved and displayed on the Textronics 4013 video terminal. The peaks are marked by the operator using movable cursors and a background and decay-corrected report is plotted on the screen, which can be recorded with the Textronics 4610 hard copy unit (see typical output, Figure 9). The HPLC data analysis is rapidly accomplished at the conclusion of a cyclotron run. Dr. Don Dohan is currently updating the operating system with multi-terminal capability which will permit on-line data analysis as well as additional input terminals for organ distribution data.

3.2.8 Development of Experimental Software

The experimental software system now in use consists of two packages: program LUCAS developed by Dr. Don Dohan and Mr. Joe Chasko for data acquisition and CRT console communication, and program PLOT written by Mr. Joe Chasko as an interactive graphics system for the analysis of chromatographic data.

3.2.9 Program LUCAS

The function of LUCAS is to acquire and store data from up to four scintillation gamma ray detectors via the buffered four-channel scaler, and to monitor the video terminal for commands and experimental information. The gamma detectors monitor the radioactivity associated with the effluent from each of three chromatographic columns. After processing the electronic signals through single channel analyzers the counts are input to three channels of the scaler. The fourth channel may be connected to a radio gas chromatograph, an additional remote HPLC system, or to a well detector for organ distribution studies. The latter requires additional software development before routine use.

The scaler has an internal timer which can be set to transmit data to PDP 11/40 at a selected interval which is normally two seconds. The program runs the command string interpreter continuously until it senses the transmission of a data string from the scaler, at which time it interrupts execution to process the data. The program reads the internal clock of the PDP 11/40 and encodes the time and counts from the four channels into one line of data which is held in an output buffer. When the buffer contains one disk block of 16 lines of data, the block is stored in the open data file on the RK05 disk. At

the conclusion of an experimental run, the last partial buffer is transferred, the file closed, and the name and length of the file entered in the disk file directory. One RK05 disk pack with all system programs resident can store more than thirty hours of data gathered from four detectors at two second intervals. (See program flow chart, Figure 10).

In between interrupts from the scaler, LUCAS continuously checks the console terminal(s) for typed characters. Characters input from the terminal are stored in a command line buffer until a carriage-return command causes execution to branch to a command string interpreter. LUCAS spans the typed line for the command and branches to the appropriate program section for execution (see Figure 11 for a summary of commands). Several commands control file maintenance and data acquisition. The LOG command is used to identify an HPLC sample and signal injection and also to record experimental information. It inputs the HPLC column identifier and the sample volume and description which are encoded with the internal-clock time for storage in a log file. The log file contains experimental and chromatographic conditions with a list of injection times, column and sample identification (see Figure 12 for a segment of a typical Log file). These entries are subsequently used by PLOT to retrieve and display the chromatograms for analysis.

3.2.10 Program PLOT

PLOT uses an interactive, semi-automated approach to chromatographic data analysis. This method was chosen over a fully automatic peak detection program because of the special demands of short-lived isotope chromatography. The extremely wide dynamic range (greater than 10,000:1) and variation in peak shape and background levels normally encountered can generate unreliable results using even very sophisticated peak recognition algorithms, while the visual identification of peaks by the operator from a graphics display is accurate, rapid, and reproducible.

PLOT is a flexible graphics display and chromatographic reduction program which permits automatic retrieval of chromatograms using log file entries or semi-automatic retrieval by manual entry of the injection time and column number. The latter method is useful for earlier data files taken without the log entry capability, or for data gathered from a chromatograph remote from the console terminal where the clock time of injection can be noted for later retrieval. The results of the data workup may be permanently stored in disk files in addition to the report printed by the hard copy unit (see Figure 13).

PLOT is called by the operator at the conclusion of a cyclotron experimental run by entering the names of the data and log files. The program opens the files and finds the first and last clock times in the file to help locate injection times and to avoid attempts to find times outside of the data set. It then reads the log entries, displaying and passing comment lines until an injection entry is read. This is printed and PLOT asks if a workup is desired. If so, it searches the input file for the injection time, and if found, reads a segment of data into memory. It normally reads 180 data points or six minutes, a typical chromatogram, but it can be longer if specified. The program scans the data for the maximum value to scale the plotting and then plots the chromatogram across the full screen on a square root or cube root scale to enhance small peaks and increase the accuracy of background level specification. The experimenter marks the peak boundaries with the movable vertical cursor and the background levels with the horizontal cursor, using a joystick or thumbwheel controls. The background is normally constant for a single peak, but for a small peak on a large tailing peak, one can choose a sloping linear background (see Figure 14 for a typical workup).

Once the peaks have been defined, a subroutine subtracts background, decay corrects each point to the time of injection, and sums the activity for each peak and for the chromatogram. The program then plots a report with each peak marked with retention time, corrected net counts and per cent activity injected. If very small peaks are involved, the chromatogam is plotted on a square root scale. Otherwise, it is plotted linearly (compare Figures 9 and 15). The properly formatted results can be stored in a more detailed form with both gross and corrected counts in a disk file (see Figure 16 for a segment of a typical result file).

After completion of a chromatogram, the program continues with the next sample, if desired, until the log file is exhausted. The time required to work up one sample, from reading the log entry to final display is about 30 seconds for a typical chromatogram with several peaks.

PLOT has been designed to handle chromatograms with the 10 minute half-life of nitrogen 13, but it can be easily adapted to treat chromatographic data of any length and time interval using other radionuclides. With the development of a multiterminal handler for the RT-11 operating system, PLOT will be able to process data on-line immediately following the elution of a chromatogram.

Fig. 1. Schematic of the target (upper) and assembled target-block (lower).

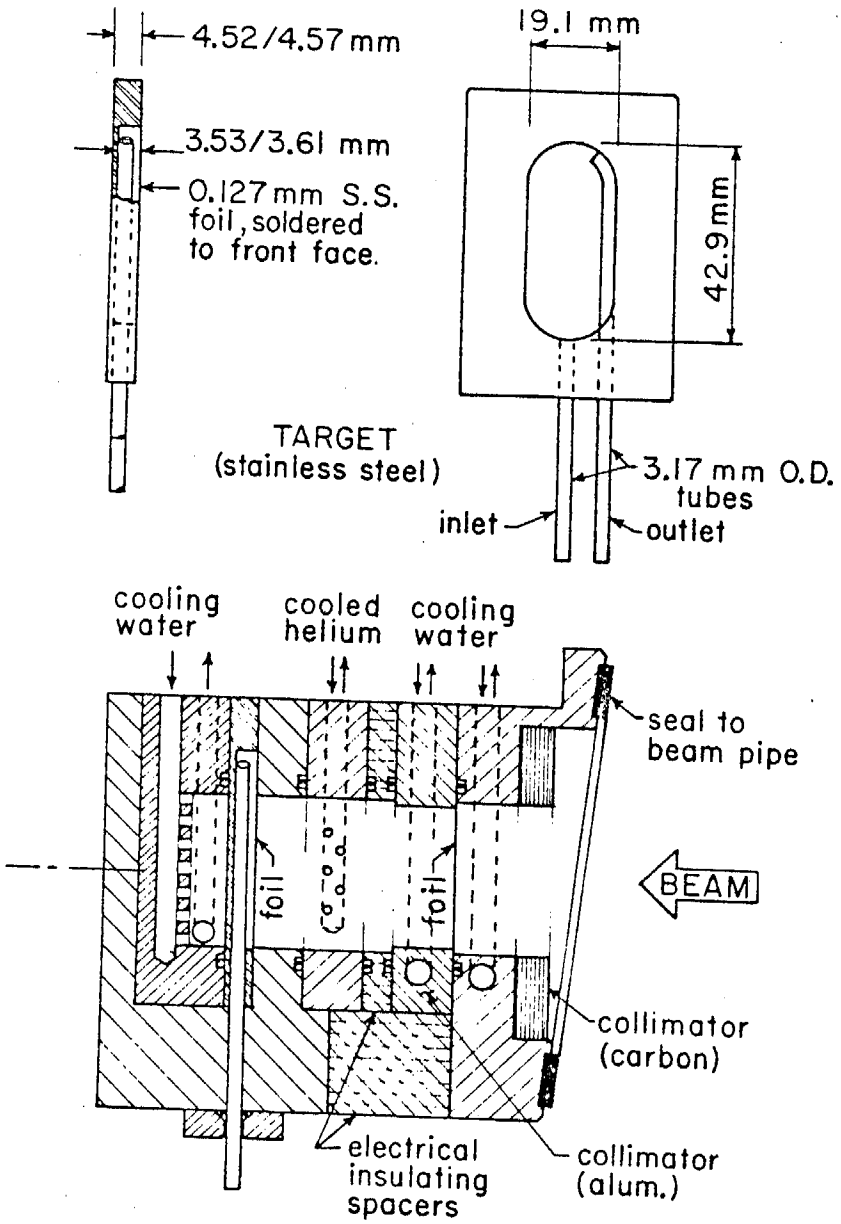
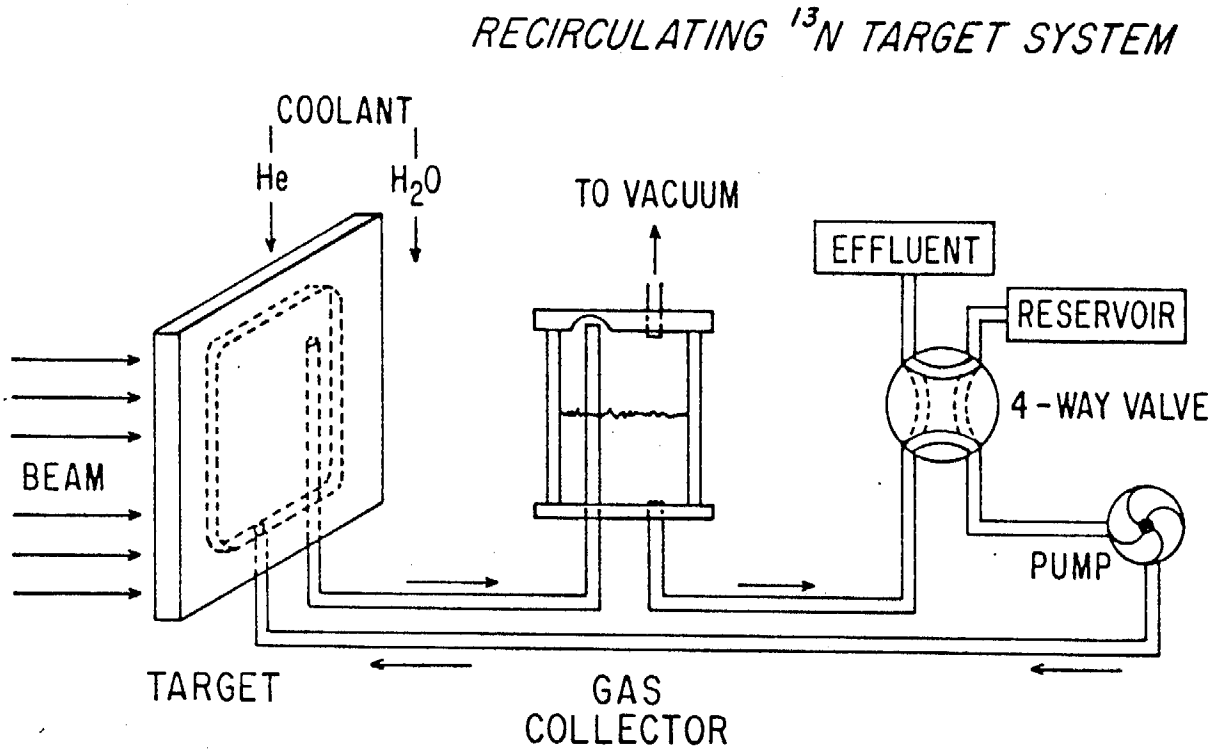


Fig. 2. Schematic of the target system.



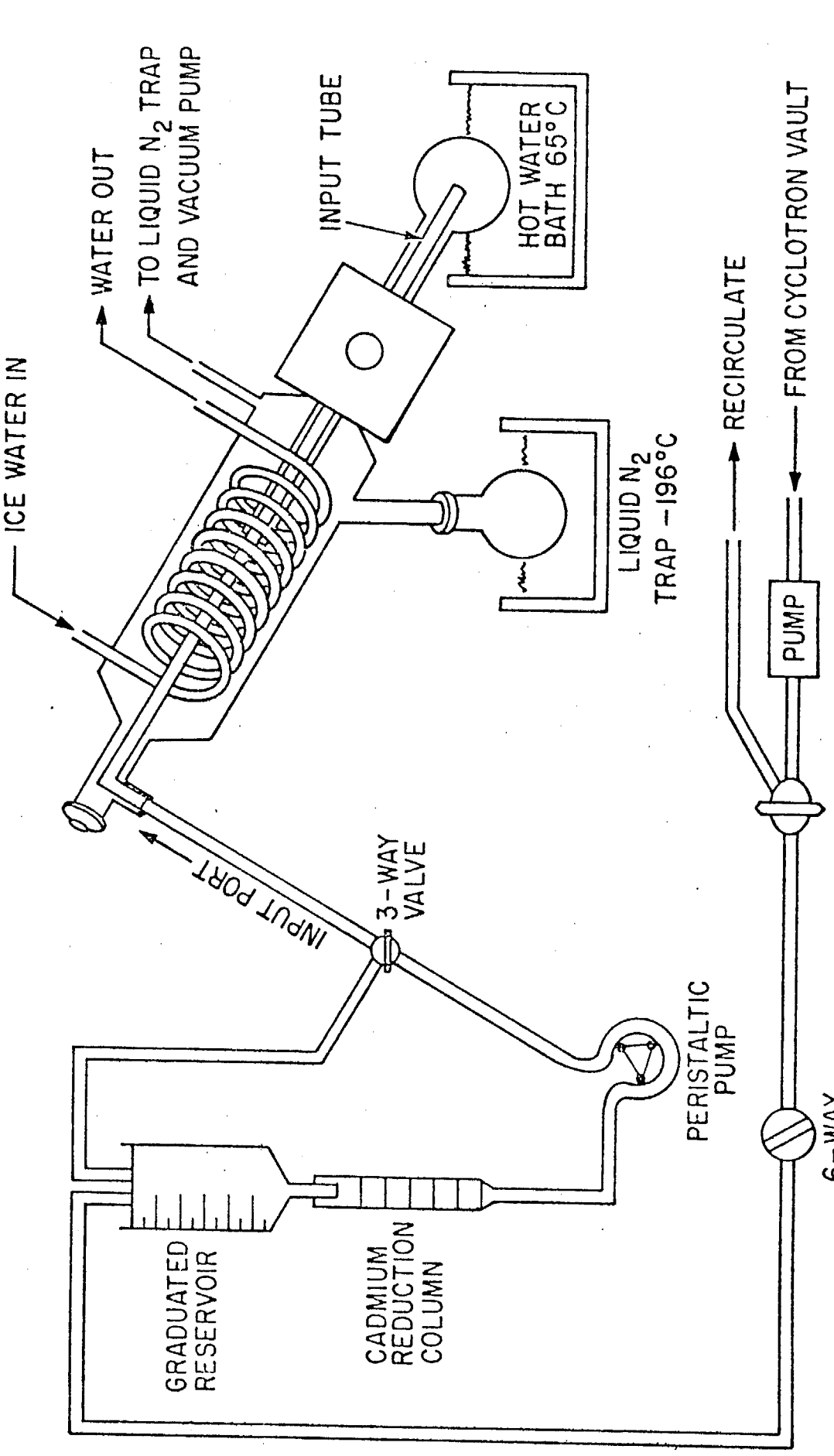


Fig. 3. Schematic of cadmium reduction system for synthesis and purification of N-13 nitrite.

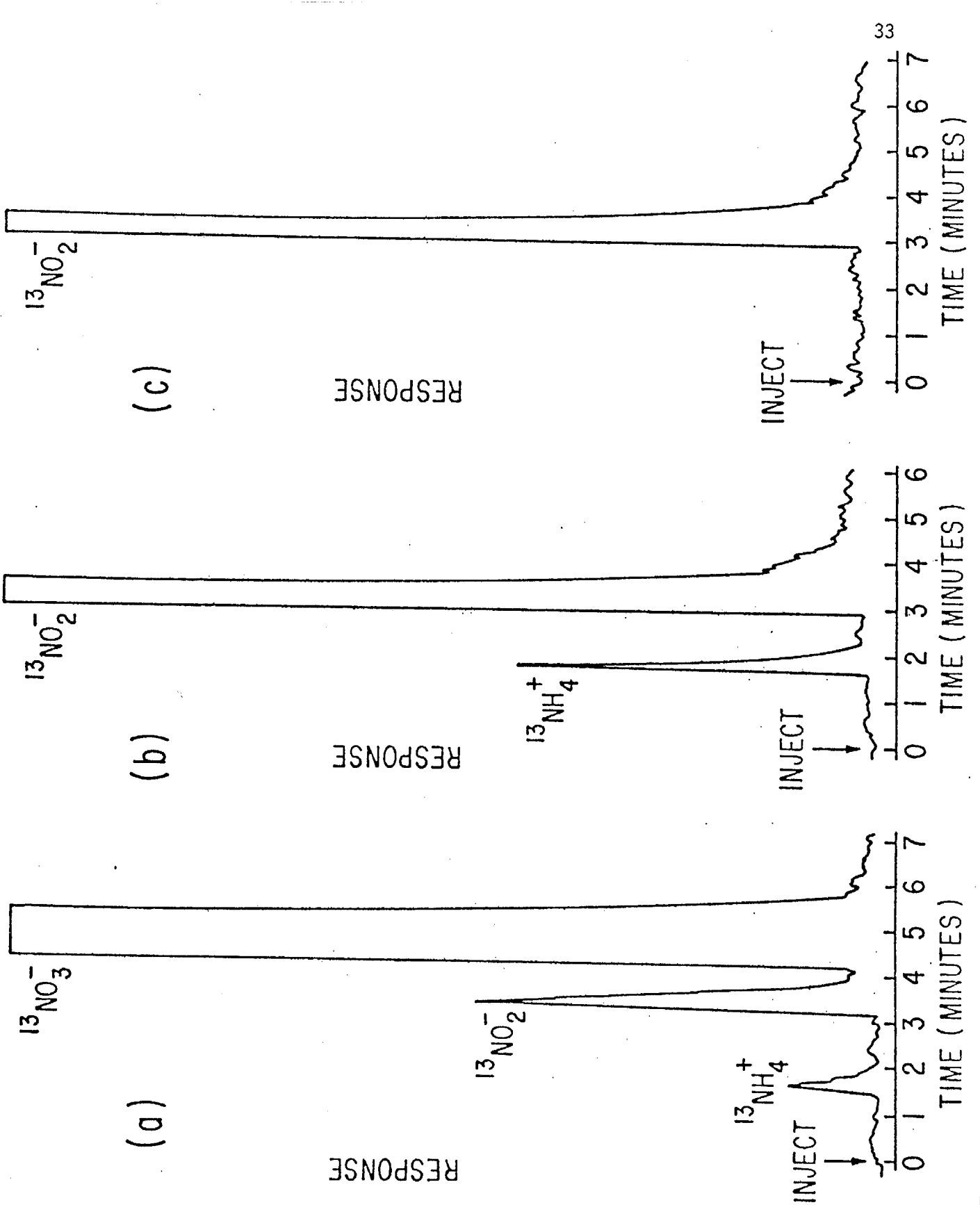
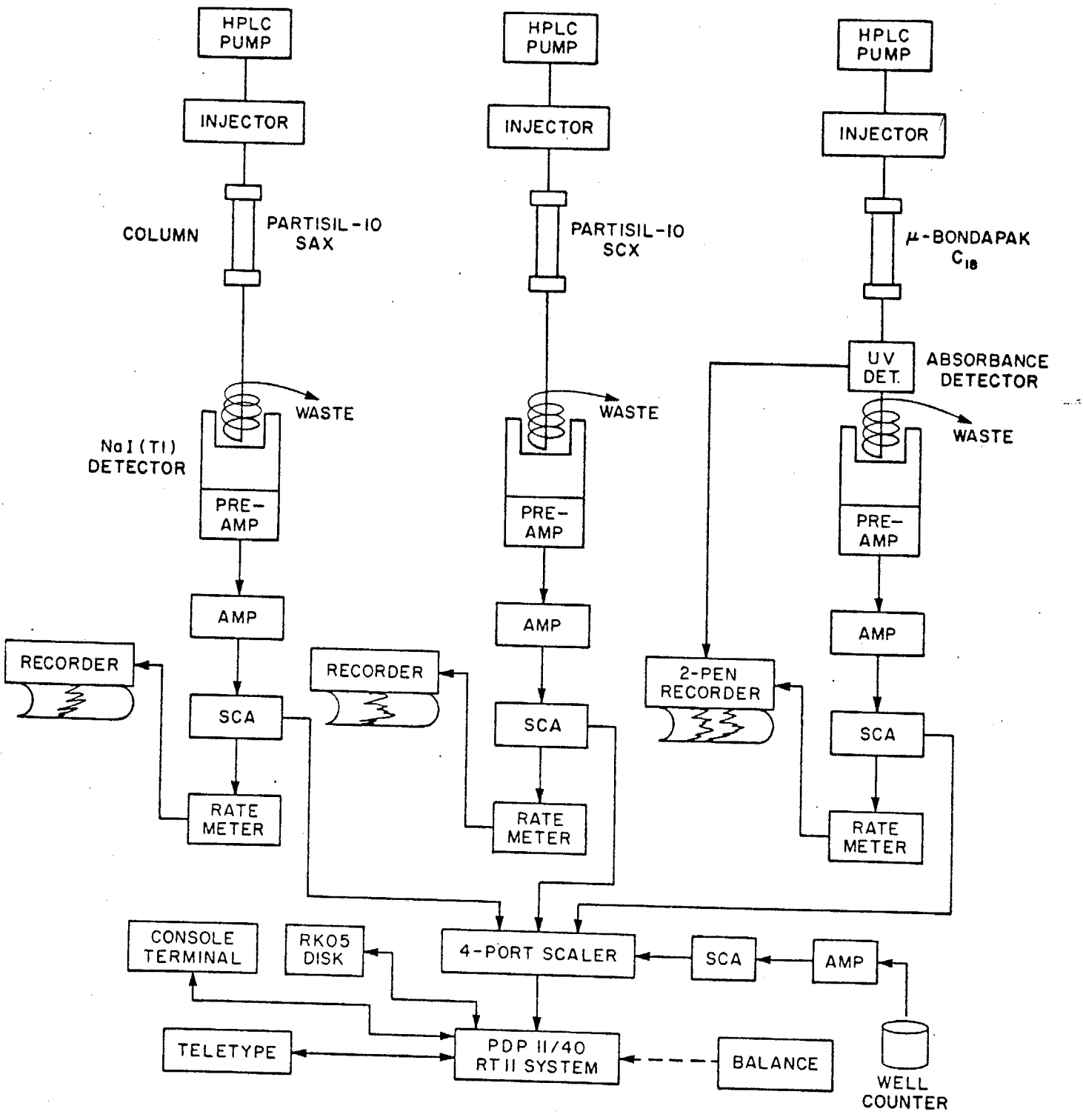


Fig. 4. Chromatograms of: a) target solution, b) reduction column eluent, and c) final solution for biological experiments.

Figure 5.

RADIOCHROMATOGRAPH AND RELATED SYSTEMS



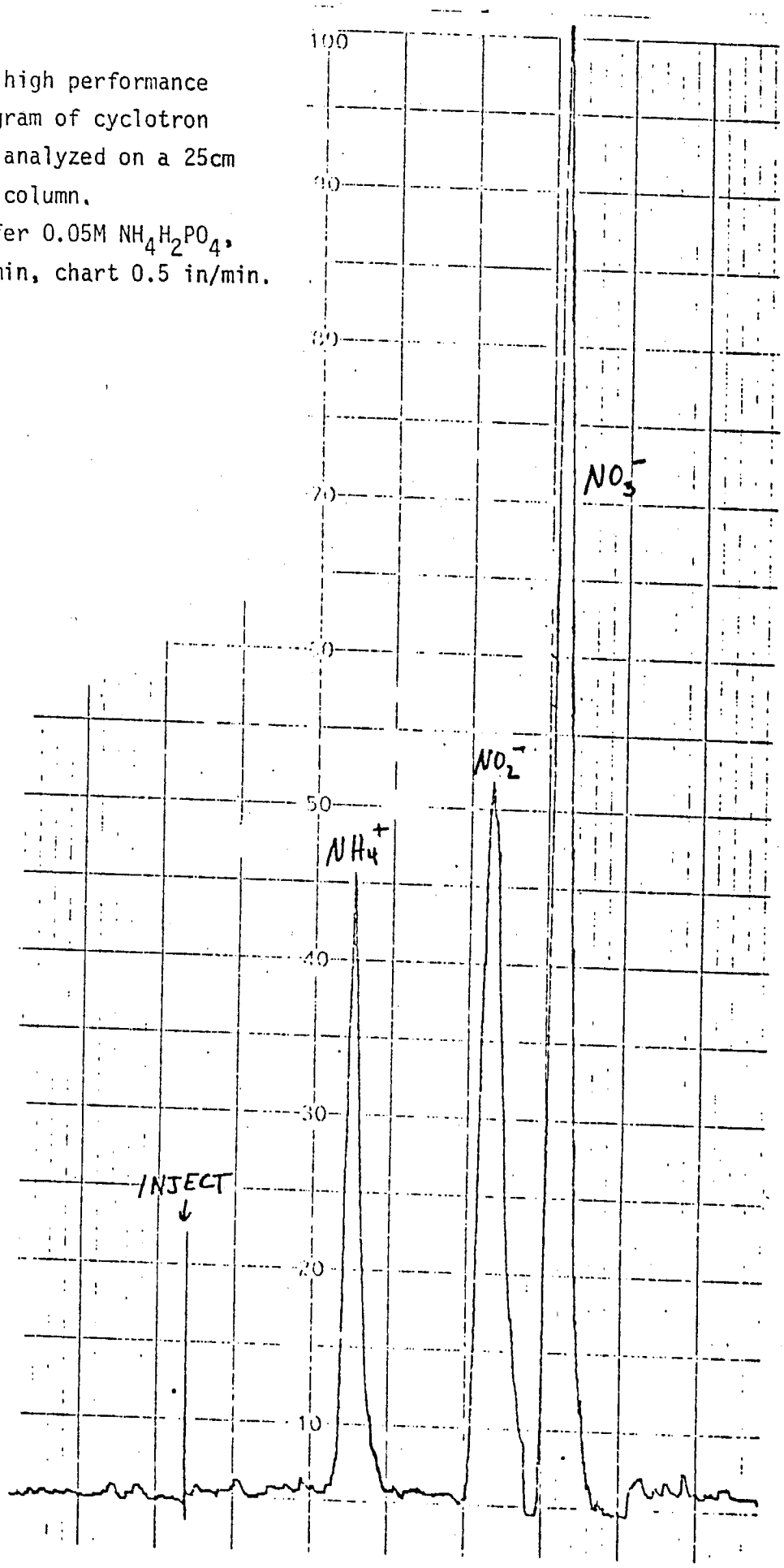


Figure 6. Typical high performance liquid chromatogram of cyclotron target solution analyzed on a 25cm Partisil-10 SAX column. Conditions: Buffer 0.05M $NH_4H_2PO_4$, pH 3.5, 1.5 ml/min, chart 0.5 in/min.

Figure 7. Typical high performance liquid chromatogram of $^{13}NH_4^+$ on a Partisil-10 SCX column. Conditions: 0.05M ammonium phosphate, pH 3.5. Flow rate 1.5 ml/min, Chart 0.5 in/min.

Peak Identification:

A = $^{13}NO_3^-$ (breakthrough)

B = $^{13}NH_4^+$

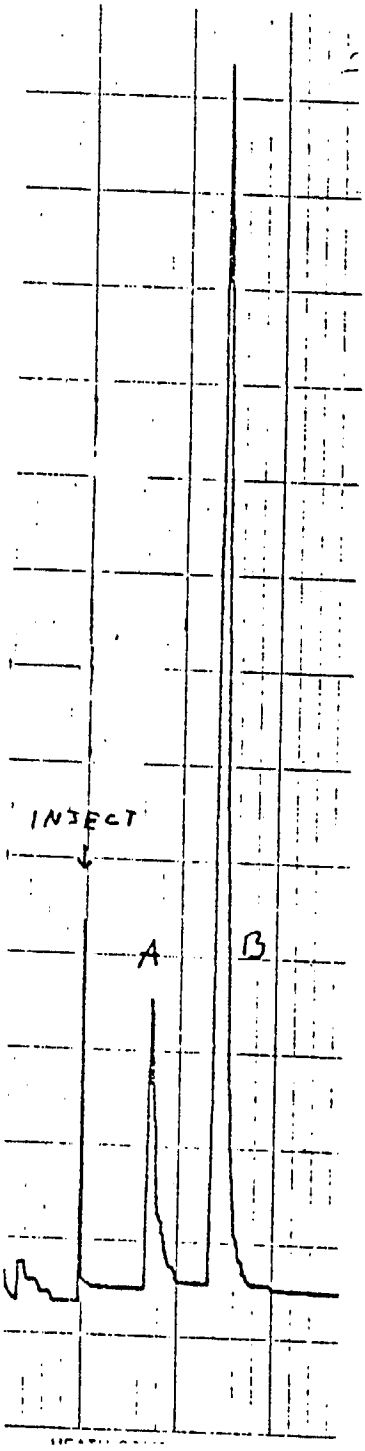
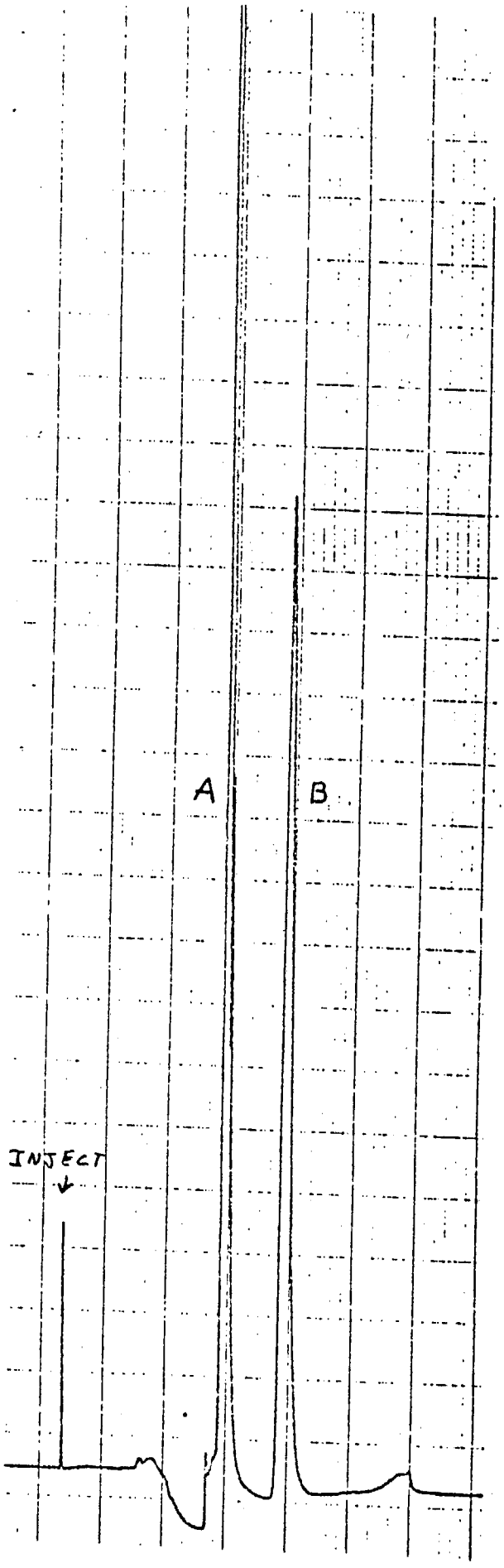
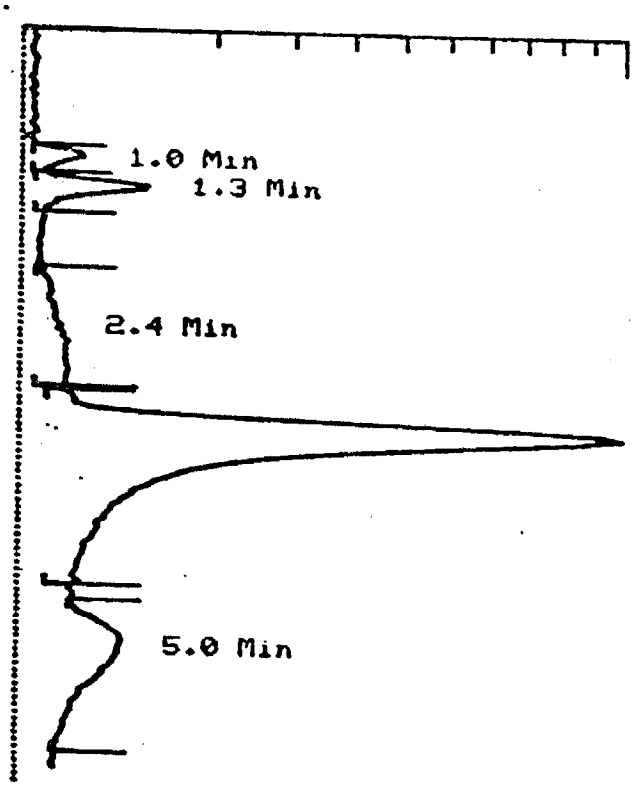


Figure 8. Typical high performance liquid chromatogram for unlabeled uv-absorbing nitrosamines C-18 micro-Bondapak column.
Conditions: Acetonitrile/ 1mM ammonium phosphate (1:10). Flow rate 1 ml/min, chart 1 cm/min.



Peak Identification:
A = dimethylnitrosamine
B = nitrosopyrrolidine

Figure 9. Sample analysis report generated by program PLOT. Radioactivity is plotted on a square root scale.



13565 CPS Full Scale

1.0 Min	957 Counts	0.39 %
1.3 Min	4023 Counts	1.62 %
2.4 Min	3291 Counts	1.33 %
3.3 Min	226697 Counts	91.50 %
5.0 Min	12801 Counts	5.17 %

6-JUN-79 Sample: R3 NO2 OFF COL

Column 1 Inject: 78935. 21:55:35 5 Peaks

Figure 10

Program LUCAS Flow Chart

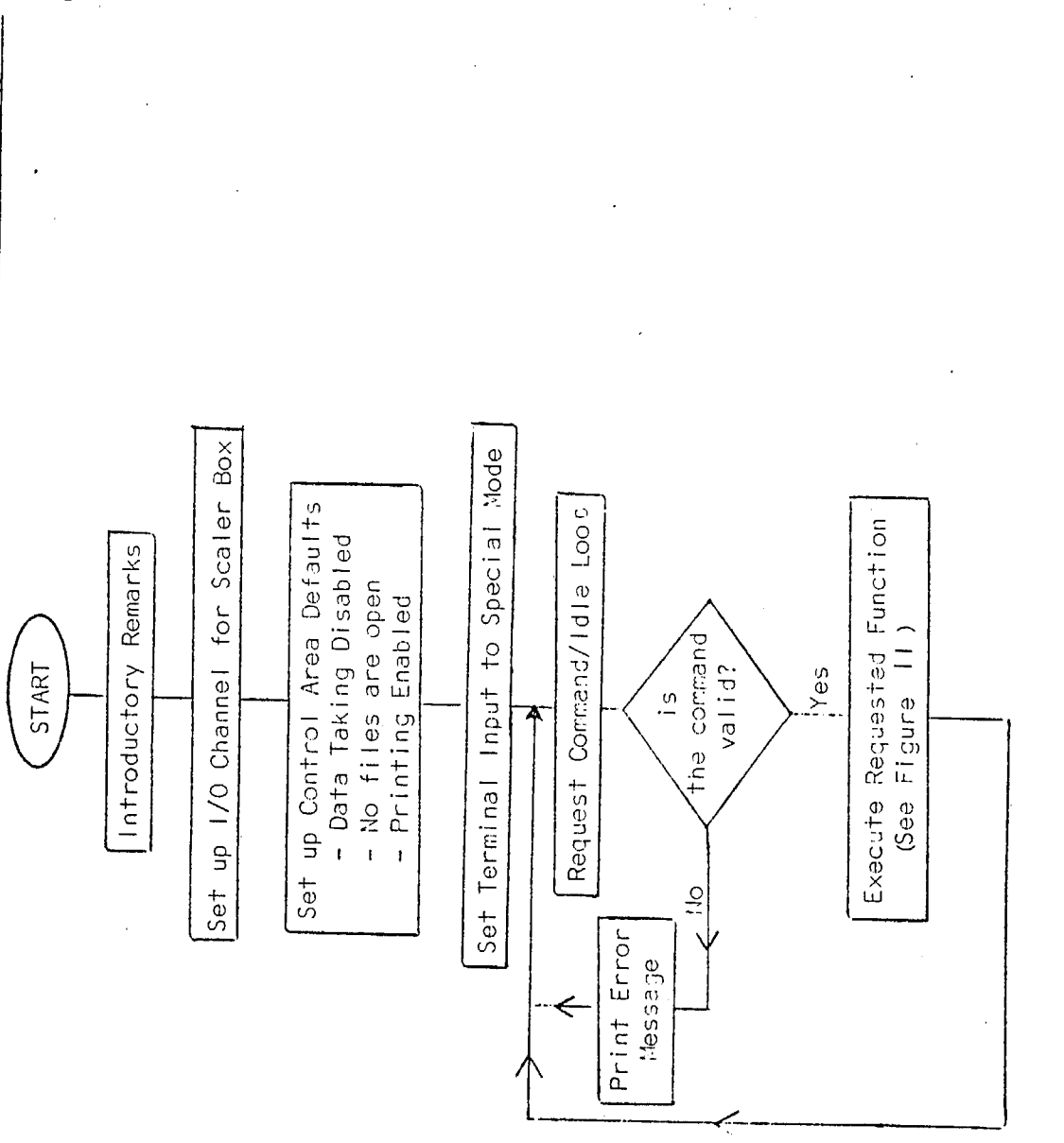


Figure II. Summary of commands for Program LUCAS.

LUCAS Commands:

OPEN or OD	Opens disk file for Lucas scaler data
OPEL or OL	Opens disk file for log entries
START	Starts taking scaler data
STOP	Stops taking scaler data
S	Starts or stops data taking
PRINT or P	Enables printing of scaler data on terminal
NO PRINT or N	Disables printing of scaler data on terminal
LOG Text	Places current Time and "Text" in Log File
CLOSE or CL	Stops taking data and closes disk files
EXIT or EX	Stops taking data, closes files, and exits program
CTRL C	Same as EXIT
HELP or H	Types this command summary

Figure 12. Segment of a log file generated during a cyclotron run using program LUCAS.

```
67443   'RUN OF 6-JUN-79 MOUSE EXP
67545   !COL A = 4.6MM SAX10 0.03M PO4 PH3 3.0 L/MIN
67591   !COL B = 3.2MM SAX10 0.03M PO4 PH3 1.5ML/MIN
67698   !COL C = 3.2MM SCX 10 0.03M PO4 PH3 1.5ML/MIN
69622   A .01ML R1 TARGET
69755   B R1 NO2 OFF COLUMN .01ML
72107   A .01ML R2  TARGET
72239   B .01ML NO2 OFF COLUMN
72425   A .01ML NO2 OFF COLUMN
72579   B .01ML NO2 FLASH
72929   A NO2 FLASH
73308   A .03ML NO2 FLASH R2
73593   B R2 N2 TRAP
73725   A .03ML NO2 FLASH R2
74233   A .03ML NO2 FLASH R2
74583   A .06ML NO2 FLASH R2
74713   B .05ML NO2 FLASH R2
75176   A ,01ML R3 TGT
75279   B R3 CD COL NO2
75647   A NO2 COLUMN .01ML
76114   A R3 NO2 PURIFIED
76195   B R3 NO2 PURE .01ML
76629   A .01ML NO2 PURE
76683   ! A > 5ML/MIN
76898   B .02ML NO2 FLASH
76975   !A > 3ML/MIN
77324   A R3 TRAP .1ML
77625   A .7ML R3 TRAP
78879   B R3 NO2 OF COL .01ML
78935   A R3 NO2 OFF COL
```

Time Column I.D. Sample name

Figure 13

Flow Chart for program PLOT

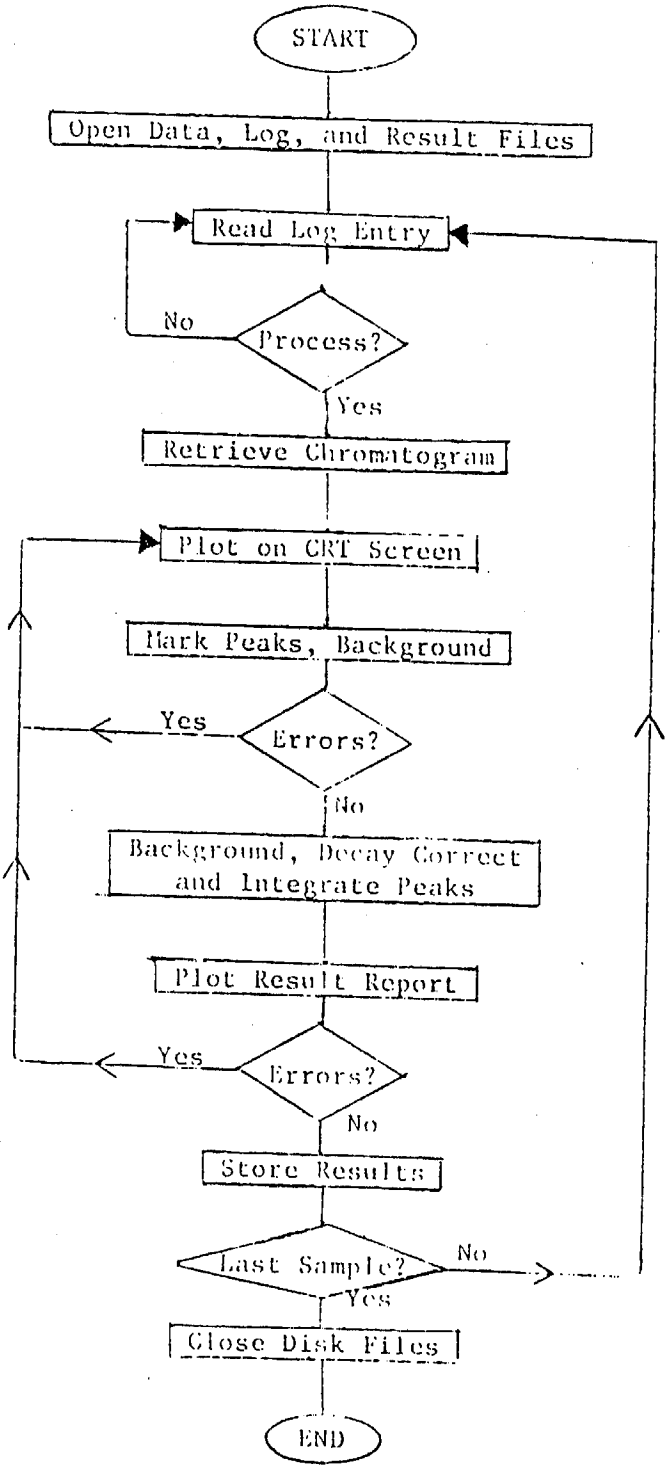


Figure 14. CRT hardcopy showing chromatogram as plotted for analysis, after the peaks have been marked.

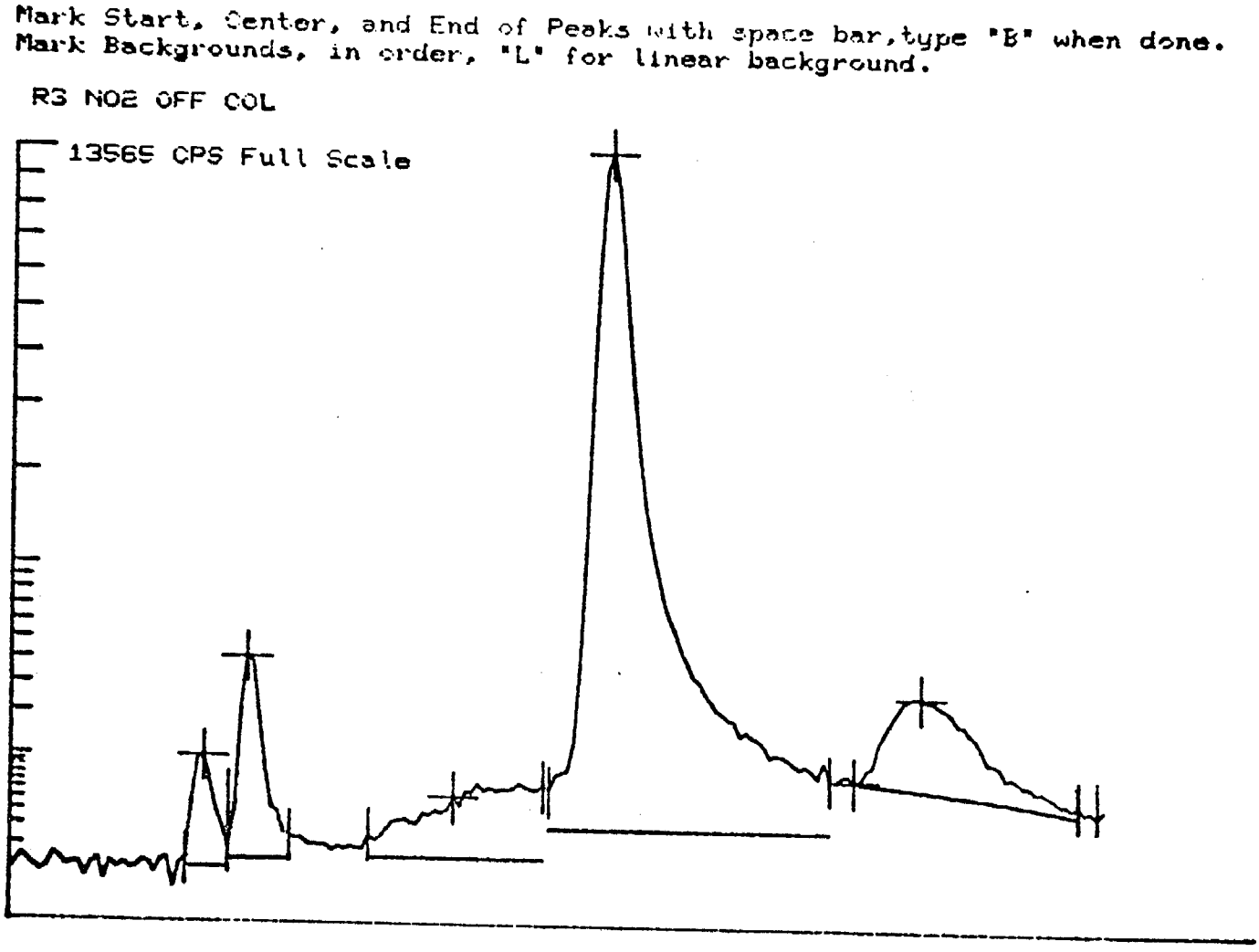
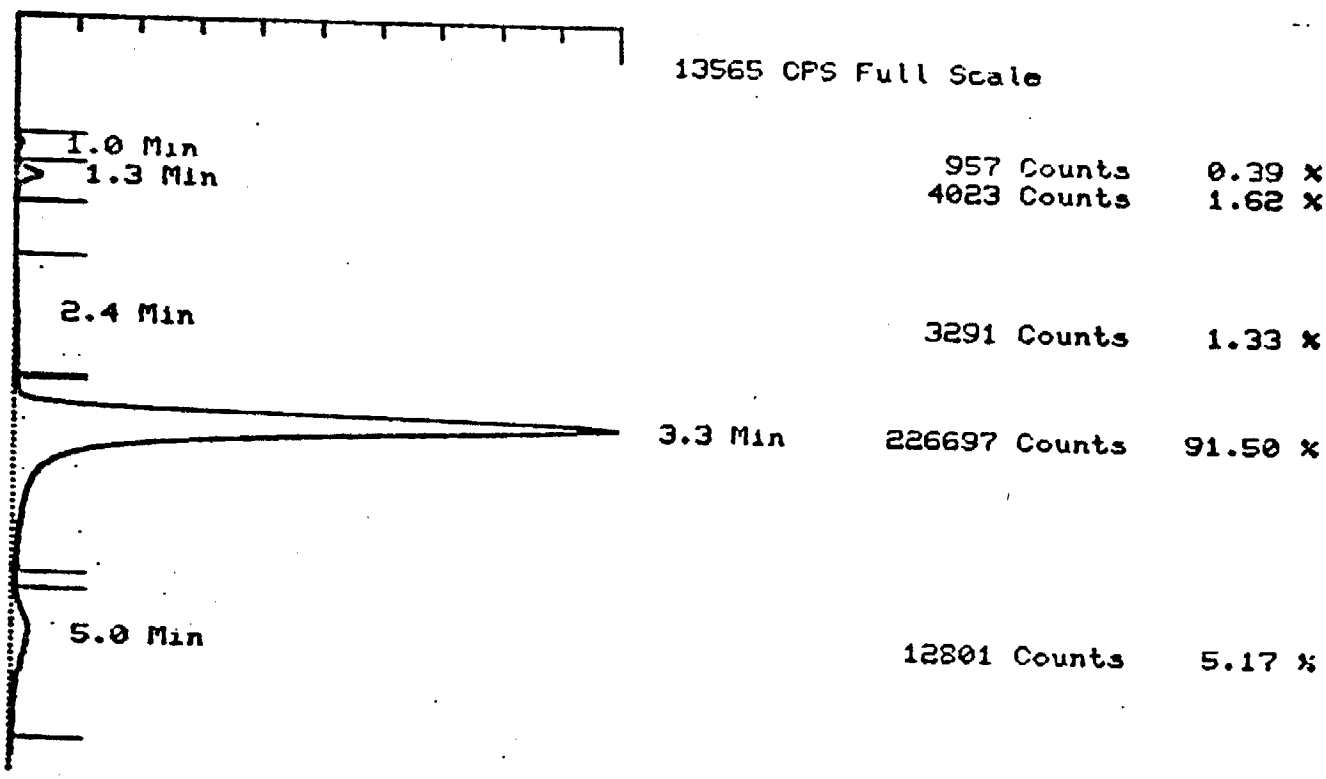


Fig. 15. Sample analysis report generated by PLOT, plotted on a linear scale.



6-JUN-79 Sample: R3 NO2 OFF COL

Column 1 Inject: 78935. 21:55:35 5 Peaks

Fig. 16. CRT hardcopy showing a segment of a Result file, with the results of two sample analyses.

6-JUN-79 Sample: .01ML R1 TARGET

Column 1 Inject: 69622. 19:20:22 4 Peaks

PEAK	RET TIME	GROSS COUNTS	CORRECTED	%ACTIVITY	IDENTITY
1	1.0	213.	204.	0.16	Ammonium
2	1.3	1363.	1460.	1.16	Peroxide
3	2.8	3937.	4609.	3.67	Nitrite
4	4.1	89786.	119435.	95.01	Nitrate

6-JUN-79 Sample: R3 NO2 OFF COL

Column 1 Inject: 78935. 21:55:35 5 Peaks

PEAK	RET TIME	GROSS COUNTS	CORRECTED	%ACTIVITY	IDENTITY
1	1.0	987.	946.	0.38	Ammonium
2	1.3	4025.	4141.	1.67	Peroxide
3	2.4	3442.	3450.	1.39	Unknown
4	3.3	182131.	226323.	91.33	Nitrite
5	5.0	14356.	12954.	5.23	Nitrate

3.2.11 Animal Experimental Methods

Because of the many routes by which NO_x^- ions may insult normal physiology, we divide our experiments into studies of two radiochemicals administered to mice and rabbits at three different sites. [N-13]-nitrate and [N-13]-nitrite are both synthesized with >97% radiochemical purity and carrier-free at the CNL cyclotron (Section 3.2). Typical cyclotron irradiations produce about 600 mCi of $^{13}NO_3^-$ in 50 ml distilled water and this can be reduced and concentrated to yield about 150 mCi of $^{13}NO_2^-$ in 3 ml of buffered isotonic saline. These two purified radiochemical tracers have been tested individually by instilling them through small polyethylene cannulas directly into the esophagus or trachea of adult Balb/C mice (See Section 3.2.12) or by injecting them into the veins of mice or New Zealand white rabbits. Pregnant mice were given IE injections (see Section 3.3.2). The mice were sacrificed at either 5, 10, 15, 20 or 30 minutes and immediately opened to remove a cardiac blood sample. The heart, lungs, spleen, liver, large and small intestines, stomach, kidneys and urinary bladder were excised in the order listed, dabbed to remove excess blood, sealed in tared vials and weighed. The samples were then counted in a standard geometry using a NaI (Tl) scintillation detector with associated electronics. All of the count rates were decay corrected to a common time. The carcasses remaining from the dissection were also weighed and counted so that all data could be reported directly in concentration units of percentage of injected dose per gram of tissue or per total organ. Independent experiments established that over a 30 minute interval, none of either of the administered radiochemicals was eliminated from the animals.

The rabbit preparation which we have developed is technically much more demanding in that these animals are under surgical anesthesia and have several in dwelling catheters. Ketamine and rompun are used for anesthesia because of the known influence of barbituates on the hepatic metabolism of organic nitrates via the glutathionine-nitrate reductase system. The radiochemical tracers are made isotonic and introduced via a catheter in the proximal femoral vein. This site is also used to supplement the animal with anesthetic and with physiologic

fluids to maintain a normal state of hydration which we have established as a hematocrit of about 0.4 and urine output of $\geq$ 0.5/ml/min/kg. We place a Foley catheter into the urinary bladder to monitor urinary activity and withdraw samples for counting and chromatographic analysis. Finally, a 21 gauge catheter is placed in the external jugular vein and passed into the superior vena cava. It is used for withdrawing blood samples much more rapidly than can be done from a peripheral vessel, for example the ear. This is important because we need several 3 ml blood samples drawn early after injection. Blood from the ear is often inadequately mixed because of poor circulation, particularly after the first sample is removed and if a hematoma forms. The catheter is flushed after each withdrawal of blood and every effort is made to get a sample that is representative of the circulating blood. This experiment, done in a way that maintains the rights of the animal and minimizes interruption of normal physiology, requires the constant attention of a person trained to work with laboratory animals. At the present time, we have "borrowed" this expertise from our associates in the School of Veterinary Medicine, but in the future we will expand our personnel roster to support this activity. The added information available from the larger model seems to justify the added effort and expense.

3.2.12 Intratracheal Instillation in the Mouse

In the course of our investigations into the *in vivo* fate of nitrogenous air, pollutants, the instillation of small volumes of aqueous $^{13}NO_3^-$ and $^{13}NO_2^-$ into the lungs of mice was used to study the physiological distribution and metabolism of oxides of nitrogen inspired from the ambient aerosol. The advantages of this technique over the administration of labeled aerosols were the ease in administering precise quantities of labeled materials and the ability to establish a precise time of instillation. This later factor was of vital importance in the establishment of an accurate pharmacokinetic model of the lung, since uptake was found to be very rapid from the lungs (*vide infra*).

Introduction of injection tubes into the lungs of anesthetized animals by feel is an accepted technique in large rodents, but when used on mice it occasionally results in injection of materials into the stomach via the esophagus. This method also often injures the soft tissues of the pharynx and

lung in the course of an injection (7). A method was developed to allow precise instillation of labeled compounds into the lungs of anesthetized mice while directly viewing the glottis via the oral pharynx. Introduction of the injection needle into the trachea under visual guidance reduced the possibility of tissue damage as well as the possibility of erroneous esophageal injection.

In this procedure an otoscope (Welch Allyn Diagnostic Otoscope, W. A. 20100) was used for viewing the pharynx. A 2 mm polypropylene otoscope specula (W. A. 22002) was modified by cutting a slot in the side along the axis (2 cm long x 1 mm wide, starting 9 mm from the top of the specula) to allow for insertion and downward motion of a syringe needle (see Figure 17). A gas tight 25 µL Hamilton Syringe, with a 23 gauge blunted needle bent at a right angle 17 mm from the tip, was used for the intratracheal injections. Volumes of aqueous labeled nitrite and nitrate of up to 15 µL were injected with no incidence of death from asphyxiation.

All animals were given pentobarbitol (60 mg/kg) as an anesthetic. At this dosage the mice were rendered unconscious within 50 to 10 minutes and remained so for 45 to 90 minutes. While unconscious, an animal was placed in a holding apparatus (see diagram). An elastic band fitting under the front teeth and a notched wooden frame at the back of the neck immobilized the head and aligned the mouth, pharynx and trachea vertically. Once positioned, the elastic band was pinned to the holder, and the head was secured. The tongue was pulled out to its full extension and placed to the side with tweezers. The otoscope specula was lowered vertically into the mouth and pharynx until the opening in the tip of the specula was just above the glottis. The magnifying lens and internal light source of the otoscope permitted aligning the specula visually. An articulating arm was used to hold the otoscope. This procedure allowed free maneuvering of the otoscope, held it firmly in place once positioned in the pharynx, and freed the hands for injection.

The glottis was observed to open on each inhalation of the animal, exposing the opening to the trachea. The entrance to the esophagus was seen directly above the respiratory passage. The injection needle was inserted through the slot in the side of the specula and was visible through the otoscope lens. When the animal inhaled and the glottis opened, the needle was slipped into the

trachea and inserted until the bend in the needle rested upon the lower edge of the slot in the specula. This insured that the needle was as deep in the lungs as the bronchial bifurcation and prevented the needle from penetrating too deeply and injuring the lung tissue. The aqueous radiochemical was then slowly injected. The mouse was often seen to gasp; careful observation determined if any of the liquid was expelled upon gasping, since bubbling around the glottis could be seen. Once gasping ceased, the otoscope was raised out of the mouth, and the mouse was freed from the holder. With some practice, the total time of mounting, injecting and unmounting an animal was approximately 1-2 minutes.

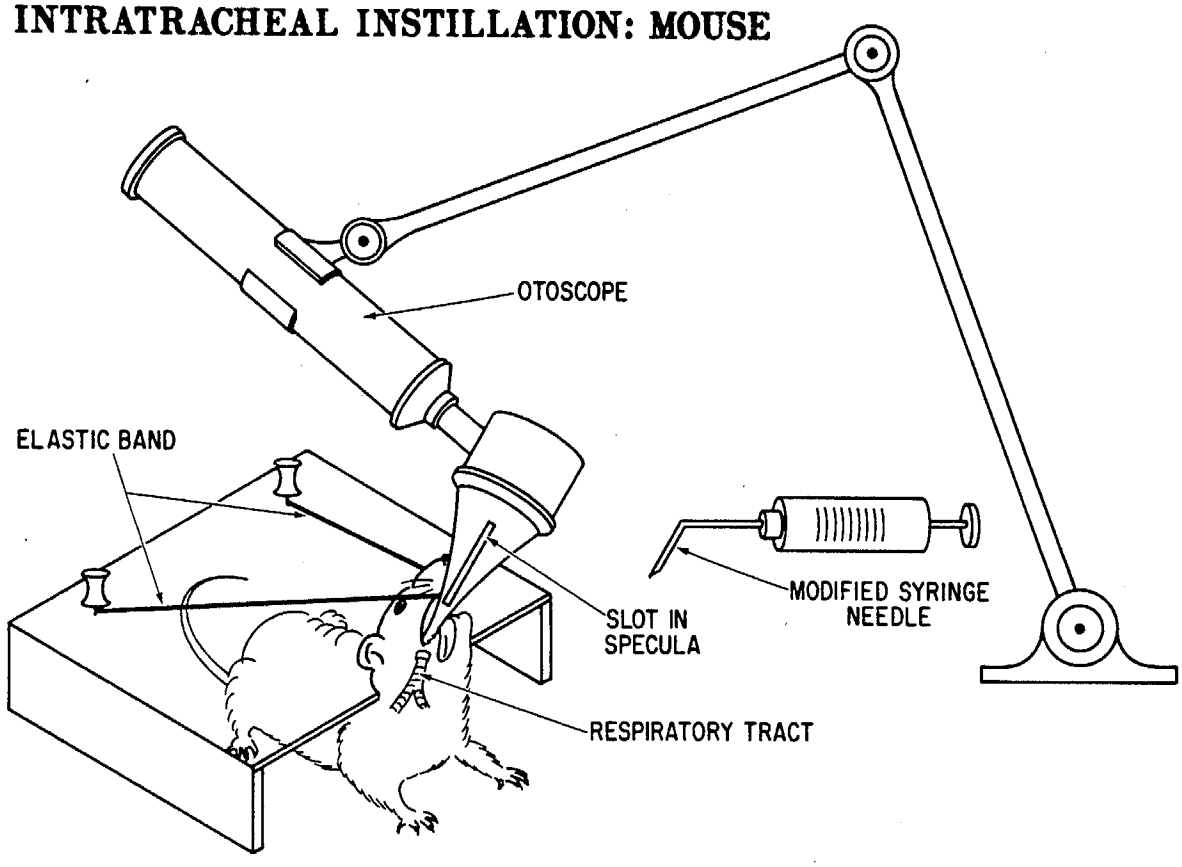


Fig. 17

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3.3 RESULTS

3.3.1 Organ Distributions

Our first series of biologic measurements involved organ distribution studies of $^{13}NO_3^-$ and $^{13}NO_2^-$ given to mice. This was completed with 75 mice studied at several time intervals ranging from 5 to 30 minutes between injection and sacrifice. We analyzed all of the data by a simple linear least squares regression model of %ID(t) = mt + b where % ID(t) is the percentage of dose in each organ at time t, and m and b are the calculated slope and intercept, respectively. The slopes for all of the various combinations of organ, tracer NO_x ion and route of administration are showing in the histogram (Figure 18). The distribution centers around zero and demonstates that there were no significant variations with time in the radioactivity 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 18, were the lung and stomach/intestines when they represented the initial organ of entry for the labeled NO_x tracer. For the IT lung data (see Illustration 4, Appendix A) the coefficient of determination, r^2 was 0.18. In other words, 18% of the total dispersion of the data for these experimental conditions could be attributed to association with time, the remainder to "between-animal" metabolic variations for this compartment. Likewise, following intraesophageal gavage, the stomach showed some tendency towards clearance (m = -0.56) with a complementary increase in intestinal activity (m = +0.52) but these slopes were not significantly different from zero at P = 0.10.

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. We have shown that the time dependent flux of tracer achieves dynamic equilibrium very rapidly when administered by any of the three routes, but it is slightly slower from the lung. In all instances these half-times are of the order of a few minutes or less and can not be determined by our data which begins at 5 min after injection.

In view of their time independency, the best format for comparing all the data is the mean ($\bar{x}$) and standard error of the mean (SE_x) given in Table 1. The most remarkable feature of this table is that, except for bladder data,

Figure 18. Time independency 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.

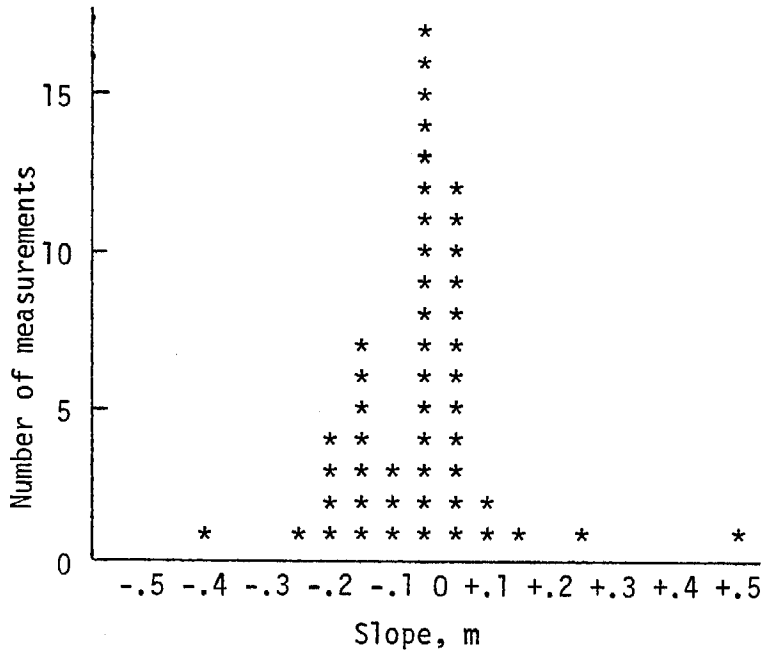


Table 1. Percent[a] of Total N-13 Radioactivity from NO_2^- and NO_3^- in the Organs of the Mouse[b]

Organ	Ion	Administration Mode[c] IE	IV	IT
Bladder	NO_2^-	1.00 ± 0.48 (11)	0.81 ± 0.44 (9)	2.92 ± 1.99 (7)
	NO_3^-	2.52 ± 1.34 (15)	1.95 ± 1.06 (6)	0.86 ± 0.47 (9)
Large Intestine	NO_2^-	3.64 ± 0.61 (11)	5.84 ± 0.94 (11)	8.90 ± 2.26 (9)
	NO_3^-	3.39 ± 0.47 (16)	6.64 ± 0.68 (10)	6.48 ± 0.88 (17)
Small Intestine	NO_2^-	9.72 ± 2.54 (11)	8.48 ± 1.08 (11)	8.40 ± 0.59 (9)
	NO_3^-	8.06 ± 1.16 (16)	7.64 ± 0.87 (10)	8.46 ± 1.06 (17)
Stomach	NO_2^-	33.1 ± 5.70 (11)	2.39 ± 0.33 (11)	3.48 ± 0.68 (9)
	NO_3^-	24.8 ± 4.76 (17)	4.48 ± 0.65 (10)	4.12 ± 0.47 (15)
Liver	NO_2^-	6.55 ± 0.77 (11)	9.68 ± 1.03 (11)	9.93 ± 1.06 (9)
	NO_3^-	5.84 ± 0.47 (16)	7.74 ± 1.24 (11)	9.77 ± 1.06 (17)
Kidneys	NO_2^-	2.49 ± 0.27 (11)	3.16 ± 0.21 (11)	3.06 ± 0.38 (9)
	NO_3^-	2.90 ± 0.33 (16)	3.55 ± 0.66 (10)	3.60 ± 0.43 (16)
Heart	NO_2^-	0.83 ± 0.10 (9)	1.15 ± 0.08 (8)	1.22 ± 0.33 (5)
	NO_3^-	0.93 ± 0.15 (10)	2.00 ± 0.38 (8)	1.55 ± 0.19 (14)
Lungs	NO_2^-	1.18 ± 0.13 (11)	1.67 ± 0.38 (11)	5.26 ± 1.60 (9)
	NO_3^-	1.54 ± 0.20 (16)	1.70 ± 0.16 (10)	5.10 ± 1.18 (17)
Carcass	NO_2^-	39.4 ± 4.74 (11)	64.8 ± 2.82 (10)	59.0 ± 3.70 (9)
	NO_3^-	48.2 ± 4.00 (16)	60.6 ± 2.51 (10)	60.2 ± 3.04 (16)

[a]Data given as $\bar{x} \pm SE_{\bar{x}}$ (number of data points) for measurements from 5-30 minutes

[b]Balb-C

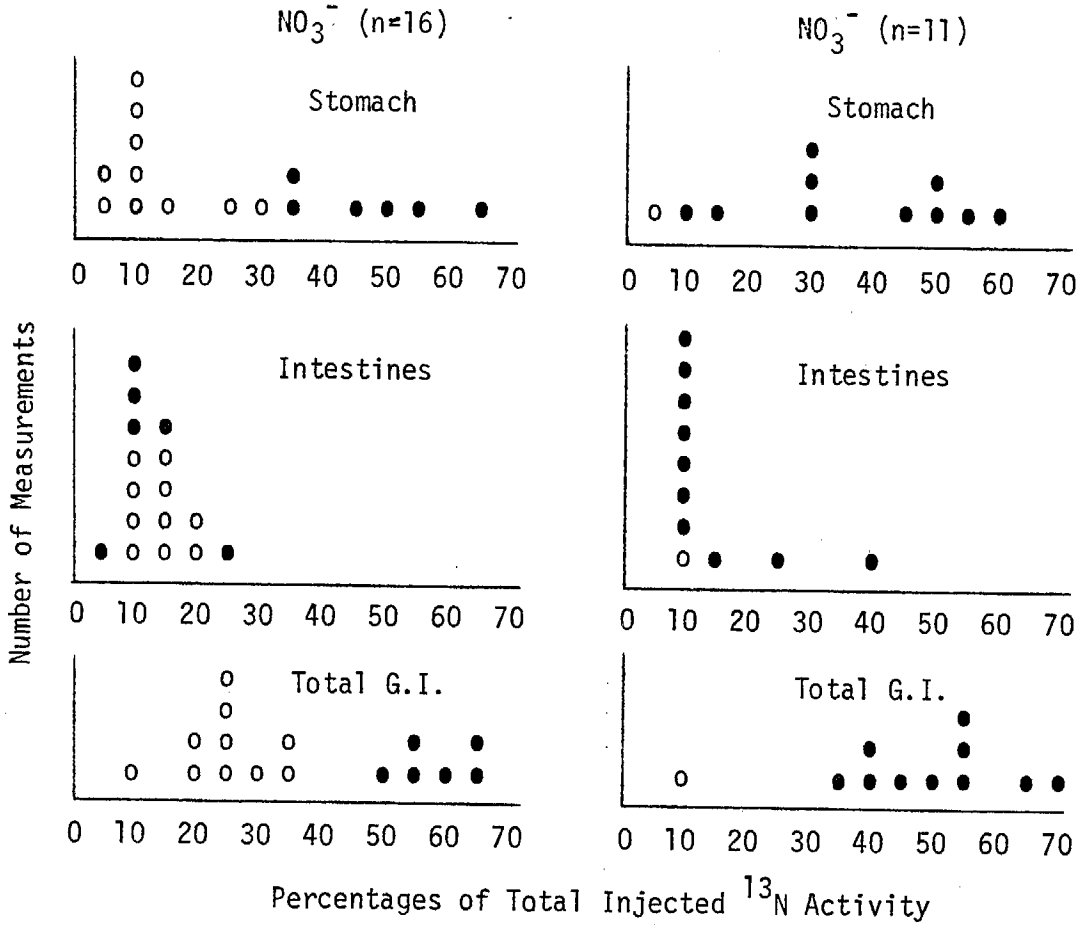
[c]IE = intraesophageal, IV = intravenous, IT = intratracheal

there are no differences between N-13 distributions following nitrite versus nitrate administered by a given route. The mechanistic and health consequence implications of this unexpected finding cannot yet be fully appreciated but some speculation is offered in Section 3.4.

The IT/IV rations for the percentages of N-13 radioactivity in Table 1 are also approximately unity in individual organs for both NO_2^- and NO_3^- except for the lungs and bladder, the former relating to this organ as the site of injection for IT, and the latter relating to the large interanimal variability in urinary excretion. The mean lung ratio of 3 can be compared to an estimated activity ratio of 50 for the lung immediately after IT administration, demonstrating the rapid approach of these tracers to equilibrium distributions. We conclude that intratracheally instilled NO_3^- and NO_2^- distribute into essentially the same physiologic pool as intravenously injected ions of the same identity.

The intraesophageal distributions we measured were quantitatively different from the IV and IT animals for two reasons. A relatively larger portion of the IE administered activity stayed at the site of entry than for IT and IV animals and the amounts remaining in the stomach were unusually variable. In the nitrate data set, one animal had only 2.5% in the stomach at 5 min whereas another had 62% remaining there at 10 min. The nitrite animals were equally variable, ranging from 2.1% to 59%. In graphing IE data, we developed the impression that the experiments involved two distinct sets of data and used the analysis shown in Figure 2 to confirm this. Approximately equal numbers of mice had 10% of the injected dose in their stomachs as had 20% or 30% or even 60%, and this was true for both nitrite and nitrate experiments. Such a broad distribution is atypical of biodistribution kinetic measurements which are usually essentially gaussian as typified by the distributions for the intestines, also shown in Figure 19. When we summed the stomach and intestinal activities to determine a "Total G.I." activity, it became apparent that both our nitrate and nitrite animals exhibited similar bimodal distributions. In one group of animals, 65 to 90% of the administered activity left the gastrointestinal tract within five minutes after injection; whereas, in the other group, only 30 to 60% left rapidly. In both cases the remainder stayed in the G. I. tract for at least 30 min although it gradually migrated from the stomach to the large and small intestines. Two distributions were apparent for NO_3^- and probably also for NO_2^- and are distinguished by open and closed data points in the illustration.

Figure 19. Equilibrium distributions following intraesophageal instillation of NO_x ions. The open and closed data points had no apparent interanimal differences but are distinguished as described in the text.



In considering some rationale for the bimodal distributions, we identified several biological and procedural differences that could have been factors; however, we were not able to retrospectively isolate any single cause to explain the separation in our data sets. The presence of food in the stomach might drastically retard the absorption of water and ions from the G.I. system. Alternatively, several dehydrated animals would exhibit accelerated absorption of the injected tracer. If the gavaged samples were too large, the instillation procedure might force some of the tracer through the pylorus and physically accelerate its movement into the intestines and also its extragastrointestinal absorption. Nitrite tracer has lower volume specific activity and thus requires larger injections, so that the latter might explain the animals represented by the right skew of the intestinal NO_2^- (middle right portion of Figure 2). Definitive elucidation of the active versus passive nature of NO_x ion absorption from the gastrointestinal system requires further experimentation that is beyond the current scope of this project.

The bladder data for the various introductory routes of NO_x ions was noticeably more variable than the other data; however, the highest mean value for urinary elimination was only 2.9% for intratracheal NO_3^-. Thus, excretion of NO_x anions from the body via this channel is consistently small, and represents a distinct difference from other simple anions such as F^-, I^-, TcO_4^- and $SO_4^=$ which are rapidly accumulated in the urinary bladder.

An alternative presentation of the data was prepared which gives the mean relative concentrations (%ID/g) of N-13 in each organ (Table 2). The mice used in these experiments averaged 20 to 22 grams body weight so that a perfectly homogenous distribution would result in 4.6 to 5.0% ID per gram of tissue. Values higher than this represent concentration while lower values represent exclusion from those tissues. Our analysis detected no special "target organ" but showed that radioactivity concentrations were higher in organs of introduction. The highly vascular vital organs all had higher concentrations than the overall carcass and muscle, but there was no simple correlation of $^{13}NO_3^-$ or $^{13}NO_2^-$ concentrations with organ blood flow or vascular volume. There was almost no activity in adipose and hair.

Table 2. Relative Concentrations[a] of N-13 Radioactivity from NO_2^- and NO_3^- in the Organs of the Mouse[b]

Organ	Ion	Administration Mode[c] IE	IV	IT
Bladder	NO_2^-	14.3 ± 8.70 (8)	3.82 ± 1.26 (6)	16.6 ± 3.14 (7)
	NO_3^-	12.2 ± 2.38 (8)	11.5 ± 3.90 (2)	9.68 ± 3.88 (5)
Large Intestine	NO_2^-	3.72 ± 0.49 (12)	6.88 ± 0.83 (11)	5.90 ± 0.96 (9)
	NO_3^-	5.01 ± 0.60 (15)	9.63 ± 1.15 (9)	7.08 ± 1.02 (17)
Small Intestine	NO_2^-	6.34 ± 1.49 (12)	5.44 ± 0.41 (11)	5.06 ± 0.43 (9)
	NO_3^-	5.08 ± 0.56 (16)	5.47 ± 0.65 (9)	5.34 ± 0.57 (17)
Stomach	NO_2^-	66.6 ± 13.0 (11)	6.39 ± 0.72 (11)	7.11 ± 6.56 (9)
	NO_3^-	65.0 ± 11.3 (16)	13.1 ± 3.01 (9)	12.1 ± 2.40 (17)
Liver	NO_2^-	4.86 ± 0.54 (12)	7.19 ± 0.59 (11)	7.38 ± 0.64 (9)
	NO_3^-	5.06 ± 0.47 (16)	7.36 ± 0.81 (9)	7.90 ± 1.09 (17)
Kidneys	NO_2^-	6.08 ± 0.68 (12)	8.04 ± 0.61 (11)	7.70 ± 0.82 (9)
	NO_3^-	10.0 ± 1.22 (16)	10.3 ± 1.66 (9)	8.73 ± 1.22 (16)
Heart	NO_2^-	4.77 ± 0.56 (10)	7.34 ± 0.40 (8)	8.08 ± 1.74 (5)
	NO_3^-	7.52 ± 1.26 (10)	11.7 ± 1.78 (7)	9.16 ± 1.12 (11)
Lungs	NO_2^-	6.60 ± 1.07 (12)	7.03 ± 0.43 (11)	19.2 ± 7.88 (9)
	NO_3^-	8.98 ± 1.02 (16)	11.2 ± 1.08 (8)	30.1 ± 6.96 (17)
Carcass	NO_2^-	2.28 ± 0.34 (12)	4.19 ± 0.28 (10)	3.32 ± 0.25 (9)
	NO_3^-	3.64 ± 0.43 (16)	4.14 ± 0.30 (9)	3.54 ± 0.25 (16)
Muscle	NO_2^-	4.21 ± 1.65 (10)	4.05 ± 0.60 (6)	3.83 ± 0.24 (6)
	NO_3^-	4.19 ± 1.15 (12)	4.45 ± 0.15 (2)	6.63 ± 1.47 (10)

[a]Data are given as $\bar{x} \pm SE_{\bar{x}}$ (number of animals) where the percent radioactivity in each organ of each animal is divided by the organ weight in grams

[b]Balb-C

[c]IE = intraesophageal, IV = intravenous, IT = intratracheal

3.3.2 Transplacental Passage of N-13 from Nitrate and Nitrite

In spite of the caveat associated with using rodents for this type of research, we have injected 0.15 ml portions of high specific activity [N-13]-nitrate and [N-13]-nitrite into the stomach of pregnant C57 mice. Our preliminary experiments were with this species because of its easy availability. The animals were not anesthetized for this procedure and were sacrificed 15 minutes after injection by cervical dislocation. The percentages of injected dose per total organ (%ID) and per gram (%ID/g) of tissue were the same ($P < 0.10$ by student's t test) as for adult Balb/C mice used in the experiments described in other parts of this report for muscle, adipose, kidneys, bladder, lungs, stomach, large and small intestines and residual carcass. The total uterus was surgically tied and excised intact from each animal to determine the %ID and %ID/g and this was compared with the uterus of non-pregnant mature females. The placenta was then separated and %ID measured for amniotic fluid, sack, fetus, maternal placenta and fetal placenta. These results have been summarized in Table 3.

Table 3. N-13 in the Uterus of Mice at 15 min after Injection of Labeled Anions

Tissue Sampled	% Injected Dose	
	$[NO_3^-]$	$[NO_2^-]$
Whole Uterus	10.5	9.0
Amniotic Fluid (50 µl)	0.05	0.05
Washed Fetus	0.58/g	1.57/g
Fetal Placenta	1.15/g	2.45/g
Fetus + Sack	0.69/g	2.91/g

The total amount of activity that migrated into the uterus at 15 min was two-fold larger on a per weight basis than all of the other organs examined except for the stomach, the site of administration. There was no difference in the amount of $^{13}NO_3^-$ versus $^{13}NO_2^-$ in the total uterus or in the amniotic fluid; however, the concentration of ^{13}N was 2-3 times higher in the fetus when nitrite was injected.

3.3.3 Blood Components

Chemical analyses were performed primarily with rabbits which provided more sample material than mice. Gross organ distributions in rabbits are done more conveniently using the Anger gamma scintillation camera with a high-energy parallel-hole collimator. Our experiments with intravenous nitrite and nitrate confirm the findings in mice; namely, a relatively homogeneous distribution of the radioactivity throughout the animals' body as demonstrated in Figure 20.

The ^{13}N radioactivity injected as either nitrate or nitrite clears rapidly from the blood into an extravascular space. Equilibrium between the intravascular and extravascular compartments is reached within 5 minutes after injection of either N-13 radiochemical, indicating the rapidly diffusable nature of these anions. The equilibrium vascular compartment contains 17-20% of the [N-13]-NO_2^- total body activity (TBA), but is only 6-7% by weight of the total animal, the remaining activity being distributed quite evenly throughout the other soft-tissue organs as described above. Only 2-3% of TBA appears in the urinary bladder during the first 30-45 minutes after injection.

Some of the blood samples from nitrite-injected rabbits were fractionated into cells and plasma by centrifugation. We found an average of 0.66 (S.D. 0.05, n=12 from three different rabbits) of the activity in the plasma fraction and the remainder in the cells. The hematocrit remained relatively constant at 40 so that the tracer is relatively evenly distributed on a per volume basis between the cells and plasma. There was no trend in this distribution with time for samples drawn 0.5 to 48 minutes after injection.

The plasma was fractionated further by precipitation of the proteins with alcohol. We found 0.60 ± 0.05 of the activity in the supernatant and 0.064 ± 0.02 in the protein precipitate for a value 0.664 for the fractional activity in plasma. Because the precipitate contained 0.12-0.15 %, by volume, of the centrifuged mixture and the precipitate was not washed prior to counting, this value is an upper limit for precipitable activity. The cells were lysed with distilled water and fractionated in the manner described for the plasma. We found 0.24 ± 0.06% of the activity in the supernatant and 0.09 ± 0.03% in the precipitate, for a value of 0.328 for the fractional activity in cells. The higher coefficient of variation for the latter numbers reflects the larger volume of precipitate from the cellular debris. There is clearly some radioactivity associated with a high molecular weight fraction of the red cells.

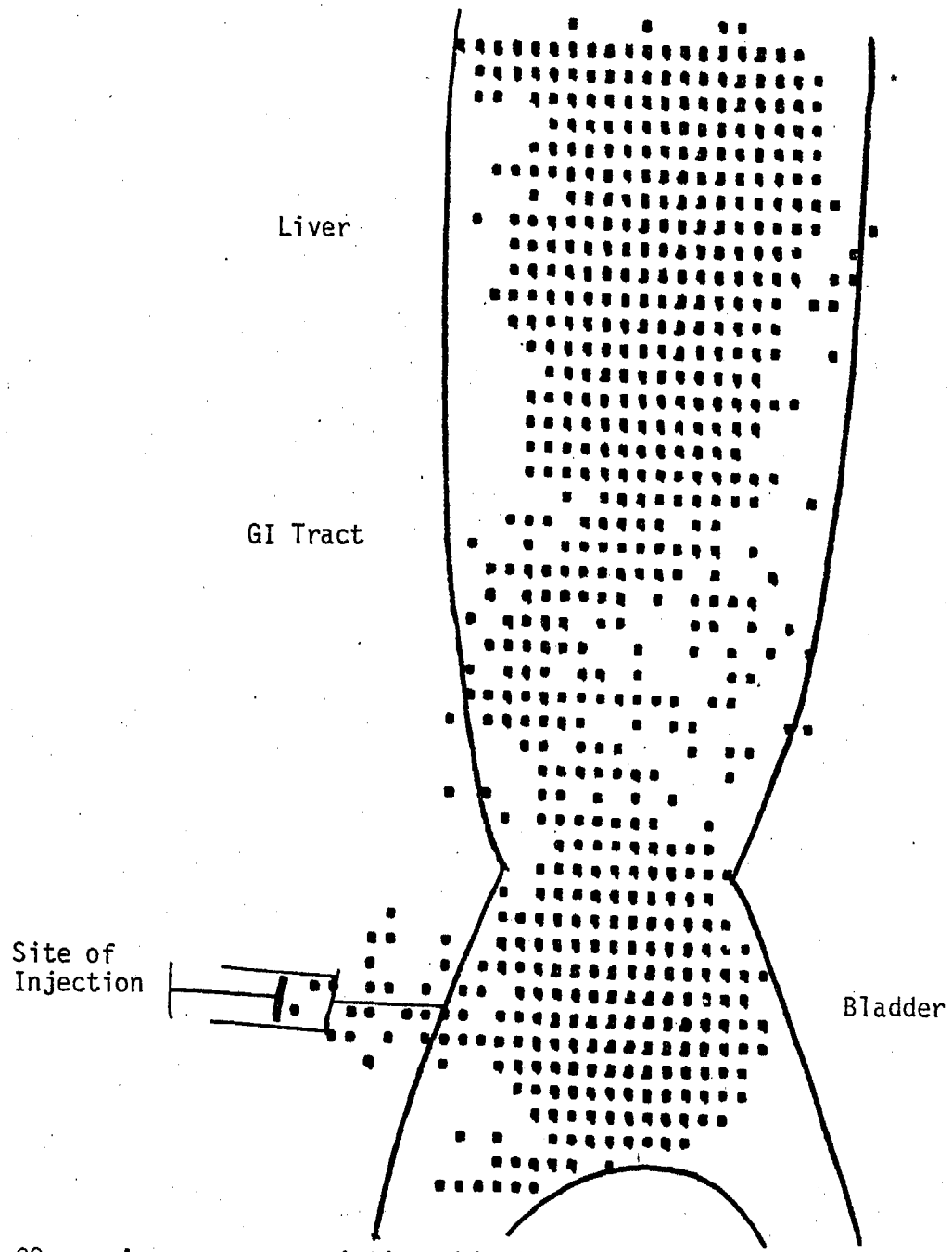


Figure 20. Anger camera scintigraphic image of a rabbit 5 minutes after injection of $^{13}NO_2^-$. Note the homogeneous distribution of radioactivity throughout the animal.

In our only rabbit in which carrier $NaNO_2$ was injected simultaneously with the tracer $^{13}NO_2^-$, there was a somewhat higher fraction of the cellular activity in the protein precipitate. The latter observation was only significant at the P=0.55 level. The distribution of N-13 from NO_3^- in the blood fractions, plasma and cells, appears in preliminary experiments to be similar to that from NO_2^-.

3.3.3 Chemical Analysis

Chemical analysis involved the separation of the various [N-13]-labeled metabolic products of NO_3^- and NO_2^- by high performance liquid chromatography. The methods which we have developed for HPLC are described in detail in section 3.2 of this report.

Plasma and urine samples were taken from one rabbit at 15 minutes after intravenous injection of [N-13]-NO_2^- and from another at 2 and 12 minutes. The first experiment showed that the 3% of injected activity that was in the urine was evenly divided between nitrate and nitrite. Thus the rabbit had oxidized some of the injected nitrite to nitrate in vivo. The chromatograph of the plasma was much more complex in that 4% of the radioactivity eluted from the chromatographic column at a position characteristic of nitrite. The remainder eluted at column breakthrough. These properties are characteristic of N-nitrosamines, but they are also consistent with urea and amino and nucleic acids. The experiments with this rabbit involved only an anion exchange column for analysis and hence did not allow us to identify the chemical form of the majority of the radioactivity. The influence of anticoagulants and anesthetics in blood on the in vivo fate of NO_2^- and NO_3^- and chromatographic separations is not yet fully understood.

We used both anion exchange and C-18 reverse phase partition chromatography for the second rabbit experiment and injected aliquots of millipore-filtered plasma supernatant from both 2 to 12 minute blood samples onto each column. Our results were consistent with the previous experiment. The yields of $^{13}NO_2^-$ and $^{13}NO_3^-$ barely exceed background, and most of the activity elutes in the column breakthrough. When analyzed on a column that specifically retains nitrosamines, we detected no activity significantly above background at the positions we had calibrated for DMN and nitrosopyrrolidine. The activity was also eluted at the void volume of this column, perhaps in two peaks.

In preliminary experiments with intravenously injected carrier-free [N-13]-nitrate, about 20% of the radioactivity appeared as compounds other than nitrate. About 10% was nitrite and the remainder unidentified due to lack of conventional optical spectrometer detectors at the time these samples were analyzed. Apparently some of the nitrate is reduced in vivo.

3.4 DISCUSSION

3.4.1 Distribution

We have found a similar rapid organ distribution of N-13 introduced as nitrate or nitrite from either the lungs following IT or the blood following IV administration. We have found a less rapid, but still fast, clearance from the stomach following IE administration. The range of estimated half periods for all these processes was 4-10 min. The percent N-13 radioactivity and radioactivity concentrations in the GI tract of IE animals was different from IV and IT animals, but once the label left the GI tract, its relative distribution pattern for IE was comparable to IV and IT experiments. Consequently, the extrapulmonary and extra GI tract fate of NO_2^- and NO_3^- may well approach congruent distribution at longer times. In any case, it does not appear that nitrate or nitrite, alone, introduced into the respiratory tract can lead to a health hazard that is different from that posed by ingested nitrate, alone. In the real ambient aerosol, these materials are well-known to appear in concert with other compounds.

3.4.2 Placental Transfer

The placental transfer experiment will require additional control data for rigorous interpretation. We must measure the extracellular fluid volume and the vascular space of the various tissues in order to identify active versus passive accumulation of these compounds. However, because these mice were of the same species, all within 3-5 days of full term and contained 12 fetuses/each, it is unlikely that the differences we have observed will be attributable to different sizes of physiologic volumes.

Our current hypothesis is that both NO_3^- and NO_2^- pass rapidly into the uterus but NO_2^- is much more reactive there and is retained, especially by fetal tissue. This is fully consistent with the other research described above; however, our research is still in progress and will require further experiments before we can make a definitive statement about the health consequences of nitrogenous air pollutants on the unborn child.

3.4.3 Blood Fraction Distribution

Early work by Greene and Hiatt (1) suggested that macroscopic concentrations of nitrate ion displaces chloride ion in physiological fluids, but was not a physiologic substitute because nitrite is not conserved by the kidneys as effectively as chloride. Later Setchell and Williams (2) observed during studies of nitrate poisoning in sheep that nitrate appeared to be equally distributed between cells and plasma. We have found the N-13 from nitrite, and likely nitrate, is distributed in a 1.6:1 ratio between extracellular fluid and the cellular component of rabbit blood. If NO_2^- is distributed initially in the same manner that the label is distributed and the label is primarily in fluids, then neither nitrate nor nitrite are physiologically controlled like chloride ion which has an extracellular:intracellular ratio of 25:1 (3). In addition, the estimated 16-17% activity equilibrium level of N-13 found in the blood of nitrite injected rabbits suggests that, for this species, the N-13 label is distributed throughout all body fluid spaces. This is because the percent of total body fluids, extracellular and intracellular, accounted for by the blood is no higher than 16-17% and is characteristically 12-13% (3).

The results of rabbit plasma chromatography after NO_2^- injection are consistent with the hypothesis that uncharged nitric oxide (NO) or some nitrosyl-derivatives have been formed in blood (4, 5). Freeman's observations (8) that NO-Hb was detectable in the blood of people, regardless of whether they smoked or not, suggests that nitrosyl-compounds are part of the natural nitrogen economy of mammalian systems. Our preliminary nitrate chromatography suggest that this ion may be reduced in vivo and connect into the nitrite reduction reactions.

3.4.4 Tentative Hypothesis

It is clear that sorting out the concentration-dependence, chemical identity, and pathogenic mechanisms of inorganic and organic nitrates and related compounds is a complex matter. However, it is possible to put forth a hypothesis consistent with our work and that of others about the relationship of nitrite administration and the excess incidence of lymphatic tissue tumors observed in rats (6).

The basis of the hypothesis is that a dynamic steady-state exists in mammals in which NO_3^-, NO_2^-, and NO or nitrosyl-derivatives all exist at nonpathogenic concentrations. Important factors in this postulated endogenous steady-state are both intrinsic mammalian biochemical systems and symbiotic biochemical interactions with GI tract bacterial flora. The known adverse effects of the chemical moities in the nitrate through nitrosyl sequence (2,4,6,7) are mediated or eliminated when the concentrations of these moities are below some critical level at which normal biological detoxification or reparative processes are overwhelmed.

The introduction of nitrite or other nitrogenous precursors of NO has been shown both in vivo and in vitro to yield nitrosyl hemoglobin or other nitrosyl iron complexes (4,5). These paramagnetic chemical species are believed to be agents that stimulate the formation of guanylate cyclase which leads to increased cellular concentrations of cyclic 3', 5' guanosine monophosphate (CGMP). The role of cyclic GMP as an effector of cell proliferation is a matter of active investigation at this time, but there are numerous reports that CGMP stimulates DNA synthesis in lymphoid cells (9). Enhanced proliferation of lymphoid cells has been demonstrated in vitro with extremely low (10^{-11} Molar) CGMP concentrations (9).

If the previously set hypothesis emanating from the present investigation with tracer N-13 is combined with the preceding information drawn from the biochemical literature, one is led to the speculation that feeding substantially superambient levels of nitrite to experimental animals may overwhelm the biochemical mechanism which maintains physiologically compatible systemic concentrations of nitrosyl-derivatives. This may result in excess production of CGMP, among other things, which presumably first affects those tissues most sensitive to the mitogenic influence of CGMP. Lymphoid cells are believed to comprise such a tissue. Consequently, Newberne's observations of abnormal proliferation in the lymph tissue of rats fed high levels (250-2000 ppm) of nitrite is consistent with our line of reasoning which was drawn from independent sources. Newberne's work is still under review by the U.S. Food and Drug Administration; therefore we cannot say with assurance whether it supports or denies our hypothesis. However, the emergence of a hypothesis which is testable in some aspects is regarded as a step forward in dealing with health effects questions related to nitrogenous air pollutants in general.

Our present assessment of the mammalian nitrogen economy for NO_x^- species, as determined under no-added-carrier conditions, is at variance with some experiments where above ambient dosages of material have been used. Future work will include higher N-13 radioactivity concentrations for longer studies with larger animals and the use of N-13 pulse techniques to probe the physiologic transport of NO_x^- in animals maintained on macroscopic dosages of these materials. This work will serve the direct purpose of relating the high dosage to our tracer metabolic studies. In addition, this information is anticipated to be useful in examining our hypothesis about the nature of the mammalian nitrogen economy by testing the variation of metabolic fate as a function of dosage.

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Section 4
LIST OF PUBLICATIONS AND PRESENTATIONS

1. McElfresh, M. W., J. C. Meeks, and N. J. Parks, "The Synthesis of ^{13}N-Labelled Nitrite of High Specific Activity and Purity", J. Radioanal. Chem. 53(1-2): 345-352 (1979).
2. Parks, N. J., K. A. Krohn, N. F. Peek, J. Chasko, and M. McElfresh, "The In Vivo Fate of Some N-13 Labeled Air Pollutant Derivatives", Symposium on the Use of Short-Lived Radionuclides in Biology and Chemistry, Division of Biological Chemistry and Nuclear Chemistry, American Chemical Society Annual Meeting, Washington, D.C., September 9-14, 1979 (Attached, as Appendix A).
3. Krohn, K. A., "The Use of Isotopic Nitrogen as a Biochemical Tracer", Symposium on the Use of Short-Lived Radionuclides in Biology and Chemistry, Division of Biological Chemistry and Nuclear Chemistry, American Chemical Society Annual Meeting, Washington, D.C., September 9-14, 1979 (Attached, as Appendix B).

APPENDIX A

THE IN VIVO FATE OF SOME N-13 LABELED AIR POLLUTANT DERIVATIVES

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Presented at the

Symposium on the Use of Short-Lived
Radionuclides in Biology and Chemistry
sponsored jointly by the Divisions of
Biological Chemistry and Nuclear Chemistry
American Chemical Society Annual Meeting
Washington, D. C., September 9 - 14, 1979

ABSTRACT

An increased loading of the tropospheric aerosol with oxides of nitrogen (NOx) derived from fossil fuel combustion is well known. These compounds may be incorporated in vivo into derivatives which have an adverse health impact. A major component of NOx emissions is NO_2 which can result in the formation of NO_2^- and NO_3^- either in the ambient aerosol or following inhalation or ingestion. In previous animal studies with superambient dosage levels, NO_2^- ion was shown to react with secondary amines in the digestive tract to form N-nitrosamines, a class of potent carcinogens, and to participate in the formation of met-hemaglobin. In our present experiments N-13 labeled $*NO_2^-$ and $*NO_3^-$ has been given to rodents and rabbits by intratracheal, intra-esophageal, and intravenous routes at carrier free (i.e. no added carrier) concentrations in order to avoid perturbation of "normal" physiological detoxification processes. Equilibration of N-13 activity with most body pools and compartments is achieved in 5-10 minutes for both $*NO_2^-$ and $*NO_3^-$ regardless of introductory route. Urinary excretion of N-13 activity from $*NO_2^-$ administered to the test animals was < 10% during the 60 minute duration of the experiments and was found equally distributed between $*NO_2^-$ and $*NO_3^-$ by HPLC. After $*NO_2^-$ injection, analysis of chemical species labeled with N-13 in various blood fractions revealed less than 20% of the activity remained as $*NO_2^-$; the residual activity eluted mostly at the injection peak on an anion exchange column. The latter did not correspond to any N-nitroso compounds for which standards were available.

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INTRODUCTION

Primary nitrogenous air pollutants, oxides of nitrogen (NOx), can give rise to ionic nitrate and nitrite compounds through atmospheric reactions (1 - 3) or through in vivo biological reactions following inhalation of these materials (4 - 6). Organic nitrate and nitrosyl compounds can be formed via similar pathways (7 - 10). The health impacts of these pollutant derivatives is presently a matter of concern, and poorly understood (9 - 17). This lack of understanding results partly from a paucity of appropriate experimental technology and partly because epidemiological and toxicological indications that a problem may exist are relatively recent (12, 13, 16). In addition, human exposure to some of these materials is not uniquely associated with air pollution, and it is not known whether the biological sequelae to exposure by inhalation and ingestion are similar or dissimilar. Some key relationships that must be characterized are presented schematically in Illustration 1.

NITROGENOUS COMPOUNDS: SOURCES AND PATHWAYS

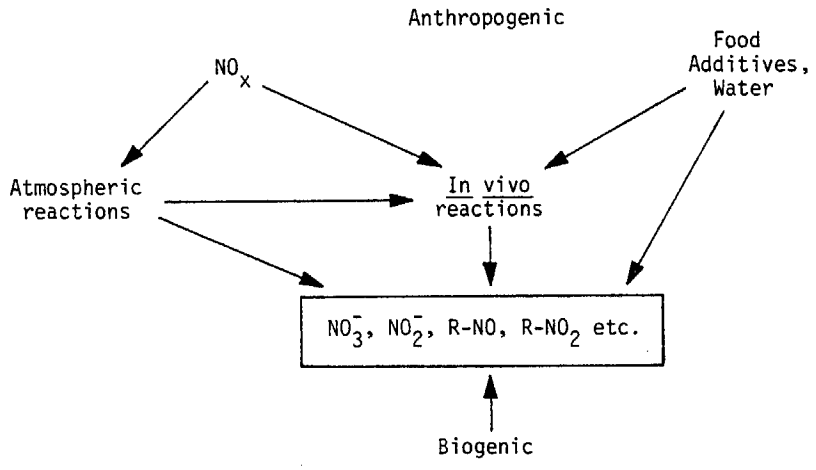

Illustration 1

There are two source categories. The first, the anthropogenic (human activity-related) category, includes atmospheric NOx emissions associated with fossil-fuel combustion, and materials introduced into the food chain by contemporary agricultural and food preservation practices. The second, the biogenic category, includes natural sources such as lightning induced NOx formation, gaseous emissions from living matter (18), water-borne mineral nitrates, and some green vegetables in which potentially hazardous nitrate concentrations can occur (20). Nitrogenous chemical species introduced into the biosphere from anthropogenic sources are the major concern with respect to increased potential for adverse health effects. However, valid risk-assessment ultimately depends on adequate knowledge of natural background concentrations both in the environment and in vivo of these same chemical species or their reaction products that are derived from biogenic sources.

Various reaction pathways lead from NOx to a spectrum of nitrogenous compounds (Box, Illustration 1) in the atmosphere (21 - 23). Examples are the reaction of photochemically produced hydroxyl radicals with nitrogen dioxide or nitric oxide to form nitric or nitrous acid. Subsequent reaction with ammonia or dissolution in aqueous aerosol droplets has been proposed for the stabilization of atmospheric nitrates (24). Similar stabilization of nitrite may compete with photodecomposition of nitrous acid because nitrites have been found in ambient aerosol samples (2, 3). In addition to the well known reaction of hydroxyl radical with acyl peroxyl radicals to form peroxy acyl-nitrates, the formation of nitrosamines, nitramines, amides (7, 8) and organic nitrates have been found in either smog-chamber or atmospheric samples (25). A second direct source for human exposure to nitrates and nitrites is ingestion of food which contains these compounds as preservatives or water

contaminated by nitrate fertilizers (20). Foods are likely the primary source of carcinogenic nitrosamines (7, 26), but the biologic consequences of inhaling these materials include carcinogenesis (27).

The in vivo reactions of inhaled primary pollutants, NO and NO_2, and important secondary pollutants such as nitric acid, particulate nitrates, and perolyacetylnitrate (PAN) are generally the subject of speculation or are unknown. Nitric oxide and NO_2 both appear to form nitrosyl-hemoglobin (NOHb) in mouse blood (9). This information, taken together with evidence for involvement of the NOHB complex in liver enzyme activation (28) by NO and NO_2^-, may partly explain liver injury and altered enzyme activity in guinea pigs exposed to 1 ppm NO_2 for 6 months (29). Other evidence indicates that a steady state level of NO is maintained in human blood irrespective of exogenous exposure to NO and NO_2 by smoking (30). In previous studies at U. C. Davis, Goldstein et al. (4) found parallel increases in blood and lung radioactivity during exposure of rhesus monkeys to N-13 labelled NO_2 (0.6 ppm) and substantial extrapulmonary distribution of N-13. The extrapulmonary activity was hypothesized to result from NO_2 dissolution in the aqueous phase of the respiratory tract with subsequent formation of nitric and nitrous acids. These acids were presumed to be neutralized by physiological buffers to yield nitrates and nitrites that were cleared by the blood. This hypothesis is supported by earlier work of Kaut et al. (5, 6) who found measurable levels of nitrite in the blood of rats and rabbits exposed to superambient levels (25 ppm) of NO_2. Many ingested or sublingually absorbed organic nitrates also yield nitrite as a metabolite (17). The chemical fate of inhaled organic nitrates is unknown, but in some cases, may be similar. Some directly inhaled nitric acid or nitrates may be reduced endogenously to

nitrites by bacterial action as proposed by Witter et al. (31, 32) to explain the fate of ingested N-13 nitrate. Alternately, Tannenbaum has argued that endogenous bacterial oxidation of reduced nitrogen compounds accounts for most in vivo nitrite and nitrate (33, 34). Nitrite is known to oxidize hemoglobin to methemoglobin, thus diminishing the oxygen carrying capacity of the blood (10). Also, nitrite is known to react with secondary amines in the acidic environment of the stomach to form carcinogenic nitrosamines (15).

In the preceding discussion, some inferences drawn about the likely chemical fate of inhaled nitrogenous pollutants are based on high exposure levels which may markedly perturb normal physiological processes. Questions about the in vivo fate of these remain largely unanswered. This is primarily because little is known about "base-line physiological" concentrations of even the simple ions, NO_3^- and NO_2^-, and their metabolic pathways.

Various factors which bear on the health impact of nitrogenous pollutants or their metabolites are summarized in Illustration 2.

HEALTH IMPACT CONSIDERATIONS

1. Compromised physiological function (respiration, methemoglobin, biochemical).
2. Carcinogenesis (nitroso-compounds, nitrites).
3. Dosage - response relationships.

Illustration 2

The first item--compromised physiological function--includes compromised lung function (common with oxidants) (21), reduction of the oxygen carrying capacity of the blood by the formation of methemoglobin, and indication of chemical changes in liver tissue (29, 36). The second factor--direct carcinogenesis--is listed because there is evidence that nitrite can react with food-derived secondary amines in the acid environment of the stomach to form nitrosamines, a known class of carcinogens. In addition, a recent report by Newberne (16) has reported that ingestion of nitrites by rats causes a excess of lymphatic cancers. The possibility of in vivo reduction of nitrate to nitrite represents an additional complication. However, it must be pointed out that Newberne's studies used excess nitrite burdens much higher than likely to accrue from ambient pollutant levels. Thus, dosage-response relationships become a critical concern with respect to health-risk assessment, i.e., what is the relationship of integrated chronic dosage of the given pollutant at ambient concentrations to the probability of an unacceptable biological response.

Our research directly addresses the question of metabolic sequelae after exposure to labelled nitrates and nitrites at concentrations which do not result in a pharmacologic perturbation of normal physiological processes. This novel nuclear application in toxicology is possible because the potential toxins are synthesized under "no carrier added" (NCA) conditions. The problem of physiologic perturbation which is encountered with tracer studies using the macroscopic quantities of material required for detection of the stable nitrogen-15 is avoided.

This paper deals with the organ distribution of N-13 following intravenous (IV) and intratracheal (IT) instillation into mice, and blood-clearance kinetics following intravenous injection into rabbits. A comparison to IV

and IT data of the fate of N-13 following intraesophageal (IE) introduction is in preparation and will be reported elsewhere (40).

EXPERIMENTAL

The cyclotron target system previously described by Parks and Krohn (38) was used to produce N-13 (T, 1/2, = 10 min) labeled NO_3^- directly by bombardment of water with 20 microamp beams of 18 MeV protons. The radioactivity concentration of N-13 was ca. 8 mCi/mL as NO_3^- and ca. 0.40 mCi/mL as NO_2^- plus NH_4^+ at end-of-bombardment (EOB). Only radiochemical preparations with greater than 95% of the N-13 as NO_3^- were used for animal experiments. Labeled NO_2^- was prepared by reduction of NO_3^- with an improved Cd reduction techique described by McElfresh et al. (39) from our laboratory. An evaporation step used to remove any NH_3 formed in the reduction procedure resulted in solutions with an N-13 radioactivity concentration greater than 16 mCi/mL. The concentration of radionuclidic impurities in the target solution were 1.46 mCi/mL of O-15 (T, 1/2, = 2 min) and 0.01 mCi/mL of F-18 (T, 1/2, = 110 min) at EOB. Physical decay reduced the O-15 activity to less than 1% of the N-13 activity by the time biological samples were assayed. Each biological sample was assayed twice in order to permit corrections for F-18 activity. Chemical identification was performed with a high performance liquid chromatograph fitted with radiation detectors.

The experimental animals, BALB-C mice and New Zealand white rabbits, and the routes of introduction are shown in Ilustration 3. Intratracheal

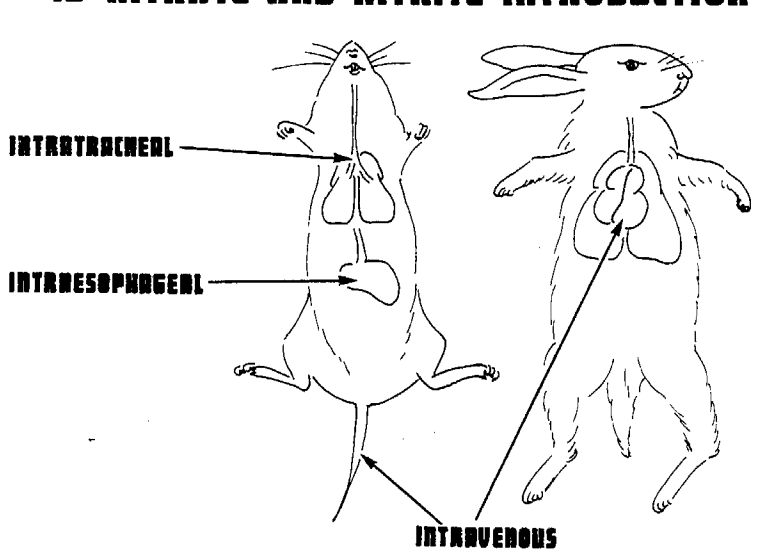

Illustration 3

instillation of 10 to 15 µL of solution was accompanied with a modification (41) of techniques reported by Ho and Furst (42). The essence of the procedure involved using an otoscope with a modified specula which permits viewing the pharynx and also serves as a guide for a 23 gauge cannula. Intravenous injections were made into the tail vein of mice or through a jugular cannula in rabbits.

In the present experiments, some ancillary in vitro studies with human blood (another species) have been carried out. These experiments were done with freshly drawn blood from a healthy adult male. Sufficient aqueous solution containing NCA labelled NO_2^- was added to cause lysis of the cellular components. The resultant solution was incubated for 10 - 20 min prior to precipitation of the protein fraction and chromatography of the supernatant.

Clearance from the lungs of N-13 activity introduced as nitrate and nitrite by the intratracheal (IT) route is shown in Illustration 4. Point one

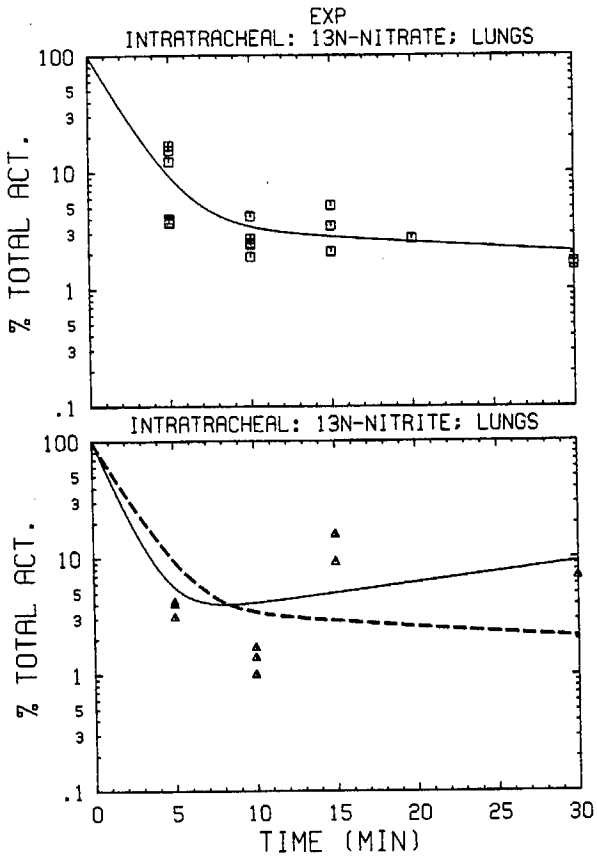

Illustration 4

is that approximately 90% of the label is rapidly cleared from the pulmonary tract; the half-period of this initial clearance was estimated to be about 1 minute by a non-linear least-squares fit of a simple two exponential equation to the data (corrected for radioactive decay).

Point two is that an approproximate equilibrium of N-13 activity is observed after 5 minutes. The locus of the curves (solid lines) describing the second exponential term obtained by regression suggests an asymptotic approach to equilibrium values for nitrate data, but possible accumulation of the N-13 label associated with nitrites. However, the dispersion of the data

for nitrite leads to a probability distribution for the second exponential rate constant that has statistically significant ($p > 0.1$) overlap with zero in both cases and is not significantly different from that defining the locus of the nitrate data (broken lines) shown for comparison. Therefore the mean and standard error ($\bar{x} \pm SE$) of 5.1 ± 1.2 and 5.3 ± 1.6 for nitrate and nitrite, respectively provide an adequate basis for comparison of lung activity data after clearance of the initial bolus.

The translocation of nitrate and nitrite to the stomach where reaction of nitrite with dietary secondary amines (such as dimethyl amine) commonly found in food can lead to the formation of carcinogenic nitrosamines has been examined. An additional concern regarding stomach translocation derives from various reports which indicate a high risk for methemoglobinemia following reduction of nitrate to nitrite in the GI tract of infants. The results of lung to stomach transfer found in the present experiments are shown in Illustration 5.

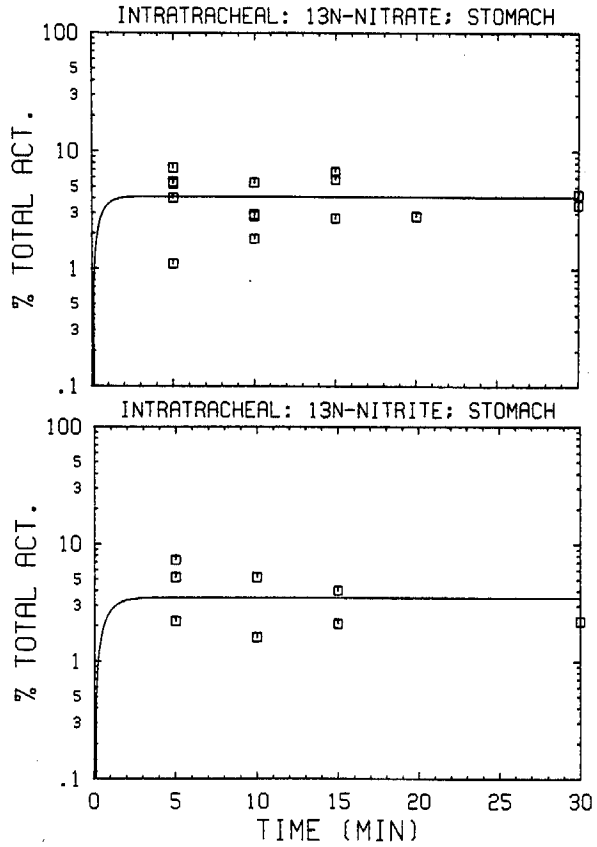

Illustration 5

As a first approximation, these data were described with a rising exponential equation of the form: % total Activity = A[1 - exp(-k)t]. With this mathematical model and data for N-13 activity in an organ other than the site of introduction, we again obtain results indicating that an approach to an equilibrium distribution occurs with a half-period of about one minute.

A similar phenomena was observed following intravenous injection of NOx$^-$ ions into the lung, an organ system of particular interest in air-pollutant studies. The comparison of nitrate and nitrite values in the lungs (shown in Illustration 6) reveals trends that are indistinguishable with median values of 1.6 ± 0.1% and 1.7 ± 0.4%, respectively.

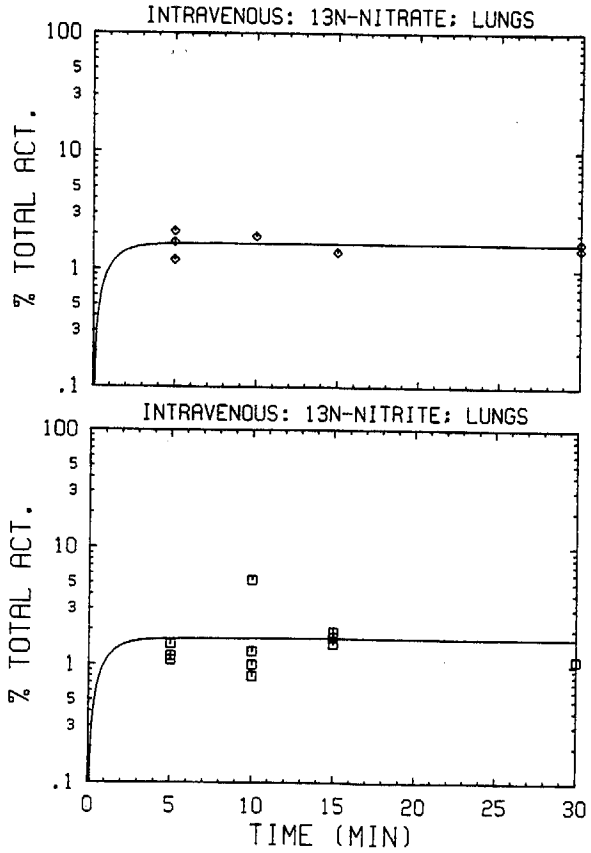

Illustration 6

The comparison of lung data for the IV route versus the IT route (Illustrations 7 and 8) shows about a factor of two higher average concentrations of activity in the lungs at 5 - 30 minutes after IT instillation over that derived from the blood following injection. In the case of nitrate, the IT data appear to asymptotically approach the equilibrium level of the IV data. Although the variation is somewhat greater for the IT data with the more reactive nitrite ion, the pattern of clearance within 5 minutes to values within the range of the IV data is similar.

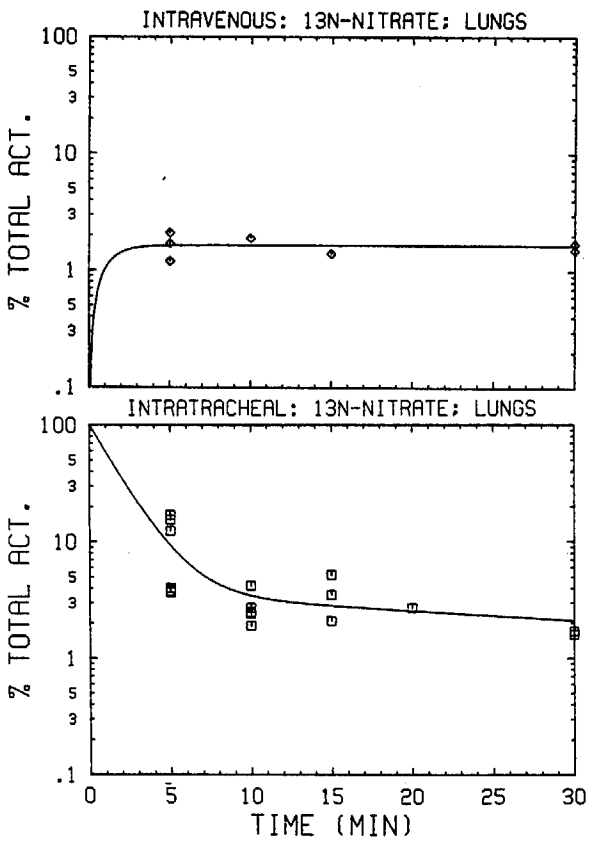

Illustration 7

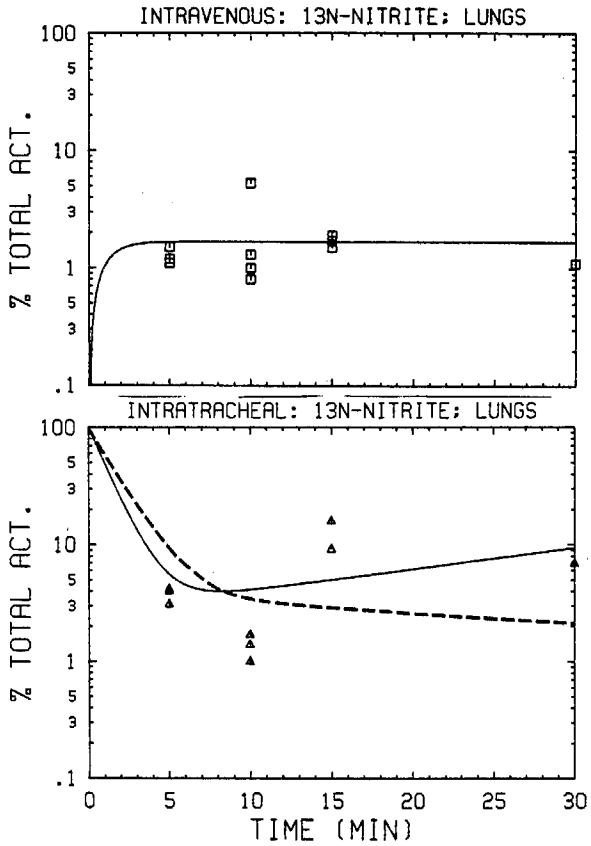

Illustration 8

The clearance of NOx$^-$ from the blood is represented by the plot of percent $^{13}NO_2^-$ (NCA) in blood versus time (Illustration 9). Earlier work (43) by others has shown that the pharmacokinetics of GI tract and plasma

clearance of macroscopic NOx^- is species dependent; however, we find with tracer levels of nitrite that the time-scale for establishing blood equilibrium levels in rabbits is remarkably similar to clearance in mice.

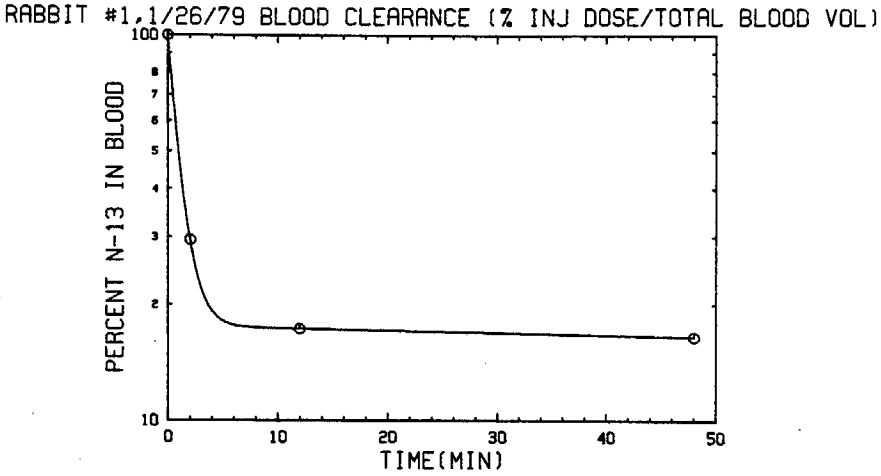

Illustration 9

Additional cross-correlation of animal models is being done because some experimental measurements are difficult to perform with mice.

The clearance of more than 80% of the N-13 activity from the blood (Illustration 9) was not accounted for by urinary excretion. A maximal estimate of blood volume (44), 70 mL/kg body weight, was taken for the calculation of percent N-13 in blood. Thus the 16 - 17% equilibrium level (Illustration 9) is an upper limit. The estimated lower limit is 12%.

The urinary activity levels for mice (Illustration 10) appeared to be log-normally distributed and thus the data is presented in terms of the geometric mean (G) and the geometric standard error (SE_g) range for

intravenous, intratracheal, and intraesophageal (IE) introductory routes. The apparently lower urinary excretion activity of nitrite N-13 may result from more in vivo reactions as compared to nitrate. The chemical form of the small activity fraction cleared into rabbit urine after the labeled nitrite IV

PERCENT URINARY EXCRETION

	NO_3^-			NO_2^-	
		IV			
$\overline{G}$	1.092		$\overline{G}$	0.472	
$\pm SE_g$	0.307	3.883	$\pm SE_g$	0.133	1.676
		IT			
$\overline{G}$	0.716		$\overline{G}$	0.309	
$\pm SE_g$	0.155	3.310	$\pm SE_g$	0.007	14.061
		IE			
$\overline{G}$	0.851		$\overline{G}$	0.687	
$\pm SE_g$	0.176	4.126	$\pm SE_g$	0.132	3.571

*BALB-C Mice (10 - 30 min)

Illustration 10

injection was approximately equal parts nitrate and nitrite as determined by anion exchange chromatography. Our preliminary observations indicate that only a small fraction of the total body N-13 activity (TBA) exists in the plasma as free nitrate and nitrite.

As noted previously, about 17% of TBA after N-13 nitrite injection was in rabbit blood at equilibrium. In freshly drawn blood, this was distributed as

approximately 12% of TBA in plasma and 5% of TBA in the cellular precipitate of centrifuged samples treated with an anti-coagulant. Plasma proteins were precipitated with methanol. The supernatant:precipitate radioactivity ratio was (10.6% TBA):(1.4%TBA). The cellular precipitate was resuspended in methanol which caused rupture of the cell membranes in addition to protein precipitation. The supernatant:precipitate ratio was (3.6%TBA):(1.25%TBA).

The plasma supernatant was chromatographed by HPLC on both a strong anion exchanger column, which separates NO_2^- and NO_3^-, and a column suitable for separating organic hydrocarbons and common nitrosamines. The radioactive N-13 was predominantly associated with the injection peak or "column breakthrough" and was not retained on either column. A fraction (ca. 0.20) of the plasma activity remained as NO_2^-. This preliminary result from the rabbit in vivo study suggests that NO_2^- in plasma is rapidly destroyed with the labelled N-13 being reduced in the process.

The ingestion or injection of macroscopic quantities of nitrite is expected to produce methemoglobin and nitrate (10, 35) as two of the possible products. Evidence consistent with this idea was obtained by in vitro studies where NCA-nitrite was added to fresh human whole-blood contained in an evacuated tube under conditions causing rupture of the cell membranes. This released cellular components for reaction with nitrite; a 14 minute incubation at 37° gave the liquid chromatogram on a strong anion exchange column as shown in Illustration 11. As shown, about 50% of the activity is in the NO_3^- form after 14 min. Thus, in vitro, the dominant reaction in hemolyzed blood is oxidation of NO_2^-. If this oxidation requires chemical constituents inside the cell, then only the 5% of TBA associated with the cellular fraction of blood is available for reaction at a given time.

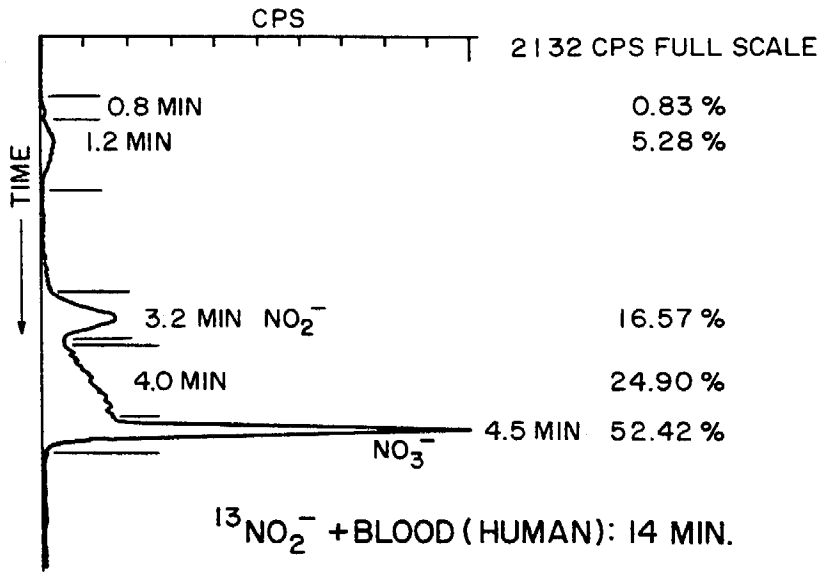

Illustration 11

DISCUSSION

We have found a similar rapid organ distribution of N-13 introduced as nitrate or nitrite from either the lungs following IT or the blood following IV administration. Early work by Greene and Hiatt (44) suggested that macroscopic concentrations of nitrate ion displaces chloride ion in physiological fluids, but was not a physiologic substitute because nitrate is not conserved by the kidneys as effectively as chloride. Later Setchell and Williams (46) observed during studies of nitrate poisoning in sheep that nitrate appeared to be equally distributed between cells and plasma. We have found the N-13 from nitrite (NCA) is distributed in a 1.6:1 ratio between extracellular fluid and the cellular component of rabbit blood. If NO_2- is distributed initially in the same manner that the label is distributed and the label is primarily in fluids, then neither nitrate nor nitrite are physiologically controlled like

chloride ion which has an extracellular:intracellular ratio of 25:1 (47). In addition, the estimated 16-17% activity equilibrium level of N-13 found in the blood of nitrite injected rabbits (Illustration 9) suggests that, for this species, the N-13 label is distributed throughout all body fluid spaces. This is because the percent of total body fluids, extracellular and intracellular, accounted for by the blood is no higher than 16-17% and is characteristically 12-13% (47).

The results of rabbit plasma chromatography after NO_2^- injection are ambiguous but suggest that uncharged nitric oxide (NO) or some nitrosyl-derivatives may have been formed in blood (9, 28). Some oxidation of NO_2^- appeared to occur from our in vitro experiments with human blood (Illustration 11). Freeman's observations that NO-Hb was detectable in the blood of people, regardless of whether they smoked or not, suggests that nitrosyl-compounds are part of the natural nitrogen economy of mammalian systems.

It is clear that sorting out the concentration-dependence, chemical identity, and pathogenic mechanisms of inorganic and organic nitrates and related compounds is a complex matter. However, it is possible to put forth a hypothesis which is consistent, in general, with our work and that of others about the relationship of nitrite administration and the excess incidence of lymphatic tissue tumors observed in rats (16). The basis of the hypothesis is that an equilibrium system exists in mammals in which NO_3^-, NO_2^-, and NO or nitrosyl-derivatives all exist at nonpathogenic concentrations. The known adverse influence of the various chemical species are balanced by biological reparative reactions. Excess NO_2^- feeding may lead to excess NO which forms nitrosyl-compounds known to stimulate liver enzymes (28, 29) which are important in cellular transformation processes and can possibly (28) lead

to the type of increased tumorigenic potential observed by Newberne (16) that did not appear to be associated with nitrosamines.

Because our probe of mammalian nitrogen economy for NOx^- species under NCA conditions is at variance with experiments where above ambient dosages of material have been used, the future work will include higher N-13 radioactivity concentrations for longer studies with larger animals and the use of N-13 pulse techniques to probe the physiologic transport of NOx^- in animals maintained on macroscopic dosages of these materials.

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APPENDIX B

THE USE OF ISOTOPIC NITROGEN AS A BIOCHEMICAL TRACER

by

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ABSTRACT

Research on N-cycle chemistry has been concerned with such diverse problems as food and fiber production, metatolic pathways and environmental control but has been impeded by the unavailability of a convenient tracer. The stable isotopes, N-14 and N-15, have been used as tracers; however, their value is limited due to the requirement for macroscopic amounts of carrier that often exceed in situ concentrations and the fact that natural N-isotope fractionation leads to variations in abundance. Nevertheless, stable tracers have been used effectively in many studies, including following N-cycle processes in soils and unraveling metabolic pathways. The longest lived radioisotope of nitrogen, N-13, has a half-life of 10 minutes which is generally described as too short for biological experiments. While its T-1/2 is inconveniently short and the isotope must be used at the nuclear accelerator where it was produced, we have found N-13 uniquely valuable in instances where a very high specific activity N tracer is required to measure rapid kinetics and metabolic pathways. It offers 10^8-fold increased detection sensitivity over stable isotope methods. One example of its use involves $^{13}NO_3^-$ to measure denitrification rates in flooded rice fields. A second example involves the use of simple N-13 labeled metabolites, ammonia, nitrite, nitrate, to determine their in vivo distribution kinetics and biochemical transformations in animals. The latter requires invasive sampling of body consitutents and their analysis on an HPLC modified with a radioactivity detector to measure the chemical form of the metabolized N-13 label. Metabolic products of only 10^{-5}% yield have been detected.

Of those elements which comprise most of our living world, carbon, hydrogen, nitrogen and oxygen, there are no gamma-emitting radionuclides of convenient half-life for widespread distribution and large scale application in the research laboratory. There are no gamma-emitting isotopes of hydrogen at all and the longest lived gamma-emitting nuclides of carbon, nitrogen and oxygen have half-lives of 20 min, 10 min, and 2 min, respectively. These are all inconveniently short and require that the isotope be used at the accelerator site where it was produced. However, a short half-life can be an advantage in the sense that these nuclides can be prepared at very high specific activity and they decay rapidly. The latter characteristic allows experiements to be spiked with multiple consecutive batches of radioisotope, resulting in serial studies following perturbations of the chemical or biological system under investigation.

Stable isotopes of each of these elements have played important roles in elucidation of their chemistries. Specifically, N-14 and N-15 have been used as stable tracers for research on N-chemistry concerned with food and fiber production, metabolic pathways and environmental control. Their value is limited due to the requirement that macroscopic amounts of carrier often exceed in situ concentrations, and natural nitrogen isotope fractionation leads to variances in natural abundance. Nevertheless, stable tracers have been used effectively in many studies, including N-cycle processes in soils (1) and unraveling metabolic pathways (2,3).

HISTORY OF ^{13}N TRACER STUDIES

The first radioisotope of nitrogen to be identified was ^{13}N, a positron emitter with a 10 min half-life. Subsequent investigations have

confirmed this isotope as the longest lived radioisotope of nitrogen. The original observation of the nuclear synthesis of N-13 was reported in 1934 by Joliot and Curie (4). They irradiated boron nitride with alpha particles and found that by heating the BN with caustic soda, gaseous ammonia was produced. The activity could be conveniently separated from the boron by simple distillation. Thus this represents not only the first synthesis of radioisotopic nitrogen, but also the first synthesis of a nitrogen compound, $^{13}NH_3$.

Nitrogen-13 was first used as a biologic tracer by Martin Kamen and his colleagues at the Crocker Cyclotron, Berkeley, in 1940 (5). These investigators irradiated charcoal (carbon) with 8 MeV deuterons to make radioactive ^{13}N-N_2 gas which was contained in a dessicator and used to quantitate the assimilation of N_2 by barley plants. They estimated that the plants assimilated 10^{-4} to 10^{-5} of the radioactive N_2 and considered their experiments as positive evidence for nitrogen fixation by nonleguminous plants. Their pioneering research is only marred by the fact that their conclusion was wrong; barley plants do not fix nitrogen. Perhaps these researchers were led astray by traces of either $^{13}NH_3$ or $^{13}NO_3^-$ in their radiochemical product. Either of these radiochemicals would be assimilated by barley.

The next contribution to the N-13 literature came from the laboratory of Joseph Varner, but is recorded only in the proceedings of a paper presented at the University of Pennsylvania in 1959 (6). Varner and Carangal bombarded thin graphite targets with deuterons at the Ohio State University cyclotron and prepared N-13 labeled NO_3^-, NO_2^-, NH_3, and N_2. These compounds were prepared for studies of inorganic nitrogen metabolism; however, there is no further report of their use in Varner's laboratory.

In spite of their lack of follow-up, many of the ^{13}N radiochemical methods used today originated with Varner's benchmark studies.

In 1961 Nicholas et al. reported the use of N-13 for studying nitrogen fixation in bacterial cells (7). They bombarded coarsely granulated charcoal with 15 MeV deuterons from the MRC cyclotron at Hammersmith, England. Radioactive N_2 gas was continuously swept from the target by carrier argon and with a 40 microamp deuteron beam the activity produced ranged from 30 to 60 millicuries per liter. Various microorganisms contained in 50 ml Erlenmeyer flasks were exposed to the N-13 during agitation in a water bath at 30°C. Aliquots of supernatant were removed periodically for analysis of N-13 uptake. The effects of various experimental parameters were noted and the authors commented on the possibility of determining the chemical composition of early fixation products. Since that time, the research interests of the MRC Hammersmith cyclotron have changed toward a more medical emphasis and these initially exciting observations have not been followed up.

Researchers at the University of Mannitoba cyclotron introduced the $^{14}N(p,d)^{13}N$ nuclear reaction to produce $^{13}N_2$ for rapid screening of the fixation potential of microorganisms (8). Their target was melamine plastic powder (40% N) which was irradiated with 50 MeV protons to give a gaseous yield of 5 millicuries per liter of gas. The high proton irradiation energy was chosen because of physical design limitations inherent in their cyclotron. The experimental arrangement for preparing the nitrogen gas and exposing microorganisms was similar to techniques described earlier. They exposed a number of subarctic soil bacteria to $^{13}N_2$ and found that several had N-fixing potential. These researchers were enthusiastic about

the potential of tracer nitrogen techniques for N-fixation research, particularly as a means of rapid screening of fixation potential. The fact that N-13 has not achieved wide use as a screening agent for fixation potential is probably attributable to the advent of the acetylene reduction technique for equivalent measurements.

In the mid 1960's, cyclotrons dedicated to biomedical research became available in several hospitals and ^{13}N labeled radiopharmaceuticals, principally $^{13}N_2$ and $^{13}NH_3$, were produced. While its short half-life limited general clinical utility of N-13 radiopharmaceuticals, nitrogen gas found some use for lung ventilation studies, and N-13 ammonia has been used as a myocardial imaging agent (9,10). The pioneering work in development of N-13 radiopharmaceuticals was done by Welch in the United States (11) and Clark in the United Kingdom (12).

Nitrogen-13 research was initiated at the University of California, Davis in 1971 by Parks, Peek and Goldstein (13). They produced $^{13}NO_2$ gas by the $^{16}O(p,\alpha)^{13}N$ nuclear reaction in a high pressure oxygen target. Cryogenically recovered $^{13}NO_2$ was used in a study of the intrapulmonary and extrapulmonary distribution of inhaled nitrogen dioxide, a major air pollutant (14). The intrapulmonary location and concentration of the inspired NO_2 was detected continuously by Anger camera monitoring of the ^{13}N radiation. The ^{13}N concentration of arterial blood was also measured by serial sampling and counting and the blood values were correlated with those of the lung. These researchers found that during normal inspiration 50 to 60 percent of the NO_2 was retained by the animal. The absorbed NO_2 or its reaction products remained within the lungs for prolonged periods after exposure. The labeled pollutant was also carried to extrapulmonary sites via the bloodstream.

In 1973 we began an experimental measurement of denitrification kinetics using $^{13}NO_3^-$ (15). Again the $^{16}O(p,\alpha)^{13}N$ reaction was used, but on a H_2O target. The radiochemical form of the ^{13}N was nitrate with no measureable nitrite or ammonia. The nitrate produced in this way was used as a tracer for direct quantitative measurements of denitrification rates in soils from flooded rice fields and in natural lake systems. Core samples were transferred with minimal agitation and under anaerobic conditions to the cyclotron laboratory where $^{13}NO_3^-$ was added, again anaerobically, and the rate of gaseous ^{13}N evolution was monitored over short time intervals. The high specific activity of the $^{13}NO_3^-$ allowed us to make these kinetic measurements without changing the nitrate concentration in situ and the rate of denitrificaiton could be measured over a period of 10 to 20 minutes. By contrast, state-of-the-art denitrification measurements using stable tracer $^{15}NO_3^-$ involve several-fold enrichments of the sample nitrate content and result in significant overestimates of the actual denitrification rates.

The same research group used tracer ^{13}N to measure the rate of inorganic N-uptake by phytoplankton in an effort to evaluate the relative importance of nitrogen cycling mechanisms with regard to phytoplankton productivity in aquatic ecosystems (16). Using the ^{13}N tracer methodology they measured nitrate uptake rates of 54.2 ± 14.6 microgram-N/l/hr in a eutrophic pond and rates as low as 4.5 ± 2.2 ng-N/l/hr in an oligotrophic lake. The precision of these measurements as well as the wide range over which they have been effectively used point out the advantages of short-lived radio-isotopes for tracer biochemistry.

A research effort has also been initiated at the Michigan State University Plant Research Laboratory and Cyclotron Laboratory to make ^{13}N-N_2

and to measure the kinetics and mechanisms of nitrogen transformations after fixation by blue-green algae (17, 18, 19). This laboratory used the $^{13}C(p,n)^{13}N$ reaction using 11 MeV protons on an isotopically enriched ^{13}C target (20). The irradiated material was removed from the target, combusted in an automated Dumas apparatus, and the resulting $^{13}N^{14}N$ gas compressed into a 1 ml vial. The technique produced a usable product of 20 millicuries per microamp. ^{13}N fixed by algal filaments was localized by a technique which permitted track autoradiography with short-lived isotopes (17). Electrophoretic and thin layer chromatographic methods were also developed for rapid isolation and characterization of the first organic products of N_2 fixation by blue-green algae (18).

Although the experimental demands of working with short-lived radiotracers are often severe, hot atom chemists have used them profitably for many years. In the historical portion of this review we have seen how pioneering research by Kamen, Varner, Volk and others has extended the methodology of short-lived radiotracers to biological research. In this book several research groups will discuss their experience with ^{13}N, with the recurring theme that the scientific rewards justify the additional demands inherent in these experiments, particularly when novel or uniquely precise results are achieved. The complexity of ^{13}N research which requires cooperative effort between cyclotron physicists, radiochemists and experimental biologists as well as access to sophisticated hardware will invariably limit the use of N-13 for research. The technology for making ^{13}N, synthesizing simple ^{13}N compounds and separating ^{13}N metabolic products is all available, waiting to be applied to a host of important problems in nitrogen biochemistry.

EXPERIMENTAL METHODS IN ^{13}N RADIOBIOCHEMISTRY

Nuclear Reactions for Producing ^{13}N. Nitrogen-13 can be produced in useful yields from nuclear reactions on either carbon, nitrogen or oxygen; thus the reaction used by each laboratory will be dictated primarily by accelerator characteristics, conveneince of target design and the radiochemical nature of the ^{13}N that can be produced directly in the target.

The $^{12}C(d,n)^{13}N$, $^{13}C(p,n)^{13}N$, $^{12}C(p,\gamma)^{13}N$, $^{14}N(p,pn)^{13}N$, and $^{16}O(p,\alpha)^{13}N$ nuclear reactions have all been used for the production of labeled compounds of nitrogen. The third and fourth of these reactions have serious disadvantages that eliminate them from contention as important sources of radionuclidically and radiochemically pure tracer nitrogen. In order to compare other reactions, Austin et al. calculated yields expected from bombardment of thick targets of pure isotope (20). The (p,α) reaction on oxygen-containing materials has been used successfully in many laboratories; however, the product is always contaminated with traces of ^{18}F and the initial radiochemical product spectrum may contain a variety of oxides of nitrogen, demanding rigorous purification to yield the desired ^{13}N chemical (15, 21, 22).

Both the $^{13}C(p,n)^{13}N$ and the $^{12}C(d,n)^{13}N$ nuclear reaction yields are large and convenient solid targets of high purity ^{12}C and ^{13}C are available. A bombarding energy below 14.5 MeV is below the threshold for producing any radioactivity with half-life greater than one second other than ^{13}N. The (p,n) reaction on ^{13}C yields more activity than the (d,n) reaction on ^{12}C (20); however, both yield curie quantities of ^{13}N and thus the selection of a reaction is generally based on whether proton or deuteron beams are more conveniently available from a given accelerator.

Cyclotrons have been the accelerator of choice for production of ^{13}N because of their high beam currents. However, for short-lived isotopes that will not be stored, Van de Graaff accelerators have been used successfully in many laboratories to produce as much activity as is needed for immediate ^{13}N radiobiochemical tracer experiments. A group from New Zealand has reported their experience with a 2 MeV Van de Graaff (23). They achieved a yield of only 0.2 millicuries with a one microamp beam. The Australian AEC laboratory irradiated graphite with 2.5 MeV deuterons and produced $^{13}N_2$ at a continuous flow rate of 8 millicuries/min taking advantage of their 200 microamp beam current (24). The 5 microamp beam of 5.4 MeV deuterons at the University of Kentucky Van de Graaff has been used to irradiate CH_4 gas at one atmosphere pressure to give 21 millicuries of ^{13}N following a 25 min bombardment (25). All of the above mentioned laboratories have been restricted by their accelerators to low energy reactions using carbon targets. Nickles and co-workers (26) at the University of Wisconsin have used their 10 MeV tandem Van de Graaff with a 1.5 microamp beam to irradiate water and initiate the $^{16}O(p,n)^{13}N$ nuclear transformation at a rate of about 6 millicuries at saturation. Our experience has been that this level of radioactivity is adequate for most of the experiments described in this review.

Radioanalytical Chemistry of ^{13}N. Nitrogen can combine to make simple molecules in oxidation states from -3 to +7. Thus when nucleogenic ^{13}N reacts in a chemical target there is a potential for multiple primary chemical products. It is thus essential that anyone synthesizing ^{13}N compounds measure the radiochemical composition of the newly formed ^{13}N.

Their methodology involved preparation of a methanolic extract of blue-green algae, 50 microliters of which was spotted on a thin layer plate of cellulose (0.1 mm thick X 5 X 20 cm). The separation was improved if the lipid-soluble compounds in the methanolic extract were first displaced from the region of spotting by about 3 minutes of chromatography in the short axis of the plate using chloroform/methanol. The plate was then dried, sprayed with buffer, and electrophoresed at 3000 volts. In order to unambiguously identify all of the metabolic products, these researchers developed two buffering systems in which the mobility of the various metabolic products were quite different. The electrophoretic mobilities of nitrite and nitrate were nearly equal under the analytical conditions described above.

Because of the frequently expressed concern that the $T_{1/2}$ of ^{13}N is too short for interesting biochemistry, it is significant to note that the incubation time in these experiments was of the order of seconds to minutes. After longer times the spectrum of ^{13}N-labeled products became so complex that thin layer chromatography had to be done following electrophoresis. The incorporation of ^{13}N into metabolic products was sufficient to allow analytical chemistry of the product as late as 2 hours after the cyclotron irradiation. In fact, the $T_{1/2}$ of ^{13}N was nearly ideally suited to these interesting and significant biochemical experiments.

Table 1 lists each of the oxidation states of nitrogen and some simple N-containing molecules, along with associated pKa's and suggests the potential of ion exchange chromatography for separating these molecules. With the advent of high pressure solvent delivery systems for column chromatography and new packing materials of very uniform size, porosity

Varner was the first to describe many of the chemical tests that are available for measuring the radiochemical composition of ^{13}N systems (6); however, much of his analytical methodology is superceded by the rapid growth of high performance analytical techniques during the last decade.

Perhaps the simplest application of analytical chemistry to separation of ^{13}N products involved trapping of the gaseous end products of denitrification. Gersberg et al. (15) used molecular sieve at liquid nitrogen temperature to trap all of the gaseous products following incubation of $^{13}NO_3^-$ with denitrifying bacteria. Tiedje et al. (27, 28) have done similar experiments except they employed differential trapping of $^{12}N_2O$ and ^{13}N to arrive at a more complete picture of denitrification kinetics and mechanisms.

One of the most important applications of ^{13}N involves identification of metabolic products. Most of the biological experiments that are contemplated with ^{13}N involve incubating a simple ^{13}N-labeled molecule with a biological system, plant or animal, and allowing natural biochemical processes to proceed for some interval of time. The biology is then abruptly interupted and the labeled metabolic products are identified. In this way one can unambiguously identify the transformation of the ^{13}N label from a simple inorganic precursor to a complex organic product. By interupting biology at different times of incubation and measuring the radiochemical composition of the labeled products, one is able to unravel both the kinetics and metabolic pathways of nitrogen biochemistry.

The first approach to the rapid separation of complex mixtures of metabolic products of ^{13}N was the high voltage electrophoresis system pioneered by Wolk and colleagues at Michigan State University (18, 19).

and coating thickness, we have techniques that are rapid, even compared to the $T_{\frac{1}{2}}$ of ^{13}N, and are conducive to quantitation by simply flowing the column effluent across the face of a scintillation detector and applying simple corrections for radioactive decay and background. This technique can be done over a very wide range in peak size.

Gersberg, Krohn et al. (15) and Tilbury and co-workers (22) first used high performance ion exchange chromatography to separate simple inorganic forms of nitrogen-13. Most of the systems used currently for separation of ^{13}N compounds involve duplication with only minor variations of the system reported by Tilbury and demonstrated by the chromatogram of figure 1. Thayer and Huffaker (29) have systematically studied the effect of varying operating parameters on this chromatographic separation and concluded that when NO_3^- is the major component of a sample that is nearly pure water, 0.05 M phosphate buffer at pH 3.5 was the ideal mobile phase. Alternatively, assays involving biological extracts where protein is abundant require lower pH and ionic strength to separate the organic acids and proteins from nitrate and nitrite. They recommended 0.045 M phosphate at pH 2.9 and use of a guard column containing HC-pellosil to remove sample components that would otherwise irreversibly bind to the analytical column and reduce its useful lifetime.

The Synthesis of N-13 Labeled Radiochemicals. In this section we will discuss the chemical targets and synthetic procedures applicable to preparing simple ^{13}N-labeled compounds. More than one method will be given for many of the compounds, reflecting the fact that some groups have developed their production methods around oxygen and/or water targets while others have preferred carbon targets. The choice between these two target systems is

frequently dictated by engineering parameters specific for each accelerator. There are some fundamental differences in the chemical form of nucleogenic ^{13}N produced in these two environments that have lead us to prefer proton irradiated water targets for most of our radiochemical syntheses.

N-13 Nitrate. The product of recoiling N-13 in water has been the subject of some controversy in the literature (15, 21, 22, 26). Welch's original work (30) reported radiochemical yields of greater than 99.3 percent NO_3^- when the target was irradiated with doses in excess of 1 eV per molecule, but at lower irradiation doses he identified some $^{13}NH_3$. While one cannot be certain that calculations of dose deposited in chemical targets are exactly comparable from one laboratory to another, we found nearly quantitative yields of $^{13}NO_3^-$ at even lower absorbed radiation doses (15, 21). In contrast, Tilbury (22) and co-workers found high radiochemical yields of NH_3 at relatively low radiation doses. Gately and Nickles (26, 31) found that in their experience with a tandem Van de Graaff accelerator, the radiochemical yield was nearly all $^{13}NO_3^-$. There is no question about the radiochemical composition as measured in these different laboratories. The analytical chromatography is essentially identical in each laboratory and is the system described above and illustrated in figure 1. The different results are most probably attributable to the degree of aeration of the water and to the chemical materials to which the aqueous target solution is exposed during and immediately after irradiation, including the composition of any solder in the target, and to whether or not the target solution is static or recirculating. At the present time we do not know the primary radiochemical product spectrum

associated with a nitrogen atom recoiling through water. This problem is discussed further in another chapter in this volume (32).

For many kinds of research $^{13}NO_3^-$ prepared directly in water targets is of acceptable purity; however, it can be purified by addition of hydrogen peroxide to oxidize any residual nitrite to nitrate, followed by adjustment of the pH and rotary evaporation to distill off any $^{13}NH_3$. The rotary evaporation process also serves to increase the concentration of the $^{13}NO_3^-$. Any excess H_2O_2 can be destroyed by reaction with a small amount of catalase. This technique has been developed and applied most extensively by Meeks (33).

Lindner, et al. (34) developed a loop system for irradiation of water to produce ^{13}N. Their system differs from the one described above in that the loop contains a 40-percent shunt provided with a small mixed-bed ion exchange column for on-line separation and collection of $^{13}NH_4^+$, $^{13}NO_2^-$ and $^{13}NO_3^-$ produced in the water. At the conclusion of a run the target solution was circulated for a few minutes for complete collection of the products on the ion exchanger. About 85 percent of the radioactivity was displaced from the columns within two minutes by 50 ml of concentrated KI. The primary reaction product was 10-30 percent $^{13}NH_3$ and 90-70 percent oxidized ^{13}N under their irradiation conditions.

^{13}N-nitrite. The $^{13}NO_3^-$ prepared in any of the water targets described above can be quantitatively reduced to $^{13}NO_2^-$ using a cadmium column developed by McElfresh et al. (35). A small column of cadmium filings cleaned with water and treated with copper sulfate can be stored for months under 0.15 M NH_4Cl. The column is washed with 300 ml of distilled water prior to addition of 40-60 ml of irradiated target solution containing

$^{13}NO_3^-$. A peristaltic pump is used to pull the target solution through the column at approximately 80 ml/min and transfer it directly to a rotary evaporator preloaded with 0.15 ml of 0.1 M NaOH. The base raises the pH to ≥11 so that any $^{13}NH_3$ from the target solution or from excessive reduction in the cadmium column can be distilled out of the $^{13}NO_2^-$ preparation. Routinely $^{13}NO_2^-$ of radiochemical purity ≥97 percent can be obtained directly from elution of cadmium column and rotary evaporation results in a final solution of ≥99.2% $^{13}NO_3^-$.

These researchers also measured an average H_2O_2 concentration of 0.5mM in the target water following a 20 min irradiation with 20 MeV protons at 20 microamps beam current. The H_2O_2 was completely removed by a single pass through the reduction column. The nearly quantitative conversion of nitrate to nitrite by cadmium reduction column makes it preferable to the batch procedure using copper dust (36). The entire synthesis from collection of irradiated target water to delivery of sterilized $^{13}NO_2^-$ solution for biological experimentation takes less than 10 min and under our irradiation conditions results in useful yields of about 200 millicuries in 2 ml.

There have been no reports of the synthesis of $^{13}NO_3^-$ or $^{13}NO_2^-$ from proton or deuteron irradiation of carbon targets. Amorphous carbon targets require elaborate chemical conversions and/or collections to obtain ^{13}N in an oxidized form. Nuclear reactions on CO_2 give gaseous oxidized products such as $^{13}NO_2$, ^{13}NO and $^{13}N_2O$ but the yields are low and the radiochemical product spectrum is complex.

^{13}N-Ammonia. The $^{13}NH_3$ yields associated with the cadmium reduction column (35) suggest that this technique might be applicable to the synthesis of $^{13}NH_3$; however, the experimental parameters have not yet been optimized to establish this as a practical synthetic method. Direct production of $^{13}NH_3$ has been achieved in good yields with the $^{12}C(d,n)^{13}N$ reaction on methane gas (37), but the product invariably contains a small percentage of methylamine and some HCN (38). Similar products were found following irradiation of metal carbides (39).

Chemical reduction of labeled oxides of nitrogen formed in water targets provide a relatively simple synthesis for $^{13}NH_3$. The reduction reaction can be followed immediately by a micro-Keldahl distillation to purify and concentrate the $^{13}NH_3$. Two reductants have been described for rapid conversion of oxidized ^{13}N to $^{13}NH_3$. In our research program we have concentrated on the use of DeVarda's alloy (40); however, other laboratories have advocated the use of $TiCl_3$ converted in situ to $Ti(OH)_3$ (41). The chemistry and techniques of both of these reductions are described in the literature, and neither offers a clear-cut advantage. The DeVarda's alloy reaction is easy to set up with standard-tapered glassware but we have had difficulty achieving reliable yields because of the variable but always violent rate at which this reduction proceeds. The titanous chloride reaction is more difficult to set up but is more reproducible and well behaved once established (42).

^{13}N-Nitrogen. Several groups have prepared ^{13}N at relatively low specific activity by taking advantage of the high cross section for the reaction $^{13}N + N_2 = {}^{13}NN + N$. Welch (43) reported on the production of ^{13}NN by deuteron bombardment of CO_2 in which the ^{13}N atoms were proposed to react with trace N_2 impurity by this scheme. Clark and

Buckingham (12) described a method for providing saline solutions of $^{13}N_2$ in which target graphite was bombarded in the presence of CO_2 sweep gas. Various experimental parameters involved in production of ^{13}N-nitrogen were evaluated.

Biological studies requiring much higher specific activities of $^{13}N_2$ have lead to new production schemes. Austin et al. (20) used the $^{13}C(p,n)^{13}N$ reaction on isotopically enriched (97%) amorphous carbon targets which were carefully degassed to prepare high specific activity nitrogen. After irradiation the powder in the target was mixed with CuO and KNO_3 in an automated Dumas combustion apparatus and the resultant gas automatically Toeplered into a 1 ml vial. This procedure could be completed in high yield in 15 min and the nitrogen prepared in this way was useful for studies of N-fixation biochemical pathways described earlier in this review (17, 18, 19) and detailed by Meeks elsewhere in this volume (44).

^{13}N-Nitrogen can also be prepared from water targets irradiated with protons. Nickles et al. used $^{13}NO_3^-$ from a water target to prepare $^{13}NH_3$ steam distilled from DeVarda's alloy and found that the ammonia could be oxidized with hypobromite ion to yield very high specific activity ^{13}NN. If a lower specific activity gaseous product was required, a small amount of carrier NH_4Cl was added prior to treatment with OBr^-. Excess OBr^- was removed by an ion retardation resin. Activities of 40-50 millicuries in a few ml's of solution remain at 20 min EOB. Gas radiochromatography verified that all radioactivity was present as molecular nitrogen. The techniques of Austin (20) and of Nickles (45) produced ^{13}N nitrogen of very high specific activity although neither produced carrier-free nitrogen

which would be doubly labeled $^{13}N^{13}N$ and would not be useful for biological experimentation due to the uncertain chemical consequences of the decay of on ^{13}N atom on the chemical form of the second ^{13}N atom.

The most convenient method for production of ^{13}NN was first reported by Suzuki and Iwata (46). They investigated recoil reactions of ^{13}N in various aqueous solutions irradiated with protons and found that an aqueous solution of 0.1 M $NH\ OH_4$ resulted in the production of 100 millicuries of ^{13}NN with a radiochemical purity >99.9 percent. The specific activity of the ^{13}NN obtained at EOB was 80 millicuries per millimole for 1 microamp of beam. We have irradiated 0.1 M NH_4OH in the same recirculating aqueous target system described above and found a radiochemical yield of 75% ^{13}NN which was easily removed in the gas phase and trapped in a spirometer (40). Sixteen percent $^{13}NO_3^-$, 6% $^{13}NO_2^-$ and 3% $^{13}NH_4^+$ remained in the aqueous target solution. We have not measured the specific activity of the ^{13}NN produced; however, total radiation-induced decomposition of the NH_4OH would give a maximum of 3 millimoles of carrier N_2 and the specific activity would be approximately 200 millicuries per millimole. By contrast the specific activity of Austin's preparation was 20,000 millicuries per millimole of N_2; whereas, the calculated specific activity of carrier-free N-13 would be 4×10^{10} millicuries per millimole.

N-13-Nitrous Oxide. Nickles and Gately (47) have described two similar methods for synthesis of $^{13}N_2O$ by pyrolysis of NH_4NO_3 in sulfuric acid. Both methods began with $^{13}NO_3^-$ produced by proton bombardment of water, but differed in that in one the precursor was $^{13}NO_3^-$ and in the other $^{13}NH_4^+$ was synthesized first via DeVarda's alloy. When tracer $^{13}NO_3^-$ was used directly, it was reduced in volume to 1 ml by rotary

evaporation, then one millimole of NH_4NO_3 was added, followed by 25 ml of H_2SO_4. Nitrous oxide was evolved at 260°C in a pyrolysis apparatus swept by N_2. When $^{13}NH_3$ from DeVarda's alloy was used, it was mixed with 4 millimoles NH_4NO_3 and 25 ml of cold H_2SO_4. A temperature was selected where the rate of evolution of gas approximately balanced the rate of decay of the $^{13}N_2O$ collected. When either of these synthetic routes were used, the $^{13}N_2O$ was contaminated with 30±10% $^{13}N_2$ produced directly from a competing pyrolysis reaction, not by breakdown of $^{13}N_2O$. No ^{13}NO or $^{13}NO_2$ was observed in radio gaschromatograms of the product. When the pyrolysis at 220°C was done in the presence of a five-fold excess of $(NH_4)_2SO_4$, the contamination by $^{13}N_2$ was reduced to <2%. Total processing time averaged 18 minutes after EOB and resulted in delivery of 20 percent of the initial ^{13}N activity in 100 ml of carrier-added N_2O. The $^{13}N_2O$ produced in this manner is used for measurement of cerebral blood flow at the University of Wisconsin (47).

^{13}N-Nitrogen Dioxide. Parks et al. (13) bombarded a 15 atmosphere high-purity O_2 target with 15 MeV protons at 5-10 microamps and found that the ^{13}N atoms were incorporated into $^{13}N_2$ (65%), $^{13}N_2O$ (8%) and $^{13}NO_2$ (28%). The target was a stainless steel cylinder heated to 475°C and the target oxygen was circulated by a stainless steel diaphragm pump which permitted selectable flow rates. The effluent gases were separated by a trap at -45°C which retained NO_2 but not the more volatile N_2, N_2O and NO components. Approximately one millicurie of cryogenically recovered $^{13}NO_2$ was produced per microamp under steady state production conditions. The concentration of radiolytically produced NO_2 carrier was approximately 0.5 ppm in O_2.

^{13}N-Amino Acids. In addition to its use in studies of ammonia metabolism, $^{13}NH_3$ is useful as a synthetic precursor for preparation of labeled amino acids. Because amino acids are optically active and organic syntheses invariably result in racemic mixtures, enzymatic reactions provide the best synthetic route to ^{13}N-labeled amino acids. Enzymatic pathways are characterized by speed, a high degree of chemical selectivity under mild reaction conditions, and a product involving only the optical isomer of interest. It is not the purpose of this review to detail the many amino acid syntheses that have been described in the literature which was recently reviewed exhaustively by Straatmann (48).

Other Organic Molecules. Another interesting class of ^{13}N labeled organic molecules can be formed from $^{13}NO_2^-$ precursor. This work has been pioneered by Digenis and his co-workers (49,50) and is described in another chapter in this volume (51). They have synthesized several interesting N-nitroso and C-nitroso compounds with the goal of elucidating mechanisms of toxicity for these important families of compounds.

Direct recoil labeling of organic molecules with ^{13}N atoms has been investigated without success. Studies of ^{13}N reactions with liquid phase acetic acid, acid aldehyde and ethanol (52) as well as with larger biologic molecules like inulin and insulin (53) and α-chymotrypsin (54) lead only to radiolysis and labeled fragmentation products. It is unlikely that this approach to ^{13}N labeling will ever be successful for preparation of ^{13}N radiochemicals because of the high valence of nitrogen.

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