THE FATE OF NITRIC OXIDE IN THE MAMMALIAN SYSTEM USING N\textsuperscript{15} AS TRACER AND ISOTOPIC DILUENT

By: GUSTAVE FREEMAN, M.D.
MICHAEL ANBAR, Ph.D.

Prepared for:
STATE OF CALIFORNIA
AIR RESOURCES BOARD
1108 14th STREET
SACRAMENTO, CALIFORNIA 95814
Attention: CHIEF, RESEARCH SECTION

CONTRACT ARB 2-291

SRI Project LSH-2189

Approved by:
W. A. SKINNER, Executive Director
Life Sciences Division

C. J. COOK, Executive Director
Physical Sciences Division

LIBRARY
AIR RESOURCES BOARD
P. O. BOX 2815
SACRAMENTO, CA 95812
ABSTRACT

During the past year, the general fate of nitric oxide (NO) in the mammalian system and the metabolism of the NO-hemoglobin complex were investigated using the nonradioactive nitrogen isotope, $^{15}$N, as an analytical and epidemiological tool.

Using rats, we demonstrated that (1) NO binds preferentially to erythrocytes, (2) the biological half-life of hemoglobin-bound NO is about one-quarter of an hour, (3) the diffusion rate of nitric oxide across the red cell membrane is rapid, and (4) high concentrations of NO introduced into the circulating blood can be tolerated by the rat.

In studies of human blood, we demonstrated the presence of a low but measurable concentration of NO-hemoglobin complex that was independent of the smoking histories of the donors.

This report was submitted in fulfillment of SRI Project LSH-2189 and Contract ARB 2-291 by Stanford Research Institute under the sponsorship of the California Air Resources Board. Work was completed as of September 14, 1973.
CONTENTS

ABSTRACT ................................................. ii
ACKNOWLEDGMENTS ................................. iv
CONCLUSIONS ............................................. 1
RECOMMENDATIONS FOR FUTURE ACTION .......... 2
RESEARCH REPORT ....................................... 3
   Introduction ....................................... 3
   Preliminary Work ................................. 3
   Experimental Phase .............................. 3
   Discussion ....................................... 4
PRESENTATION ........................................... 6

APPENDICES
A   A PROPOSAL FOR RESEARCH ..................... 7
B   QUARTERLY REPORT NO. 1 ...................... 15
C   QUARTERLY REPORT NO. 2 ...................... 19
D   QUARTERLY REPORT NO. 3 ...................... 23
E   EXCERPT FROM REPORT TO CALIFORNIA DEPARTMENT OF PUBLIC HEALTH .......... 31
F   PAPER PRESENTED AT CONFERENCE ON STABLE ISOTOPES IN CHEMISTRY, BIOLOGY, AND MEDICINE .......... 39
ACKNOWLEDGMENTS

The authors wish to thank Nazzareno J. Furiosi, Kenneth D. Lunan, K. Jean Koskela, and Gilbert A. St. John, as well as Laszlo Juhos and Robert Dyer for their valuable assistance in the performance of this project.
CONCLUSIONS

Based on investigations of the past year, the following conclusions are presented:

- The nonradioactive nitrogen isotope, $^{15}$N, is a suitable tracer for the isotope dilution techniques using field ionization mass spectrometry to study the biology and metabolism of inhaled NO in the mammalian system.

- The kinetics data reported by Gibson and Roughton on the competitive and relative reactivities of NO and oxygen with hemoglobin in vitro are not directly comparable to the observed behavior of NO under in vivo conditions. The high rate of reactivity and firm binding in vitro that were reported by Roughton are counteracted by the mammalian system in vivo.

- Exposure to moderately low ambient concentrations of NO (about 5 ppm) did not result in a demonstrable concentration of NO-hemoglobin in the rat.

- When higher concentrations of NO are introduced into the circulating blood of the rodent, the primary target of reaction is the hemoglobin, with a high rate of diffusion of NO through the red cell membrane.

- The biological half-life of the NO-hemoglobin complex is about a quarter of an hour.

- In human blood, unlike that in rats, small but measurable concentrations (a few nanomoles per milliliter) of NO-hemoglobin are demonstrable.

- The clearance of NO and its metabolic byproducts in vivo, after direct exposure of blood to very large amounts of NO, proceeds without acute toxic effect.

---

*Q. H. Gibson and F.J.W. Roughton. J. Physiol. 136, 507 (1957).*
RECOMMENDATIONS FOR FUTURE ACTION

Details of recommended investigative work are presented in our application for renewal of Contract ARB 2-291 (Appendix A). In our future study, we propose to:

- Pursue the metabolic fate of NO after its dissociation from hemoglobin through tissues and body fluids to the point of its excretion from the mammalian body. The observed biological clearance rate of NO in vivo will be compared with in vitro measurements of NO-hemoglobin dissociation rates in a controlled, physiological milieu.

- Investigate the possible endogenous source(s) of NO found in human blood by reviewing known pathways of nitrogen metabolism.

- Relate quantitative clinical data on NO and its metabolites to exogenous concentrations of NO from the environment in selected populations under variously controlled conditions of exposure.
RESEARCH REPORT

Introduction

In accordance with the requirements of Contract No. ARB 2-291, our main objective during the year ending September 14, 1973, was to investigate the fate and metabolism of nitric oxide (NO) in the mammalian system using the isotopic dilution technique with $^{15}$N as a tracer. The general potential of this technique as an epidemiologic analytical tool was also studied. Confirmatory studies were conducted by electron spin resonance.

To evaluate the fate of NO in the mammalian system, the two following tasks were performed:

1. The amount of NO bound to hemoglobin of freshly drawn rat blood was determined under controlled conditions; the NO-reacted hemoglobin was reintroduced into the rats, and its rate of clearance from the blood was monitored. The extent of binding of NO to hemoglobin, the effect of the partial pressure of NO on the NO-hemoglobin complex, and the rates of NO-hemoglobin clearance and nitric oxide diffusion across blood cell membranes were studied.

2. The NO-hemoglobin concentrations in human blood samples from smokers and nonsmokers were determined under random conditions relative to exposure.

Preliminary Work

Preparatory to experimentation, the field ionization mass spectrometer instrumentation and methodology were adapted and refined to meet the particular requirements of this study; and contaminating and interfering substances were identified and eliminated. Quantitative methods were developed for working with gaseous NO, avoiding premature oxidation of NO to nitrogen dioxide (NO$_2$), and for the delivery of intact NO to the desired target or reaction sites without oxidation. Quarterly Report No. 1 (Appendix B) describes in detail the progress during this period.

Experimental Phase

Although some effort for controlling or eliminating contaminating ions from the field ionization mass spectrometric system was continued, experimental work was initiated in the second quarter. The instrumental detection limits for the field ionization mass spectrometer were determined to be less than 0.2% of hemoglobin associated with NO in blood.
We also studied a series of in vitro exposures of human blood to large quantities of NO (up to almost one-half of the equivalent of its oxygen-carrying capacity). Analysis revealed that only 5 to 25% of the NO binds to hemoglobin. These results were unrelated to either exposure time or partial oxygen tension in the blood before NO exposure. Detailed results are presented in Quarterly Report No. 2 (Appendix C).

During the third quarter, the utility of the $^{15}\text{N}$ isotope dilution method with the field ionization mass spectrometric analysis was fully realized. A series of analyses were conducted employing either in vitro or in vivo exposures of both human and animal blood samples to NO. Also during this period, a series of blood samples from smokers and nonsmokers were analyzed for NO-hemoglobin under separate auspices. Small quantities of NO (about 20 nanomoles/ml, representing 0.25% saturation of hemoglobin) were detected in these samples, and no correlation was evident with smoking histories of the subjects.

No discernible NO-hemoglobin existed in blood of rats that were either not exposed to NO or were exposed to about 5 ppm of NO for up to 45 minutes. The biological half-life of NO bound to hemoglobin was shown to be 15 to 20 minutes by field ionization mass spectrometry in repeated experiments. In a single experiment, a much longer half-life of about 100 minutes was measured by electron spin resonance. Details are reported in Quarterly Report No. 3 (Appendix D) and in a report to the California Department of Public Health (Appendix E).

During the last quarter, we refined and confirmed our measurements of the biological half-life of the NO-hemoglobin complex. These determinations supported our previous evidence that the biological half-life of NO-hemoglobin was $17 \pm 4$ minutes in the rat.

It became clear that efficient operation in this kind of study depends critically on the dedicated and uninterrupted operation of an available field ionization mass spectrometry unit. However, this analytical tool is subject to the familiar vulnerability of mass spectrometry to contamination, especially when it is operating close to the limits of its detection capability.

Discussion

The first year of investigations of the biological impact of environmental NO has provided new perspectives for pursuing the study of the metabolic fate of NO. This compound is an important component of smog, and it and its derivatives or metabolic byproducts are potential health hazards. The data from our experiments clearly show that, despite the high intrinsic reactivity of NO with hemoglobin demonstrated by Roughton in his early in vitro experiments, the in vivo environment of the mammalian system is capable of coping with very high concentrations of NO introduced into circulating blood. Even at concentrations estimated to saturate about $75\%$ of the oxygen-carrying capacity of the blood, which would be
expected to cause death by anoxia, efficient detoxifying mechanisms are activated to maintain a blood environment consistent with life. Equally surprising was the observation that the detoxifying mechanism does not lead primarily to the formation of nitrite, since such a reaction would form methemoglobin, which would also be lethal.

It is conceivable—although still purely speculative—that the reaction of NO with amines or amides forms nitrosamines, and thus their danger as potential carcinogens remains an environmental health issue.

The finding of small but constant amounts of NO-hemoglobin in human blood is intriguing. The quantitative similarity among smokers and non-smokers, despite gross differences in the NO content of their environments—especially for smokers—may be explained by the rapid and efficient clearance of NO, from the blood, by detoxification. (Haagen-Smit has demonstrated the presence of several hundreds of parts per million of NO in tobacco smoke).⁶ The relative absence of similar levels of NO-hemoglobin in rats is unexplained at this time. It may reflect a relative difference in basic metabolic rate—besides other differences such as enzyme systems—between man and the rat. Also, it should be remembered that, if the steady-state concentration of NO-hemoglobin in rats had been one-tenth of that in humans, it would have escaped detection.

The penetration, biological fate, and possible injury from ubiquitous ambient NO remains obscure, and its role as an environmental health hazard should be further investigated. The research proposed in our request for renewal of Contract ARB 2-291 is presented as the logical sequel to the investigations already performed.

---

PRESENTATION

The paper, "Determination of Nitric Oxide in Blood by Isotope Dilution Analysis," by M. Anbar, R. L. Dyer, and G. A. St. John, was presented at the Conference on Stable Isotopes in Chemistry, Biology, and Medicine at Argonne National Laboratory, Argonne, Illinois, in May 1973. The study described in this paper was supported by California Department of Public Health Contract 568 and by California Air Resources Board Contract ARB 2-291. The text is presented in Appendix F.
Appendix A

A PROPOSAL FOR RESEARCH
Introduction

This proposal is for renewal of Contract No. ARB 2-291 with the Air Resources Board, State of California, to start September 1, 1973.

During this past year, a substantial part of the two-year program outlined in the original application and initiated September 1, 1972, was developed. Relationships between nitric oxide (NO) and the circulating blood in rats and in man appear to exist; thus, the origin(s) and metabolic fate of pulmonary and circulating NO in both experimental animals and man should be investigated. The ultimate aim of this research is to define the impact of environmental (inhaled) NO on the health of a population at risk.

Objectives and Background

The objectives and background are described in the original contractual statement. In view of new observations resulting from our work and that of other laboratories during the year, the following modifications of Contract No. ARB 2-291 are in order:

1. Differential spectrophotometric analysis for NO-hemoglobin (NO-Hb) alluded to by other investigators has failed to maintain its promise and need not be pressed further at this time.

2. The potential application of the isotope dilution technique, using N\textsuperscript{15}O to detect circulating NO as a basis for epidemiologic investigation, now needs to be viewed on the basis of an appropriate baseline incorporating "unexposed" negative controls.

3. The reputed stability of NO-Hb, as reported by Roughton for in vitro systems, was not confirmed by our studies in vivo, in which dissociation has proven to be fairly rapid.

4. Exposure of rats to high ambient concentrations of NO did not result in detectable blood levels of NO-Hb, whereas direct infusion of NO into the arterial circulation induced positive levels readily measurable even by electron spin resonance (ESR), which has a sensitivity considerably below that of N\textsuperscript{15}O isotopic dilution analysis.

5. Tentative values for circulating NO in a small sample of volunteers, including smokers and nonsmokers, have been uniformly positive in the order of nanomoles/ml of whole blood.

Several of the questions posed in the original contract have already been answered, at least in part. Following a period required for (1) refinement of the field ionization mass spectrometry (FIMS) instrumentation and methodology, (2) control of oxidation of NO, and (3) identification and elimination of formaldehyde (an interfering molecular species of mass 30), we determined with various degrees of certainty that:
(1) NO binds in erythrocytes preferentially, rather than in plasma.

(2) There may be an endogenous source of NO.

(3) The half-life of NO in blood is fairly short.

(4) The diffusion rate of NO across the red cell membrane is quite rapid.

Other questions remain to be answered, and partially resolved issues need to be developed to achieve more definitive conclusions.

Statement of Work

During the ensuing year, we propose to investigate primarily (1) the metabolic fate of NO that may gain access to the circulating blood; (2) the possible endogenous source(s) of NO found in blood; and (3) the distribution of values for NO-Hb or for its metabolic products in human populations that are exposed to neither ambient NO nor to tobacco smoke and in those that are exposed to either NO alone or to NO and tobacco smoke.

Methods

Isotope dilution analysis and isotope tracer methods used in conjunction with precision mass spectrometry are sensitive and precise enough to obtain meaningful answers to the questions posed. The techniques to be used are briefly outlined in this section.

As necessary, rats will be exposed to NO-containing air in a chamber kept free of NO$_2$ either by a sufficiently rapid, continuously monitored flow of NO in air or by its rapid circulation through mercury (which reduces NO$_2$ to NO quantitatively). An alternative method will be the direct, slow injection of NO into the circulation, as described in Quarterly Report No. 3 (June 15, 1973). Blood and its components will be processed without exposure to air (see Quarterly Reports), and urine drawn from the bladder will be treated similarly for determination of NO content. Concentrations of NO, duration of exposure, and sampling times will be varied quantitatively to develop behavior patterns. Negative controls will be included.

In view of the fact that NO is slowly converted in the chamber to toxic concentrations of NO$_2$, chamber gas, when indicated, will be allowed to flow at a rate consistent with a complete exchange of chamber gas in about two minutes. Prior experience indicates that animals will die of pulmonary edema at the higher concentrations of NO because of its conversion to NO$_2$. At appropriately rapid flow rates and reduced concentrations, rats can survive indefinitely (up to two to three years), depending on the concentration.
The oxidation pattern of NO will be determined again by exposing rats to $N^{15}O$-containing air. Plasma and urine will be examined for their $N^{15}O_2^-$ and $N^{15}O_3^-$ content, using $N^{14}O_2^-$ and $N^{14}O_3^-$ as carriers. The nitrite will be converted to NO for analysis by precipitation of AgNO$_2$ and heating; the NO$_2$ formed will be reduced to NO by exposure to elemental mercury. The nitrate will be reduced to NO$_2^-$ by cadmium, followed by the AgNO$_2$ procedure.

Erythrocytes, suspended in a physiological solution at 37°C, will be exposed to various concentrations of NO in air. After equilibration, the cells will be separated, washed, and hemolyzed, and the NO content will be determined by isotope dilution. The rate of decrease of NO concentration in the exposed cells after exposure to NO-free air will be followed by isotope dilution and compared with the change in concentration of NO bound in whole, hemolyzed blood. As indicated, these experiments will be conducted under different O$_2$ tensions.

Whole autologous blood that had been exposed to NO is being injected intravenously into rats. Blood samples are taken, and their NO content is determined as a function of time. These experiments will determine the rate of oxidation of NO in vivo.

Various tissues of animals exposed to inhaled NO will be separated under NO-free conditions (in a glove box) and analyzed for NO by dilution analysis.

The determination of the biological half-life of NO is rather complicated because of the potential uptake of NO by tissues, in which there may be different residence times, and because of the potentially variable alternative routes of chemical reactions with NO in the biological systems. A direct approach would be the determination, by isotope dilution, of NO concentrations in different tissues of the rat after its continuous exposure to NO-containing air for a specified standard period and subsequent residence in an NO-free atmosphere.

Blood of human smokers and nonsmokers will continue to be sampled, because NO exists in tobacco smoke in hundreds of parts per million (ppm). Specimens of blood will be drawn from volunteers selected to include a quantitatively diverse population with respect to the number of pack-years of cigarettes smoked. The methodology for these experiments will be the same as that used in a preliminary study. Additional screening will be made of smokers and nonsmokers with varied histories of smoking habits and exposure to oxides of nitrogen from local sources as well as from the ambient air of the larger community. "Negative controls" will be sought as a basis for investigating the possibility of endogenously generated NO.

Animals will be examined grossly and microscopically postmortem to determine whether injury to the lungs or other organs had taken place with reference to lesions already known to occur following exposure to NO$_2$.
All data will be analyzed statistically as indicated.

To supplement the methods described originally and outlined above (with modifications), the following definitive experiments will be conducted to:

1. Identify nonsmoking residents of rural (low NO) areas to serve as a "negative" control population for epidemiologic purposes. This will require the development of a portable sampling technique.

2. As indicated, repeat the in vitro and animal experiments already conducted to confirm and improve methods and techniques.

3. Determine the partition of NO among hemoglobin, erythrocyte ghosts, and plasma in a system in which potential oxidation of NO to NO$_2$ can be controlled.

4. Examine urine and tissues (liver, bone marrow, muscle, lung, kidney, and brain) for NO by isotope (N$^{15}$O) tracer following exposure of rats to several concentrations of NO in air.

5. Look for nitrate and nitrite in urine of similarly exposed animals. Also, N$_2$, NH$_3$, NH$_2$OH, and N$_2$O will be sought after exposing erythrocytes to N$^{15}$O.

6. Expose rats and monkeys to N$^{15}$O and process their blood, urine, and tissues (as above) to isolate residual N$^{15}$ in the different possible products investigated under Item 5.

7. Initiate examination of the degree to which and the mechanism by which adaptation may take place in individuals chronically exposed to NO. This may be studied effectively qualitatively and quantitatively in animals first, in terms of acute versus long-range effects. Such studies, however, could not be completed within the year.

8. Determine changes in maximum oxygen-binding capacity of hemoglobin in vitro resulting from prior treatment with NO. After oxygen has been removed from whole blood, it will be exposed to NO to allow substantial reaction with hemoglobin. NO will then be evacuated under reduced pressure, after which the oxygen-binding capacity may reflect either persistent binding of NO or alteration of the oxygen-binding sites on hemoglobin.
Facilities

Approximately 3,000 ft\(^2\) of laboratory space are divided into (1) exposure-chamber facility, (2) physiology laboratory, (3) pathology laboratory, (4) electron microscopy and cell biology laboratory, (5) biochemistry laboratory, and (6) tissue culture and virology laboratory. A mass spectrometer, scanning electron microscope, and a variety of other high precision instruments are available within the Institute for various kinds of chemistry, physics, engineering, electronics, and computerization. Offices are available for all professional personnel.

Four 80-cu-ft exposure chambers, each capable of holding 60 full-grown rats, are available. Each is equipped with the necessary control and safety devices. With an air-turnover rate of 40 cu ft per minute, the gas concentrations in each are maintained within 10% of the nominal value without resorting to feedback control techniques. An alarm system signals equipment failures, and necessary repairs can be made without materially upsetting exposure schedules.

The concentration of NO in each chamber can be monitored for 15 minutes once every three hours, around the clock. The concentration prevailing at the end of each monitoring period is recorded on a multipoint, strip chart recorder. The monitoring cycle, normally surveying ten chambers, can be stopped at will, and detailed studies of a single chamber can be made. In addition, each chamber will be routinely checked for NO concentrations by an alternate method twice a week, as a check on the monitor. A Bendix 8101-B analyzer is currently in use for NO.

A physiology unit contains whole-body plethysmographs for small animals and monkeys and a four-channel Grass electronic recorder for pressure-volume studies. Chemistry facilities include a Hitachi-Perkin-Elmer Model 139, uv-VIS spectrophotometer, and other essential equipment to carry out determinations of chamber concentrations, abnormal hemoglobins, pH, \(P_{O_2}\) and \(P_{CO_2}\) (Radiometer Copenhagen), and a CO diffusion analyzer system.

Complete laboratory facilities are available for biochemical research involving enzyme preparation and assay, metabolism studies, isolation and identification of natural products, and synthetic studies. The necessary instruments for paper, column, thin-layer, and gas chromatography, electrophoresis, ir, uv, and visible spectrophotometry, fluorometry, and nmr and mass spectroscopy are available. These and other instruments include the Spinco L2-50 high-speed centrifuge with fixed-angle and swinging bucket rotors, Sorvall RC-2B medium-speed centrifuge, Cary 14 spectrophotometer, Varian A-60 and HA-100 nmr spectrometers with time-averaging computers, and Consolidated Electrodynamics Corp. mass spectrometers, including models 21-110B High Resolution and 21-103C (SRI Modified) Isotope Ratio, Nuclear-Chicago Model 720 Refrigerated Liquid Scintillation Counter, Packard Tri-Carb Sample Oxidizer Model 305, and a Hewlett-Packard F and M Research Gas Chromatograph Model 5750. Also, the Life Sciences Division has a fully equipped isotope synthesis laboratory and the services of a resident organic chemist specializing in radiochemistry.
The mass spectrometer to be used for the determination of the N\(^{14}\)/N\(^{15}\) isotope ratio is an SRI-built, special-purpose instrument. This mass spectrometer comprises SRI's proprietary highly efficient field ionization source, a 22.5° magnetic analyzer (resolution > 100), and a triple collector system. The field ionization source induces minimum molecular fragmentation during the ionization process, thus eliminating the necessity for corrections for the formation of secondary products such as N\(_2\), N\(_3\)O, and NO\(_2\). The triple collector system allows the analysis of isotope ratios between 10\(^{-3}\) and 10\(^3\), with a precision better than 0.1%.

**Life Sciences Division and Mass Spectrometry Development Center**

The proposed research will be conducted in both the Life Sciences Division and the Mass Spectrometry Development Center at SRI. The Life Sciences Division encompasses more than 60 research programs using both multidisciplinary and unidisciplinary approaches. The staff totals about 215, including 48 with Doctorates, 25 with Master's degrees, and 76 with Bachelor's degrees. Disciplines represented in the scientific staff include pharmacology, toxicology, experimental psychology, general physiology, neurophysiology, food sciences and nutrition, plant sciences, basic biology, virology, immunology, microbiology, biochemistry, and organic chemistry.

The majority of the projects in Life Sciences are contracted with governmental agencies such as the National Institutes of Health, the National Aeronautics and Space Administration, the U.S. Department of Agriculture, and various branches of the Department of Defense, including the Office of Naval Research, Holloman Air Force Base, and the Atomic Energy Commission. Industrial support comes from numerous commercial companies.

The Mass Spectrometry Development Center conducts an extensive program to develop novel types of mass spectrometers and new methodologies using mass spectrometry. The Center has developed numerous mass spectrometers, including field ionization mass spectrometers applied to isotope dilution analysis and to wide-range "fingerprint" mass analysis; a high temperature sustained discharge mass spectrometer designed for high precision isotope ratio mass spectrometry of calcium ions; a surface ionization negative ion mass spectrometer for measuring halogen isotopes; and a plasma ionization negative ion mass spectrometer for the assay of \(^{14}\)C at very low concentrations.

Seven members of the Center's staff hold Doctorates in physics, and four have Doctorates in chemistry; Master of Science degrees are held by five members in chemistry and by four in physics. The staff also includes four technicians.

The range of applications of mass spectrometry actively pursued by the Center includes drug detection, identification of oil slacks, determination of subpicogram quantities of metabolites, epidemiologic survey of nitric oxide in humans, diagnosis of osteoporosis, detection of explosives, determination of the viability of tissue cultures, radiocarbon dating, and geochemical studies using the \(^{48}\)Ca/\(^{40}\)Ca isotope effect.
REFERENCES


Appendix B

QUARTERLY REPORT NO. 1
Evidence that NO may enter the circulating hemoglobin through the respiratory tract is conflicting. The application of a more sensitive technique using N\textsuperscript{15}O and mass spectrometry is being explored for the purpose of determining the fate of NO in the mammalian system and its possible application to epidemiology. During the initial quarter of Contract No. ARB 2-291 with the Air Resources Board of the State of California, the following preparatory efforts have been made.

**Isotope Ratio Measurement**

1. After setting up a vacuum manifold, steps were taken to install a pressure-measuring instrument of suitable sensitivity. Several devices have been tried, including an all-glass Bourdon gauge. Currently, NO readings are being made with a Wallace & Tiernan pressure gauge that is ideal for the purpose. Some difficulty in mounting the gauge resulted in breakage of the glass-to-metal seal between the gauge and manifold. Therefore, a much more rigid mount was installed. Since then (about two months ago), the instrument has been in continual operation. A mercury diffusion pump was added to the vacuum system, enabling the production of cleaner samples.

2. Approximately three weeks were required to calibrate two separate sample volumes by means of expanding ammonia (NH\textsubscript{3}) and condensing it into a weak boric acid solution. Titration of the ammonium borate with HCl yielded the amount of NH\textsubscript{3} available at that pressure reading. From PV = nRT, the volume of NH\textsubscript{3} was determined. The average value of over 40 titrations was used as the sample volume. More recently, a sample volume was determined by filling the volume with mercury and weighing the mercury. This method saved considerable time over the titration method and gave equally accurate volume determinations.
3. The mass analysis of NO is performed on a magnetic spectrometer with a field ionization source at an ionizing potential of 3.5 Kv and an ion energy of 7 Kv. Detection is by two ion electron multipliers with a gain of $10^6$. The multipliers are used in the pulse mode, so that each impinging ion liberates an electron pulse in the detector output. The pulses are counted on two high-frequency universal counters, after amplification and clipping. The total system pressure at the source was $4 \times 10^{-8}$ Torr and the sample pressure was $1-2 \times 10^{-8}$ Torr. It was found that, unlike ionization induced under electron bombardment, NO, ionized by field ionization, does not form $N_2$, $N_2O$, or NO$_2$. This allows quantitative assessment of the $N^{15}O/N^{14}O$ ratio with high precision and without interference by isotope fractionation or from $N_2^{15,15}$ formation.

4. A series of human whole blood samples were exposed to NO and are being scanned on our field ionization mass spectrometer. The $N^{15}O/N^{14}O$ ratios are being determined. Currently, we are testing compounds that will bind to the hemoglobin molecule (i.e., $O_2$, CO) and that can be used to replace whatever may be bound to hemoglobin prior to exposure to $N^{15}O$, other than NO; such compounds must be readily replaceable with NO. Such a compound would allow more efficient use of $N^{15}O$ as well as increase the sensitivity of the $N^{15}O/N^{14}O$ determinations. Recent tests with KCN and preliminary results seem to indicate that CN$^-$ is likely to fulfill the requirements. Further experiments are planned to extend the apparent validity of these results to our satisfaction. In that event, CN$^-$ will be used as standard treatment during the handling of blood samples.

5. A series of blood samples treated with CN$^-$ are currently undergoing mass spectrometric analysis. Preliminary results indicate that cyanide binding to hemoglobin allows the use of smaller amounts of $N^{15}O$ carrier thus increasing the isotope ratio and the sensitivity of the method. Blood samples from a common pool will be tested to determine if the $N^{15}O/N^{14}O$ ratios obtained are consistent.

6. An interesting but unanticipated mass 30 background feature, other than $N^{14}O$, has introduced some difficulties in determining
$^{14}\text{N}/^{15}\text{N}$ ratios. Experiments are planned to identify the source of this mass 30, which could be ethane. The results suggest that blood may contain trace amounts of ethane.

7. The apparatus for handling the vacuum and preparing the sample is being used on a routine basis, and only slight changes in methodology may be expected.

Our results indicate that the $^{15}\text{O}$ supplied by Isomet, Inc. was composed of 96.7% $^{15}\text{O}$ and 3.3% $^{14}\text{O}$. An increase in the $^{14}\text{O}$ was found in gas collected over $^{15}\text{O}$ equilibrated blood. From the initial 3.3%, the $^{14}\text{O}$ rose to $4.4 \pm 10\%$ in the several blood samples tested.

NO, in the presence of $\text{H}_2\text{O}$ and $\text{O}_2$, produced a level of $\text{NO}_2$ that was detrimental to the field ionization source. An experiment using a pellet of ascarite adsorbant to collect $\text{NO}_2$ resulted in a complete loss of all the nitrogen oxides in the sample. Therefore, the method now being used is to store NO over a droplet of mercury, which reconverts $\text{NO}_2$ to NO.

Methodology will be developed further during the next quarter.
Appendix C

QUARTERLY REPORT NO. 2
During the first quarter, the isotope dilution method was being developed as a useful means of determining whether inhaled nitric oxide binds to circulating hemoglobin. A series of 19 human hemoglobin-containing samples was prepared variously in vitro for study. Isotope ratio (N\textsuperscript{15}O/N\textsuperscript{14}O) measurements proved to be feasible when practiced essentially according to the procedure described in Quarterly Report No. 1 (December 15, 1972).

Contaminating Mass 30

1. As reported earlier, ethane (CH\textsubscript{3}-CH\textsubscript{3}) was suspected of being a compound interfering with mass 30/31 ratio determinations. To investigate this possibility, a one-to-one mixture of N\textsuperscript{14}O (mass 30) and D\textsubscript{6}-ethane (mass 36) was prepared and measured on the mass spectrometer. At the same ionizing voltage, a strong ion beam was registered for mass 30 and a very weak one for mass 36 (greater than a thousandfold difference). On raising the ionization voltage, only a slight increase in mass 36 resulted, whereas a much greater increase appeared for mass 30. Thus, ethane could not have been responsible for the unknown mass 30, because 30/31 ratios were determined normally at a voltage at which any contribution from ethane would be less than 0.01% of the mass 30 value.

2. Formaldehyde, H\textsubscript{2}CO, also with a mass of 30, was not initially considered to be a reasonable candidate for contamination of blood because of its tendency to react with amines. However, to test this by excluding formaldehyde, a sulfonic acid amine, 2-amino-1-naphthylene sulfonic acid (Tobias' acid), was added to the gas sampling flask along with the elemental mercury droplet already in use. This resulted in reasonably consistent ratios that remained constant during the time required for the sample to be introduced into the mass spectrometer. In contrast, in the absence of such treatment, the ratio of 30/31 increased within 15 minutes by a factor up to four, presumably because of the presence of formaldehyde, which diffuses over the metal surfaces of the inlet system much more slowly than NO. It was concluded, therefore, that formaldehyde was most likely the unknown impurity interfering in the mass 30 determinations.

3. With the above problem seemingly resolved, the purity of the N\textsuperscript{15}O supplied by Isomet, Inc., was redetermined. It proved to contain 99.81% N\textsuperscript{15}O and 0.19 ± 0.01% N\textsuperscript{14}O, a refinement of the values reported last quarter.

N\textsuperscript{14}O Recovery Studies

1. Currently, it appears that the mass spectrometer is able to detect N\textsuperscript{14}O/N\textsuperscript{15}O ratios to within ±0.01%. Thus, the smallest significantly detectable increase of the ratio would be about 0.02%. This would correspond to about 10 nanomoles of N\textsuperscript{14}O/ml of blood (when equilibrated with about 55 μmoles of N\textsuperscript{15}O), indicating that less than 0.2% of the hemoglobin in normal blood could carry NO without detection. The amount
<table>
<thead>
<tr>
<th>I.D.</th>
<th>Sample (Treatment)</th>
<th>$^{14}$O Added ($\mu$moles)</th>
<th>$^{15}$O Added* ($\mu$moles)</th>
<th>$^{14}$O Recovered ($\mu$moles)</th>
<th>Ratio of Mass* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD 5</td>
<td>Whole blood</td>
<td>3.483</td>
<td>54.897</td>
<td>2.515</td>
<td>14.973</td>
</tr>
<tr>
<td>BD 6</td>
<td>Whole blood (15 min air)</td>
<td>3.483</td>
<td>54.805</td>
<td>1.808</td>
<td>5.192</td>
</tr>
<tr>
<td>SJ 8</td>
<td>Whole blood (KCN-treated)</td>
<td>3.391</td>
<td>54.805</td>
<td>0.893</td>
<td>2.634</td>
</tr>
<tr>
<td>SJ 6</td>
<td>Whole blood (KCN-treated)</td>
<td>3.483</td>
<td>54.897</td>
<td>0.2986</td>
<td>5.989</td>
</tr>
<tr>
<td>SJ 3</td>
<td>Whole blood (KCN-treated)</td>
<td>3.483</td>
<td>54.605</td>
<td>0.3590</td>
<td>10.307</td>
</tr>
<tr>
<td>SJ 4</td>
<td>Whole blood (KCN-treated--100 sec air)</td>
<td>3.483</td>
<td>54.897</td>
<td>0.0752</td>
<td>2.159</td>
</tr>
<tr>
<td>SJ 5</td>
<td>Whole blood (KCN-treated--5.25 min air)</td>
<td>3.483</td>
<td>54.897</td>
<td>0.1856</td>
<td>5.329</td>
</tr>
<tr>
<td>SJ 7</td>
<td>Whole blood (KCN-treated--15 min air)</td>
<td>3.483</td>
<td>54.897</td>
<td>0.1043</td>
<td>2.994</td>
</tr>
<tr>
<td>SJ 10</td>
<td>Whole blood (CO-treated--30 min)</td>
<td>3.391</td>
<td>54.714</td>
<td>0.1362</td>
<td>4.018</td>
</tr>
<tr>
<td>SJ 11</td>
<td>Hb solution (CO-treated--30 min)</td>
<td>3.391</td>
<td>54.805</td>
<td>0.2521</td>
<td>7.434</td>
</tr>
<tr>
<td>BD 1</td>
<td>Hb solution</td>
<td>3.483</td>
<td>54.805</td>
<td>0.4660</td>
<td>13.370</td>
</tr>
<tr>
<td>BD 2</td>
<td>Hb solution (15 min air)</td>
<td>3.483</td>
<td>54.897</td>
<td>0.2510</td>
<td>7.219</td>
</tr>
<tr>
<td>BD 9</td>
<td>Hb solution (15 min air)*</td>
<td>3.552</td>
<td>54.897</td>
<td>0.3124</td>
<td>8.794</td>
</tr>
<tr>
<td>BD 10</td>
<td>Hb solution (15 min air)*</td>
<td>3.552</td>
<td>54.897</td>
<td>0.2975</td>
<td>8.377</td>
</tr>
<tr>
<td>BD 11</td>
<td>Hb solution (15 min air)*</td>
<td>3.552</td>
<td>54.714</td>
<td>0.5661</td>
<td>15.941</td>
</tr>
<tr>
<td>BD 3</td>
<td>Hb and ghosts</td>
<td>3.483</td>
<td>54.805</td>
<td>0.2450</td>
<td>7.049</td>
</tr>
<tr>
<td>BD 4</td>
<td>Hb and ghosts (15 min air)</td>
<td>3.391</td>
<td>54.805</td>
<td>0.8769</td>
<td>25.859</td>
</tr>
<tr>
<td>BD 7</td>
<td>Hb and ghosts (15 min air)</td>
<td>3.483</td>
<td>54.887</td>
<td>0.2745</td>
<td>7.881</td>
</tr>
<tr>
<td>BD 8</td>
<td>Hb and ghosts (15 min air)</td>
<td>3.483</td>
<td>54.805</td>
<td>0.4165</td>
<td>11.959</td>
</tr>
</tbody>
</table>

* Corrected for 0.18 ± 0.014 $^{14}$O in $^{15}$O (prepared by Isomet, Inc.).
† of $^{14}$O ions per $10^5$ or $10^6$ $^{15}$O ions.
‡ Hb from pool of 4 cc blood gives a single $^{14}$O exposure.
of N\textsuperscript{14}O on the hemoglobin of the whole blood samples tested thus far, therefore, must be less than than this because 1.0-ml samples of normal whole blood exposed to N\textsuperscript{15}O did not give rise to ratios significantly different from those of the background material supplied by Isomet. The previously observed apparent increase of 3.3\% to 4.4 \pm 10\% of N\textsuperscript{14}O gas collected over N\textsuperscript{15}O-equilibrated blood was probably due to "formaldehyde."

2. A series of experiments to determine by isotopic dilution analysis whether N\textsuperscript{14}O could be recovered from N\textsuperscript{14}O-exposed blood are summarized in Table 1.

3. Whole liquid blood (1.0 ml), its equivalent in aqueous hemoglobin solution, or hemoglobin plus erythrocyte membranes were exposed under vacuum to a known amount of about 3.5 \mu mole of N\textsuperscript{14}O (less than half of full saturation) and then equilibrated with about 55 \mu mole of N\textsuperscript{15}O. Some samples were exposed to air prior to equilibration with N\textsuperscript{15}O to determine whether the bound N\textsuperscript{14}O would be oxidized (BD 1-8 and SJ 3-8, Table 1). It appears that air may have oxidized some of the bound N\textsuperscript{14}O but at a relatively slow rate. Also, it was surprising that recovery of the N\textsuperscript{14}O, presumed to have been bound, was in the range of 5-25\%. Therefore, the basis for the unexpectedly low recoveries is being investigated.

4. To test one possibility, that O\textsubscript{2} may oxidize NO before it can bind to hemoglobin, samples SJ 10 and 11 were first exposed thoroughly to carbon monoxide (CO) and then flushed with helium to remove the O\textsubscript{2}. Recovery from both samples remained poor. A second possibility, that the N\textsuperscript{14}O-N\textsuperscript{15}O equilibration process of the dilution technique may require considerably more time than the expected 2-10 minutes, is still under investigation. However, SJ 10 and 11 were also equilibrated for 30 minutes at 37\degree C, but the recovery rate was not enhanced. A third possibility, denaturation of part of the hemoglobin due to frequent freezing and thawing while handling samples for degassing and for transfer of the NO gas, is a serious contender. Samples prepared without freezing will soon be analyzed. An additional factor to be introduced will be improved control of pH during preparation and testing of specimens.
Appendix D

QUARTERLY REPORT NO. 3
During the third quarter, the objective of determining whether the isotopic dilution method using field ionization mass spectrometry (FIMS) could detect nitric oxide (NO) in blood has been adequately achieved. A series of determinations has been made on both animal and human blood samples. Parallel studies were carried out on common samples of blood in selected cases using the less sensitive method of electron spin resonance (ESR).

Background

In 1962 (K. Sancier, G. Freeman, and J. Mills, Science 137, 754-755), we were unable to detect significant amounts of NOHb by ESR in the blood of rats exposed either to 10 ppm NO continuously for nine days or to very high concentrations of NO that became lethal by partially oxidizing to NO₂ in the chamber.

During his recent visit, Dr. T. Nakajima of Osaka advised us that workers in his laboratory applied our ESR method to mice exposed to 4 to 7 ppm of NO for 10 to 50 minutes in a similar flow-through chamber equipped with a soda-lime column for removal of nitrogen dioxide (NO₂). Using identical ESR equipment, they increased the sensitivity for detection twofold by carrying out measurements at -100°C, and they believe they can detect specific levels of NOHb in the blood of NO-exposed mice.

Under separate auspices, a series of human blood specimens from smokers and nonsmokers was analyzed at SRI by the isotope dilution technique using FIMS, and the results were reported to Dr. John Goldsmith of the Department of Health, State of California. The ranges of these values was from 17.9 to 24.4 nanomoles/cc of whole blood. No correlations between these values and the smoking histories of the donors were evident. The relative uniformity of the results suggest that either the ratios of masses 30/31 are not specific for NO (which is unlikely) or that an endogenous source of NO exists independently of exogenous contribution.

Experimental

To investigate the evidence from Japan, we conducted the following experiments using ESR and FIMS, as indicated below.

In Vitro Exposure*

1) About 2 ml of rat whole blood (citrated) was exposed to 50 ml of NO gas bubbled through the blood specimens at 3 ml/min in the absence of air.

* Samples from (1), (2), and (3) were studied by ESR only.
(2) A similarly prepared aliquot of blood was centrifuged to separate the plasma and the cells.

(3) Saline containing citrate as a control was exposed to NO similarly to (1) and (2).

(4) Human blood was diluted with an equivalent amount of buffered 0.1 M PO₄ (pH 7.2) and then bubbled overnight with N₂. This was followed by three hours of bubbling with NO gas and subsequently with N₂ again for 30 minutes. (On this sample, only mass spectrometric study was carried out.)

**In Vitro and In Vivo Exposure**

(1) Whole rat blood was treated as described in In Vitro Exposure (1), with the difference that NO exposure of the whole blood was for six minutes. One ml of the treated blood was processed immediately for FIMS, as described below, and another 1-ml aliquot was reinjected into an anesthetized rat. Ten, 74, and 300 minutes after injection, blood samples were taken from the rat and studied by mass spectrometry only.

(2) The red blood cells of an exsanguinated rat were separated by centrifugation, washed once in physiological NaCl solution, and diluted to the original volume with 0.1 M PO₄ buffer, pH 7.2. Nitrogen was then bubbled through the suspension for 10 minutes to remove oxygen. Next, N¹⁴O was bubbled through for 10 and 30 minutes in two aliquots, respectively. Finally, N₂ was again bubbled for 10 minutes to remove unbound N¹⁴O. The resulting red cell suspensions were reinjected into two anesthetized rats, and simultaneous processing for FIMS was started on an aliquot of the treated blood. Five and ten minutes after injection of the NO-treated red cells, blood samples were taken from the rats and analyzed by mass spectrometry.

**In Vivo Exposure**

(1) Under anesthesia, 0.5 ml and 5 ml NO gas samples were slowly injected into the exposed abdominal aortas of two rats (~500 g body weight each). An arterial blood sample was drawn five minutes later from each rat, followed by additional sampling at 30, 60, and 180 minutes from the rat that had received the larger dose. Samples were collected into heparinized tubes without exposure to air and were stored until they were analyzed. All blood samples were analyzed by ESR 10 minutes after collection of the last sample (180 minutes). The 5- and 30-minute samples were analyzed again 24 hours later by the same method.
(It was estimated that 5 ml of injected NO gas should result in about 77% saturation of the total hemoglobin mass, assuming a blood volume of 29 ml and an oxygen-carrying capacity of 23 ml of oxygen per 100 ml of whole blood.)

Samples were analyzed also by FIMS, as described below.

(2) Mice and weanling rats were exposed in a dynamic flow-through chamber to 4 to 5 ppm NO for 45 minutes. The air containing NO was first led through a soda-lime column, following the Japanese procedure. Flow rates in the chamber allowed a complete exchange of air every two minutes. The concentration of NO was determined continuously by a Bendix Model 8101 BX nitrogen oxide analyzer and recorded (1 mV recorder). At the end of 45 minutes of exposure, blood was taken from the rats and mice for both the ESR and FIMS study.

(3) Blood samples were obtained from two volunteers, one of whom had been smoking a pack of cigarettes per day and the other, three to four packs per day for a number of years. These samples were studied by ESR only.

Sample Preparation

Although no special sample preparation was necessary for the ESR studies, in which the whole blood, plasma, or red cell samples were introduced directly from a glass syringe into the ESR capillary, the following special sample handling technique was employed for the FIMS studies by isotope dilution.

One ml of the NO-exposed samples was injected immediately through a rubber septum covering a side arm of a flask previously filled with one atmosphere of nitrogen (N₂) and cooled to 0°C. As necessary, the sample was stored under these conditions until the vacuum line was free to receive the next sample. On the vacuum line, the N₂ was pumped off, and the sample was flushed six times with N₂ to remove oxygen while at 0°C. The sample was then quick-frozen in liquid N₂, and a known amount of N¹⁵O was added to the sample. While at liquid N₂ temperature, 1 ml of 10% sodium dodecyl sulfate (SDS) was injected through the rubber septum to denature the hemoglobin in order to release any bound N¹⁴O. The sample was then thawed and shaken to complete the denaturation and frozen again in a dry ice-acetone mixture. The isotopic mixture of N¹⁵O and liberated N¹⁴O gases was then condensed with liquid N₂ into another glass flask containing chemicals that preserved the gas sample until the mass 30/31 ratios could be determined.
Results

Tables 1 and 2 summarize the results of the FIMS (isotope dilution) and ESR analyses, respectively. In addition to the quantitative values derived by ESR, the analyses reveal a difference in the characteristics of the ESR signals between blood samples exposed in vitro and in vivo. Figure 1 shows this difference, which probably relates to structural aspects of the hemoglobin molecules that bind nitric oxide. (Further clarification of molecular structure is beyond the scope of the immediate investigation.)

Recapitulation

When viewed simultaneously by two independent methods, the evidence for the presence of trace amounts of NO bound in human blood is reasonably consistent. The results may be summarized tentatively as follows:

(1) The current evidence favors the presence of "trace" amounts of NO in the blood of all the individuals tested, and its presence can be detected by FIMS after isotope dilution.

(2) NO is bound, essentially, in the red cells rather than in the plasma, as observed by ESR in blood treated in vitro.

(3) Human blood specimens, including one from a very heavy smoker, had less than detectable levels of NO in blood by ESR. (The technique may be about two orders of magnitude less sensitive than mass spectrometry.)

(4) The half-life of NO in vivo, following an extraordinarily high dose administered intraarterially, was about 100 minutes as determined by ESR and considerably less by mass spectrometry.

(5) Although Gibson and Roughton reported firm binding of NO by hemoglobin in vitro (J. Physiol. 136, 507-526, 1957), the reactivity in rats in vivo appears to be considerably less pronounced.

(6) There is little or no evidence favoring a cumulative effect for NO in the blood of either smokers or nonsmokers, so that competition with oxygen for hemoglobin-binding, per se, may be very small.

(7) Therefore, these important questions are raised: Is there a rapid turnover of NO (or of its ionized products) from NO-containing respired air to the tissues without a significant elevation of its concentration in the circulation? If so, what is the metabolic fate of inspired NO, and what are the consequences in terms of injury or toxicity?
Table 1

SUMMARY OF NO EXPOSURE OF BLOOD BY FIELD IONIZATION
MASS SPECTROMETRIC (ISOTOPE DILUTION) ANALYSIS

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Sampling Time, Post-Exposure (min)</th>
<th>(^{15}O) Added ((\mu)moles)</th>
<th>Mass 30/31 Ratio (%)</th>
<th>(^{14}O) Recovered/(\mu)moles/(\mu)l Blood (nanomoles)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Vitro Exposure (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>36.59</td>
<td>8.00</td>
<td></td>
<td>(5.72 \times 10^3) (=) 64.8% binding based on 8.8 (\mu)moles/(\mu)l = 100%</td>
<td></td>
</tr>
<tr>
<td>In Vitro and In Vivo Exposure (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>55.10</td>
<td>1.12</td>
<td></td>
<td>17.60</td>
<td>500-g rat; Blood volume = 28.8 ml</td>
</tr>
<tr>
<td>10</td>
<td>54.88</td>
<td>0.218</td>
<td></td>
<td>9.88</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>54.88</td>
<td>0.195</td>
<td></td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>55.10</td>
<td>0.194</td>
<td></td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>(2) 10-min exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>54.80</td>
<td>5.58</td>
<td></td>
<td>128</td>
<td>401-g rat; Blood volume = 23.1 ml</td>
</tr>
<tr>
<td>10</td>
<td>54.88</td>
<td>0.25</td>
<td></td>
<td>32.9</td>
<td></td>
</tr>
<tr>
<td>(3) 30-min exposure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>54.88</td>
<td>6.66</td>
<td></td>
<td>77.0</td>
<td>475-g rat; Blood volume = 27.3 ml</td>
</tr>
<tr>
<td>5</td>
<td>54.80</td>
<td>0.30</td>
<td></td>
<td>69.3</td>
<td></td>
</tr>
<tr>
<td>In Vivo Exposure (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>--</td>
<td>--</td>
<td></td>
<td>--</td>
<td>500-g rat; Blood volume = 30.5 ml</td>
</tr>
<tr>
<td>5</td>
<td>51.96</td>
<td>0.586</td>
<td></td>
<td>205.8</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>49.58</td>
<td>0.378</td>
<td></td>
<td>99.6</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>43.36</td>
<td>0.260</td>
<td></td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td>(2)† Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>20</td>
<td>38.06</td>
<td>0.204</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>45</td>
<td>41.44</td>
<td>0.218</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>--</td>
<td>--</td>
<td>0.190</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

* Sample numbers in ( ) are those described on pages 1 to 3.

† All samples stored on ice in syringes overnight before vacuum treatment.
### Table 2

**ANALYSIS OF BLOOD NITRIC OXIDE BY ELECTRON SPIN RESONANCE**

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Temperature for ESR Analysis</th>
<th>Quality of ESR Signal</th>
<th>ESR Signal Strength (Arbitrary Units)</th>
<th>Saturation of Hemoglobin by NO (%)</th>
<th>Expected</th>
<th>Found</th>
<th>ESR</th>
<th>FIM&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vitro Exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) 2 ml whole rat blood to 50 ml NO gas</td>
<td>ambient</td>
<td>strong</td>
<td>5</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) Plasma of &quot;a&quot; Cellular portion of &quot;a&quot;</td>
<td>ambient</td>
<td>none</td>
<td>&lt;0.02</td>
<td>~100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) 2 ml citrated saline to 50 ml NO gas</td>
<td>ambient</td>
<td>none</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ml citrated saline to 50 ml NO gas</td>
<td>ambient</td>
<td>questionable</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In Vivo Exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) i.a. infusion of rat (0.5 ml NO into 500-g rat)</td>
<td>ambient</td>
<td>none</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.a. infusion of rat (0.5 ml NO into 500-g rat)</td>
<td>liquid N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>questionable</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.a. infusion of rat (50 ml NO into 500-g rat):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-min sample (24 hr later)</td>
<td>ambient</td>
<td>clear</td>
<td>0.35</td>
<td>-77</td>
<td>7.5</td>
<td>2.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-min sample (24 hr later)</td>
<td>ambient</td>
<td>clear</td>
<td>0.30</td>
<td>(0.15)</td>
<td>6.4</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60-min sample</td>
<td>ambient</td>
<td>clear</td>
<td>0.24</td>
<td></td>
<td>5.1</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>180-min sample</td>
<td>ambient</td>
<td>clear</td>
<td>0.04</td>
<td></td>
<td>0.9</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) Exposure to 4-8 ppm NO breathing for 45 min:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>ambient</td>
<td>none</td>
<td>&lt;0.02</td>
<td></td>
<td>--</td>
<td>none&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; liquid N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>questionable</td>
<td>&lt;0.01</td>
<td></td>
<td>none&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td>ambient</td>
<td>none</td>
<td>&lt;0.02</td>
<td></td>
<td>--</td>
<td>none&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; liquid N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>questionable</td>
<td>&lt;0.01</td>
<td></td>
<td>none&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Cigarette smoking: 1 pack/day</td>
<td>ambient</td>
<td>none</td>
<td>&lt;0.02</td>
<td></td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>liquid N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>questionable</td>
<td>&lt;0.01</td>
<td></td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-4 packs/day</td>
<td>ambient</td>
<td>none</td>
<td>&lt;0.02</td>
<td></td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>liquid N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>questionable</td>
<td>&lt;0.01</td>
<td></td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Sample numbers in ( ) are those described in pages 1 to 3.
<sup>†</sup> i.a. = intraarterial
<sup>‡</sup> Not greater than blank value for ratio 30/31.
A - HbNO in Whole Blood Saturated with NO
  G = 50  M = 1000

B - Blood of Rat Administered 5 cc NO
    and Sampled 5 min After
    G = 500  M = 1000

FIGURE 1  ELECTRON SPIN RESONANCE OF HbNO IN RAT BLOOD
Appendix E

EXCERPT FROM REPORT TO CALIFORNIA DEPARTMENT
OF PUBLIC HEALTH
RECOVERY OF N\textsuperscript{14}O FROM N\textsuperscript{14}O SPIKED BLOOD

A series of experiments was performed to determine by isotope dilution analysis whether N\textsuperscript{14}O could be recovered from N\textsuperscript{14}O-exposed blood. Whole liquid blood (1.0 ml), its equivalent in aqueous hemoglobin solution, or hemoglobin plus erythrocyte membranes was exposed under vacuum to a known amount of about 3.5 \textmu{}moles N\textsuperscript{14}O (less than half of full saturation) and then equilibrated with about 55 \textmu{}moles N\textsuperscript{15}O.

The reproducibility of recovery of N\textsuperscript{14}O from a number of samples of blood of the same exposure was fairly satisfactory; a variance (S.D./average) of 10% was observed. This relatively large variance in recovery of NO is tolerable when we are concerned with human blood samples, which seem to carry 10 to 20 nanomoles per ml. At these low levels of NO, the precision of the mass spectrometric determination corresponds to \pm{} 1.5 nanomole/ml. In other words, the variance in recovery is comparable to the variance in the mass spectrometric determination and is, therefore, not the limiting factor.

Some samples were exposed to air before equilibration with N\textsuperscript{15}O to determine whether the bound N\textsuperscript{14}O would be oxidized. It appears that air oxidizes some of the bound N\textsuperscript{14}O but at a relatively slow rate.

It was surprising, however, that the amounts of the N\textsuperscript{14}O found to have been bound were in the range of 5-25%. A series of experiments was run to try to find the cause of these low recoveries. Removal of O\textsubscript{2} by treating the blood sample with CO did not increase the recovery percentage, nor did chemical denaturation of the hemoglobin in the final step result in higher recovery of N\textsuperscript{14}O.

Trichloroacetic acid (TCA) was tried as the denaturation agent, but for unknown reasons, TCA treatment removed all NO from the samples.
It seems that an acid-catalyzed or acid-consuming reducing agent present in the biological sample was activated by the relatively strong TCA. Sodium dodecyl sulfate (SDS) was found to be a suitable denaturing agent.

A hemoglobin solution was equilibrated with a subsaturation level of \( ^{14} \text{N} \) at 35°C for 2 hours and 40 minutes. The residual unbound \( ^{14} \text{N} \) was transferred to a flask and a known amount of \( ^{15} \text{O} \) was added. Approximately 85% of the \( ^{14} \text{N} \) initially added to the blood sample was recovered and, therefore, was not bound by hemoglobin. The limited binding of NO to hemoglobin observed in our procedures may be due to partial denaturation of the protein in the handling of the blood before the exposure to \( ^{14} \text{N} \), although very strict precautions have been taken to handle the blood as gently as conceivable and practically no hemolysis is observed in the treated blood. Additional research is planned to determine the proper parameters for obtaining complete binding of NO to hemoglobin.

It must be emphasized that the problem of finding the conditions for quantitative binding of NO to hemoglobin in blood \textit{in vitro} has no bearing on the efficiency of our method for determination of the bound NO.
DETERMINATIONS OF NO IN BLOOD OF HUMAN SUBJECTS

The blood of six human subjects was analyzed for its NO content by the analytical procedure described above. The results are presented in Table 1. All samples of NO recovered after exposure to human blood show a significant increase in the 30/31 abundance ratio. The calculated NO content of the blood is about 20 nanomoles/ml whole blood, corresponding to about 0.25% saturation of Hb with NO. No correlation between the smoking habits of the subjects and the NO content in their blood was found. In fact, it is rather surprising that the NO content of different human subjects is so similar—a variance of ±13% may be calculated for our short series. The blood of rats showed much lower steady-state concentrations of NO, below the sensitivity of detection of our method. The higher steady-state concentrations of NO found in humans may be due to a slower metabolism compared with rats. If this is the case, we would expect a difference in NO content between smokers and nonsmokers. The latter discrepancy might, however, be resolved if it were found that smokers exhibit enhanced NO metabolization to compensate for their high rates of uptake. Another explanation for the relatively high but rather constant NO content of human blood could be the formation of endogeneous NO as a by-product intermediate of nitrogen oxidation in vivo. This is analogous to the formation of CO, which has been well established as a metabolic product. If this is the case, there ought to be only a very small variation in NO content on increased uptake of exogeneous NO. Moreover, this would make exogeneous NO a rather innocuous substance, as long as it is not oxidized to NO₂ before absorption.
Table 1
DETERMINATION OF NO IN HUMAN WHOLE BLOOD

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>30/31 Abundance Ratio x 10^2</th>
<th>N\textsuperscript{14}O Recovered\textsuperscript{a} (nanomoles)</th>
<th>Percent HbNO\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>N\textsuperscript{15}O blank\textsuperscript{c}</td>
<td>0.199 ± 0.005</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>0.240 ± 0.006</td>
<td>22.2</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>0.242 ± 0.003</td>
<td>23.6</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.238 ± 0.010</td>
<td>21.1</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.232 ± 0.001</td>
<td>18.1</td>
<td>0.21 ± 0.006</td>
</tr>
<tr>
<td>5</td>
<td>0.244 ± 0.016</td>
<td>24.4</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>6</td>
<td>0.212 ± 0.001</td>
<td>17.9</td>
<td>0.20 ± 0.003</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Average value. 54.9 µmoles N\textsuperscript{15}O (containing 109.5 nanomoles N\textsuperscript{14}O) were added to 1 ml of whole blood.

\textsuperscript{b} Calculated assuming that each milliliter of normal whole blood contains 2.2 µmole Hb each with 4 binding sites for NO. \% HbNO = nmole N\textsuperscript{14}O recovered/8800.

\textsuperscript{c} Commercial N\textsuperscript{15}O containing 1994 ppm N\textsuperscript{14}O.

\textsuperscript{d} Smoking subject.
SCOPE AND LIMITATIONS OF THE ISOTOPE DILUTION METHOD FOR DETERMINATION OF NO

Isotope dilution analysis using field ionization mass spectrometry is competitive with esr techniques. As our method now stands, it will readily detect 10 nanomoles of NO in a milliliter of whole blood, i.e., 0.1% saturation of Hb. This sensitivity can be tripled without any special effort by decreasing the amount of $^{15}$N added by a factor of 3 or by a corresponding increase of the blood sample to 3 ml. The increase of sensitivity to 1 nanomole per ml could also be achieved after investment of some additional development.

The precision of the method as it stands today is $\pm 10\%$. The main limiting factor is the yield of NO recovery. The mass spectrometric determination is precise to within $\pm 2\%$ and could be improved if necessary. The pressure measurement of the added $^{15}$N and the efficiency of gas handling may add 1 to $2\%$ uncertainty to the determination. If substantial amounts of interfering substances, such as ethane or formaldehyde, occur in the biological samples, they will add another source of error that could be completely eliminated only by a gas chromatographic separation before the mass spectrometric determination.

It seems reasonable to suggest, however, that a 10% precision is sufficient for epidemiological or physiological investigation. Thus no upgrading of precision is required at this time.

The analytical procedure developed in this study has not yet been streamlined to handle many samples per day on a routine basis. At present we estimate an average effort of 3 man-hours per sample. However, this effort can be reduced to 1 man-hour or less if routine determination of hundreds of samples is called for. This streamlining of the analytical procedures will, however, require a substantial research and development effort.
CONCLUSIONS

In this study we have demonstrated the applicability of a unique isotope dilution technique to the determination of NO in blood. We have also shown that human subjects carry small but fairly constant concentrations of NO in their blood. It is not clear at this point whether increased levels of NO in the breathing air result in significant increase in the NO level in blood. On the other hand our results strongly suggest a rapid oxidation of NO in vivo even after being bound to hemoglobin. The most likely immediate product of oxidation of NO in vivo is nitrite ions, although a direct enzymatic nitrozation of a substrate by NO$^+$ cannot be excluded. If the substrate is an amino group R-NH$_2$, the reaction will result in oxidative deamination. The physiological action of NO is thus likely to be very different from that of NO$_2$, which reacts, most probably in the lung tissue, as an oxidant rather than as a reducing agent. Further research is required to determine the mode of action of NO at the cellular level. This information is absolutely necessary before any conclusions are reached on the potential toxicity of atmospheric nitric oxide. Although our analytical method may be helpful in evaluating the environmental hazards of NO to human subjects, it ought to be supplemented with biochemical and physiological information generated by other methodologies.
REFERENCES


Appendix F

PAPER PRESENTED AT CONFERENCE ON STABLE ISOTOPES IN CHEMISTRY, BIOLOGY, AND MEDICINE
Determination of Nitric Oxide in Blood
By Isotope Dilution Analysis

Introduction

Nitric oxide, a major air pollutant, is produced in internal combustion engines and during other high temperature combustion processes. Because the rate of oxidation of nitric oxide (NO) to nitrogen dioxide (NO₂) in the air is relatively slow, a considerable steady-state concentration of NO is built up. Nitric oxide is a critical ingredient in the mechanism of smog formation, but little is known about its biological effects at low concentrations.

Gibson and Roughton demonstrated that NO binds to hemoglobin (Hb) many times stronger than does carbon monoxide (CO).¹ The mode of binding of NO to Hb has been recently investigated using electron spin resonance (esr) techniques.² The ready access of Hb in the lungs to inhaled CO suggested that NO might react similarly with Hb. Nitric oxide has not been, however, recognized as a significantly toxic material and though a lethal effect on mice has been reported,³ it was not included among the substances having a threshold limit value by the American Conference of Governmental Industrial Hygienists (1967).⁴

There is a discrepancy between the expected binding of NO to Hb in vivo and the failure to detect the characteristic HbNO optical absorption band, despite the many years of observations on blood by spectrophotometry. Negative results were also obtained in a study using the more sensitive technique of esr to detect the free radical (either NO or NO₂) in blood of rats exposed to NO.⁵ A recent study using practically the same technique indicates, however, the presence of Hb-NO in the blood of mice exposed to NO.⁶ Preliminary experiments using the same isotope dilution analysis on which the present study is based suggest that human subjects carry minute amounts of NO in their blood.⁷

Owing to its stability the concentration of the Hb-NO complex in blood is expected to increase in time, reaching a steady-state concentration determined by the rates of NO intake and of its metabolism. Considering the reported stability constant of the Hb-NO complex, the Hb bound NO must be metabolized, most probably oxidized, rather effectively; otherwise NO would have been recognized a long time ago as a highly toxic substance.

The hypothesis that NO reaches a steady-state concentration in blood suggests that the level of HbNO in blood should increase in areas with high air pollution.
or in persons who smoke excessively. The concentration of HbNO in blood, even if very low, could thus serve as a simple basis for epidemiological studies of populations exposed to NO-laden atmospheres.

It was of importance, therefore, to develop a sensitive quantitative analytical method for determination of NO in blood. We are interested in quantities of NO of the order of 10 nanomoles or less per ml blood (NO/O₂ < 10⁻³). These are concentrations too low to be reliably determined in situ by esr. Although NO can be released from blood by denaturation of Hb, this cannot be used as a base for an analytical method because NO would be readily oxidized to NO₂ by the oxygen simultaneously released. Removal of the oxygen prior to denaturation by evacuation, by repeated flushing with an inert gas or by substitution with CO is not a practical solution either, because it is impossible to guarantee quantitative removal of O₂ to one part per ten thousand which is required if NO is to be determined with a 10% precision.

A unique solution to the analytical problem outlined above is isotope dilution analysis. This quantitative method allows the handling of the minute amounts of NO without necessitating a quantitative recovery.

Whole blood, hemolized or nonhemolized erythrocytes, when exposed to a known amount of N¹⁵O will exchange any bound N¹⁴O with the excess N¹⁵O, resulting in an isotopic dilution of the latter. Part of the N¹⁵O added is consumed in the process of substituting O₂ and CO on the hemoglobin, as well as by being oxidized to NO₂ by the O₂ released. If excess N¹⁵O is added, the isotopic composition of the residual NO will still represent the isotope equilibrium value. Thus, if the amount of N¹⁵O added is known as well as the isotope ratio N¹⁴O/N¹⁵O in the residual NO, the amount of N¹⁴O present in the blood sample can be readily calculated. Since commercially available N¹⁴O contains a small percentage of N¹⁵O, this amount must be taken into account when calculating the N¹⁴O present in blood.

The same equilibrium value of N¹⁴O/N¹⁵O is obtained when the hemoglobin is denatured in the presence of N¹⁵O. The strong binding of NO to hemoglobin is
overcome by denaturation of the protein. The denaturation has the advantage of making the dilution analysis independent of the kinetics of isotopic equilibration between bound and unbound NO, which may lead to a possible error due to competition between the isotope exchange and the NO oxidation reaction. As the isotopic exchange with the NO released by denaturation is instantaneous, the much slower oxidation reaction which removes part of the NO is inconsequential. It is still advisable to remove the oxygen from the blood before denaturation by evaucation or by substitution with CO. This diminishes the losses of NO and allows, therefore, the use of smaller quantities of N\textsuperscript{15}O, thus increasing the sensitivity of the isotope dilution analysis. Furthermore, if most of the NO is being oxidized to NO\textsubscript{2}, the isotopic composition of the residual NO may be modified by isotopic fractionation. This effect is not trivial when we are considering a mixture which contains a minute quantity of the more reactive N\textsuperscript{14,16}O.

The precision of the isotope dilution method depends on the reliability of the determination of the 30/31 mass abundance ratio. In other words, it is imperative that the measured ratio represents the true N\textsuperscript{14}O/N\textsuperscript{15}O abundance ratio. This imposes stringent requirements on the ionization mode of the mass spectrometer. When the classical electron impact ionization is used, the species produced from NO, in addition to NO\textsuperscript{+}, include N\textsuperscript{+}, O\textsuperscript{+}, N\textsubscript{2}\textsuperscript{+}, N\textsubscript{2}O\textsuperscript{+}, O\textsubscript{2}\textsuperscript{+}, and NO\textsubscript{2}\textsuperscript{+}. Under these conditions the formation of N\textsubscript{2}\textsuperscript{15,15}, which would be the predominant form of N\textsuperscript{+}, would overshadow the minor peak of N\textsubscript{2}\textsuperscript{14,16}. Chemical ionization is also unsuitable for our purpose because if we used a carbon containing ionizing gas, we could not avoid the formation of minute amounts of H\textsubscript{2}CO\textsuperscript{+} and C\textsuperscript{18}O\textsuperscript{+} in addition to some N\textsubscript{2}\textsuperscript{15,15}. If we used an inorganic ionizing gas, e.g., argon, the ionization pattern would be similar to that obtained by electron impact.

The only mode of ionization suitable to our problem is field ionization. Here we produce NO\textsuperscript{+} exclusively under conditions which do not produce any N\textsubscript{2}\textsuperscript{+} even from trace impurities of N\textsubscript{2}. The multipoint field ionization source recently developed at the Stanford Research Institute\textsuperscript{2,3} was found most suitable for our purpose.
Experimental

Vacuum Apparatus and Sample Handling

All nitric oxide manipulations were carried out in vacuo using an all glass vacuum manifold and a mercury diffusion pump backed up with a medium sized (~5 cfm) Welch mechanical pump. To eliminate oxygen from the system and thus avoid the unwanted reaction $2\text{NO} + \text{O}_2 = 2\text{NO}_2$, we flushed the vacuum system with either nitrogen or helium at one atmosphere pressure. Flushing with nitrogen and helium was also used to remove oxygen from blood before $^{15}\text{N}_2\text{O}$ exposure.

The general procedure for exposing blood to $^{15}\text{N}_2\text{O}$ was as follows: One cubic centimeter of whole blood (or any other form such as red blood cells or hemoglobin solution) is withdrawn using a standard glass syringe and placed into a stopcocked glass vessel with a rubber septum stoppered side arm. This vessel is connected to the vacuum manifold with a ground glass joint (see Figure 1). The blood is then cooled to 0°C and deoxygenated with He or $\text{N}_2$ by bringing the blood to one atmosphere pressure of $\text{N}_2$ and then evacuating. This is then repeated for a total of six exposures to $\text{N}_2$. During this type of degassing, the blood has a tendency to bubble during evacuation. This can be kept to a minimum by closing the stopcock whenever boiling starts. After the fourth exposure to $\text{N}_2$, the sample is removed from the manifold and rotated by hand to coat the inner surface of the glass vessel, so as to give maximum surface exposure of the blood to $\text{N}_2$. It has been shown that no NO is lost from the sample during this procedure. The flushing procedure has been preferred over freeze-thawing because the latter procedure may result in partial denaturation of Hb and in consequence, loss of NO.

After the final evacuation from $\text{N}_2$, the sample is frozen in liquid nitrogen. The sample loses some water during degassing, but this is held to a minimum by avoiding prolonged pumping.

The $^{15}\text{N}_2\text{O}$ is expanded from its flask into a calibrated volume (B, Figure 1), which is connected via a stopcock to a second calibrated volume (A, Figure 1), which includes the measuring capsule of a Wallace and Tiernan pressure gauge. The pressure of the $^{15}\text{N}_2\text{O}$ gas can be read to within 0.2 torr. Knowing the pressure and volume permits calculation of the number of micromoles of $^{15}\text{N}_2\text{O}$ added to the blood sample.
FIGURE 1  VACUUM APPARATUS FOR SAMPLE PREPARATION
After reading the pressure of \(^{15}\)N\(^{15}\)O, the \(^{15}\)N\(^{15}\)O is condensed (using liquid nitrogen) into the flask containing the frozen degassed blood and the stopcock is closed. One ml of 10% sodium dodecyl sulfate (SDS) is then added using a syringe and needle via the rubber septum. The SDS is introduced under vacuum and while the blood is still at liquid nitrogen temperature. The blood sample flask is then removed from the manifold and thawed. Since hemoglobin does not bind NO when denatured, all bound \(^{14}\)N\(^{14}\)O is released, forming a mixture of \(^{14}\)N\(^{15}\)O and \(^{15}\)N\(^{15}\)O. Attempts to use trichloroacetic acid (TCA) as denaturing agent were unsuccessful, resulting in the total loss of NO. It seems that TCA induces oxidation or binding of NO by one of the cellular constituents.

After equilibration the flask is replaced on the vacuum line, and the blood is frozen in dry ice-acetone, which freezes out water vapor but not nitric oxide. The isotopic mixture of nitric oxide is then condensed with liquid \(N_2\) into a previously evacuated small stopcocked receiving flask that can be removed from the system. This flask is designed to fit onto the vacuum inlet of the mass spectrometer and is now ready for isotopic ratio determination. This flask contains a droplet of mercury plus a small amount of Tobias' acid to remove \(NO_2\) and formaldehyde.

There were two substances, which interfered with our analytical procedure, \(NO_2\) and formaldehyde. \(NO_2\), formed by oxidation of NO during the procedure or subsequently by trace amounts of oxygen which leaked into the gas sampling vessel, was found to attack chemically the microcones of our multipoint source and thus to diminish the ionization efficiency. We overcame this artifact readily by adding a droplet of elemental mercury to the gas sampling vessel. Mercury reduces \(NO_2\) to NO forming \(HgO\) thus preserving the nitric oxide while keeping it from accidental oxidation.

It was also found that human blood undergoing the treatment described above releases small quantities of formaldehyde, of the order of 50 nanomoles per ml blood. Formaldehyde, \(H_2CO\), having mass 30 thus interfered with the determination of NO in spite of its lower volatility. This artifact was eliminated by adding to the gas sampling flask a small amount of 2-naphthylamine 1-sulfonic acid (Tobias' acid) which removed the formaldehyde quantitatively forming a Schiff base.
We have also looked into the possible interference of ethane, \( \text{C}_2\text{H}_6 \), also mol. wt. 30. Gas chromatographic analysis of the gas sample set the upper limit of ethane present at 0.1 nanomole/ml human blood, which is equivalent to < 0.3% of the NO concentration found in the same blood. This observation does not exclude the presence of ethane in human blood, only that it is absent when the blood is denatured after repeated flushings with pure nitrogen. In any case, ethane should not be considered as an interfering substance in the NO dilution analysis.

**Mass Spectrometric Isotopic Ratio Determination**

The mass spectrometer used in this study was a Labmade 45° magnetic sector instrument fitted with a SRI multipoint porous field ionization ion source\(^9\) and two nearly identical detectors. One detector is fixed and the other is movable in two dimensions to center the ion beam in the detectors (see Figure 2). The detectors are continuous dynode electron multipliers operated in pulse mode. The pulses are amplified, shaped, and fed through a discriminator set to respond to all true ion events but to reject random pulses down to a rate of \( \sim 0.5/\text{sec} \), which constitutes the natural background dark current of the detectors. The counts are registered in an electronic ratio counter, which displays the mass 30 count while counting a preset mass 31 count. A second counter is used to display mass 31 count to determine the rate of count and to aid in aligning the two ion beams initially.

The NO sample is introduced into the ratio measuring mass spectrometer through a stainless steel leak valve. The gas passes directly through the ionizing field of the source into a pumped volume at a rate sufficient to raise the total pressure in the vicinity of the source by \( 3 \times 10^{-8} \) torr over base pressure, which is \( \sim 3 \times 10^{-8} \) torr. At this flow rate, \( ^{15}\text{N}^+ \) ions reach the detector at a rate of 3,000 to 10,000 per second. A ratio determination is made by counting the number of \( ^{14}\text{N}^+ \) ions detected while \( ^{5}\text{N}^{15}^+ \) ions are counted. This measurement was repeated ten times and the ratios are averaged. The variance of the ratios was about 2%.
FIGURE 2  SCHEMATIC OF THE MAGNETIC SECTOR MASS RATIO SPECTROMETER
The flow of NO is then interrupted, and the background ion count at mass 30 is measured for 100 seconds. Knowing the time required to reach the $10^5$ counts at mass 31, we subtract the equivalent background counted of mass 30 from the rough ratio average. This correction seldom exceeded 5% of the counts at mass 30. A conversion factor is applied to the resultant ratios to equalize the detectors in terms of counting efficiency. This factor is arrived at by measuring a sample with a known ratio of $^14$N/$^15$N O/N$^15$N O. The variance (S.D./average) when the same sample was measured several times on the same day was about $\pm$ 3%. This variance increased to $\pm$ 5% when the same sample was measured on different days over a period of several months.

The reproducibility of recovery of $^15$N O from a number of samples of blood of the same exposure was fairly satisfactory; a variance of 10% was observed with a recovery of about 95%. This variance is not too surprising if we remember that the volumetric measurement of 1 ml of a heterogeneous fluid like blood may involve a 5% error. Further the boiling of the whole blood, when the nitrogen is being removed, may result in partial hemolysis and denaturation of hemoglobin with a consequential loss of NO. The relatively large variance in recovery of NO is tolerable when we are concerned with human blood samples, which seem to carry 10 to 20 nanomoles per ml. At these levels of NO, the precision of the mass spectrometric determination corresponded to $\pm$ 1.5 nanomole/ml. In other words, the variance in recovery was comparable to the variance in the mass spectrometric determination and was not, therefore, the limiting factor. The sensitivity and the precision of the mass spectrometric determination can, however, be increased by diminishing the amount of $^15$N O added. Under the latter conditions, the reproducibility of $^14$N O recovery would become the limiting factor of precision.

Scope and Limitations of the Isotope Dilution Method for Determination of NO

Isotope dilution analysis using field ionization mass spectrometry is competitive with esr techniques. As our method now stands, it will readily detect 10 nanomoles of NO in a milliliter of whole blood, i.e., 0.1% saturation of Hb. This sensitivity can be tripled without any special effort by decreasing the amount of $^15$N O added by a factor of 3 or by a corresponding increase of the blood sample to 3 ml. The increase of sensitivity to 1 nanomole per ml could also be achieved after investment of some additional development.
The precision of the method as it stands today is ± 10%. The main limiting factor is the yield of NO recovery. The mass spectrometric determination is precise to within ± 2% and could be improved if necessary. The pressure measurement of the added \( ^{15} \text{N} \) O and the efficiency of gas handling may add 1 to 2% uncertainty to the determination. If substantial amounts of interfering substances, such as ethane or formaldehyde, occur in the biological samples, they will add another source of error that could, however, be completely eliminated by a gas chromatographic separation before the mass spectrometric determination.

It seems reasonable to suggest, however, that a 10% precision is sufficient for epidemiological or physiological investigation. Thus no upgrading of precision is required at this time.

Results and Discussion

The blood of six human subjects was analyzed for its NO content by the analytical procedure described above. The results are presented in Table 1. All samples of NO recovered after exposure to human blood show a significant increase in the \( ^{30}/^{31} \) abundance ratio. The calculated NO content of the blood is about 20 nanomoles/ml whole blood, corresponding to about 0.25% saturation of Hb with NO. No correlation between the smoking habits of the subjects and the NO content of their blood was found. In fact, it is rather surprising that the NO content of different human subjects is so similar—a variance of ± 13% may be calculated for our short series. On the other hand, the blood of rats showed no nitric oxide at all. If rats' blood contains a steady-state concentration of NO, it must be less than 1 nanomole/ml blood, i.e., below the sensitivity of detection by our method. The higher steady-state concentrations of NO found in humans may be due to a slower metabolism compared with rats. If this is the case, we would expect a difference in NO content between smokers and nonsmokers. The absence of such a significant difference may suggest that smokers exhibit enhanced NO metabolism to compensate for their higher rates of NO uptake. Another explanation for the relatively high but rather constant NO content of human blood could be the formation of endogeneous NO as a by-product or intermediate of nitrogen oxidation in vivo; this would be analogous to the well established physiological formation of CO. If this is the case, there
Table 1

DETERMINATION OF NO IN HUMAN WHOLE BLOOD

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>30/31 Abundance Ratio x 10^2</th>
<th>N\textsuperscript{14}O Recovered\textsuperscript{a} (nanomoles)</th>
<th>Percent HbNO\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textsuperscript{15}O blank\textsuperscript{c}</td>
<td>0.199 ± 0.005</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>0.240 ± 0.006</td>
<td>22.2</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>0.242 ± 0.003</td>
<td>23.6</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.238 ± 0.010</td>
<td>21.1</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>4\textsuperscript{d}</td>
<td>0.232 ± 0.001</td>
<td>18.1</td>
<td>0.21 ± 0.006</td>
</tr>
<tr>
<td>5\textsuperscript{d}</td>
<td>0.244 ± 0.016</td>
<td>24.4</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>6</td>
<td>0.212 ± 0.001</td>
<td>17.9</td>
<td>0.20 ± 0.003</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Average value. 54.9 \textsuperscript{15}O moles (containing 109.5 nanomoles \textsuperscript{14}O) were added to 1 ml of whole blood.

\textsuperscript{b}Calculated assuming that each milliliter of normal whole blood contains 2.2 \textsuperscript{15}O mole Hb each with 4 binding sites for NO. % HbNO = nmoles \textsuperscript{14}O recovered/8800.

\textsuperscript{c}Commercial \textsuperscript{15}O containing 1994 ppm \textsuperscript{14}O.

\textsuperscript{d}Smoking subject.
ought to be only a small, and probably nondetectable, variation in NO content on increased uptake of exogeneous NO by humans.

The findings in humans have to be correlated with observations on animals. The low concentrations of NO found in blood of mice exposed to NO\(^6\) cannot be the result of limited uptake of NO by the lungs as suggested,\(^9\) because NO, being as small a molecule as O\(_2\) or CO, must be able to diffuse through the air-blood barrier at the alveolus and into the erythrocytes. If no NO whatsoever were found in blood one could speculate that it is completely oxidized on contact with or during diffusion through the air-blood barrier. As it seems, the low level steady-state concentrations of NO in mice and man indicate that NO undergoes rapid oxidation inside the erythrocytes in vivo. This assumption is corroborated by preliminary experiments in an ongoing study on rats carried out by our isotope dilution technique. These experiments indicate that in the rat, erythrocyte-bound NO has a halflife of a few minutes.\(^10\) On the other hand, samples were exposed to N\(^{15}\)O and then to air before equilibration with N\(^{15}\)O to determine whether the bound N\(^{15}\)O would be oxidized in vitro. It appears that air oxidizes some of the bound N\(^{15}\)O but at a slow rate, with a halflife of hours. It seems that the rapid disappearance of NO in vivo is a more complex process which may involve certain ATP dependent oxidases. The in vitro oxidation, which is oxygen dependent, may be a charge transfer process involving oxygen and NO bound to the same Hb molecule. Alternatively it may involve the same reactions as the in vivo metabolization of NO which are slowed down under the non-physiological conditions.

The fast metabolization of NO observed in the rat supports the suggestion that the steady-state concentration of NO observed in normal humans is the result of endogenous formation of NO, at least in humans. It is too early to speculate on the metabolic pathway which leads to the formation of NO in vivo.

Let us present some speculations on the basis of our preliminary findings. We do not know at this point what fraction of inhaled NO is oxidized at the air-blood barrier. Such an oxidation, if it occurs, is not extensive or it does not produce NO\(_2\). Otherwise NO would be exhibiting toxicological effects identical to those of NO\(_2\).\(^11\) We have also found\(^10\) that gaseous NO injected intravenously into rats is by far less acutely toxic than NO\(_2\). This NO was metabolized, however,
with a halftime of a few minutes. These results suggest that NO$_2$ is not a major product of the metabolism of NO but that the latter is oxidized by a single electron transfer process. If this is the case, we may be confronted with two potentially detrimental effects. First, a certain oxidase reacts in a non-characteristic peroxidative mode, which may result in the occurrence of additional unwanted single electron transfer processes. Second, the most likely product of oxidation of NO is the NO$^+$ ion which in the presence of secondary amines or peptides will rapidly produce nitrosoamines or nitrosoamides:

\[
\begin{align*}
R_1R_2NH + NO^+ & \rightarrow R_1R_2NO + H^+ \\
R_1CONHR_2 + NO^+ & \rightarrow R_1CON(NO)R_2 + H^+
\end{align*}
\]

These products may be toxic and possibly carcinogenic.

Nitrogen dioxide, on the other hand, which acts as an oxidant, produces NO$_2^-$ ions as the major product. The toxicological effects of the two nitrogen oxides are thus completely different. Although NO has no or very mild acute toxicological effects, it may have worse long term detrimental consequences. This would indicate that air pollution control of NO is as important as that of NO$_2$. On the other hand, if our hypothesis on the endogenous formation of NO in humans is corroborated, the inhalation of additional NO even from moderately polluted air may be inconsequential.

Further research is required to determine the mode of action of NO at the cellular level. This information is absolutely necessary before any conclusions are reached on the potential toxicity of atmospheric nitric oxide. It is evident that our preliminary results obtained by isotope dilution analysis open up a large number of questions most of which will have to be answered by aids of isotopic nitrogen.
REFERENCES


