Measurement of nitric oxide, nitrite and nitrate using a chemiluminescence assay: an update for the year 2000

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This review deals with the measurement of nitric oxide (NO) using the chemiluminescence assay. A preliminary discussion is offered to explain the chemical basis for this assay and emphasize the importance of measuring NO. After reviewing some practical aspects and caveats of the chemiluminescence assay, we review its application in a variety of research and clinical settings, such as measurement of breath NO and serum nitrates. The importance of avoiding confounding effects of dietary nitrates when assessing the NO system in humans is discussed and new data are provided on the confounding effects of atmospheric NO on measurement of breath NO. The utility and ease of the chemiluminescence assay for these applications is contrasted with the challenge of using chemiluminescence for correlation of vascular tone and NO production, studies in which real-time measurements are required. Although the chemiluminescence assay is one of the most reliable, rapid and reproducible assays available, it is optimal for gas phase measurements and may be suboptimal for measurement under certain circumstances. This chapter complements several more general reviews of the methodology for measuring NO.

Abbreviations

cGMP: cyclic guanosine monophosphate
NO, NOS: nitric oxide, nitric oxide synthase
NO_2^-, NO_3^-, NO_x: nitrite, nitrate, the collective products of NO oxidation
$ONOO^-$: peroxynitrite
PMT: photomultiplier tube
DEA/NO: diethylamine NONOate
ppm, ppb: parts per million or billion, respectively
NaI, KI: sodium and potassium iodide
EDRF: endothelium derived relaxing factor
EDHF: endothelium derived hyperpolarizing factor
L-NMMA: N^G- monomethyl–L–arginine
DMSO: Dimethylsulfoxide
PMT: photomultiplier tube

Introduction

Interest in NO measurement originated from studies of its role as an environmental contaminant in Earth's air and water [1]. NO and its oxidation products are common air pollutants, produced by automobile engines and various industrial processes. Moreover, nitric oxide is produced at pathological levels (hundreds of ppm) in cigarettes [2]. In Paris, France, ambient NO levels vary from 10-100 ppb, depending on atmospheric conditions and traffic congestion (Fig. 1). Ambient NO levels tend to be high in the morning, during rush hour, and low when traffic levels decrease (Fig. 1). The NO pathway is also of interest to microbiologists because NO is an intermediate in the reduction/oxidation of nitrogen that occurs in various bacteria [3,4].

However, it was the identification of NO as endothelium derived relaxing factor (EDRF) [5,6] and the discovery of its many physiological and pathophysiological roles in mammalian biology that has elevated the importance of NO measurement. Measurement of NO is important for many vascular biologists, physiologists and increasingly, for physicians as well. NO is one of the most important signalling molecule in vascular biology. Industrial [7] and therapeutic applications [8] have employed NO itself or have exploited NO's several signal transduction pathways (*e.g.* guanylate cyclase, cyclic guanosine monophosphate (c-GMP), cGMP kinase, and the large conductance, calcium-sensitive potassium channel [9]). For example, sildenafil (Viagra®), a selective type V phosphodiesterase inhibitor, has been very effective as an oral therapy for erectile dysfunction in men. Its mechanism of action amplifies and prolongs the biological effect of penile NO, by sustaining tissue cGMP levels [10]. NO or sildenafil can also prolong the survival of certain vegetables or cut flowers [7]. NO is made in the human airway, particularly the nasopharynx. NO in human breath is a useful marker of inflammation and can be used to evaluate patients with asthma [11-13].

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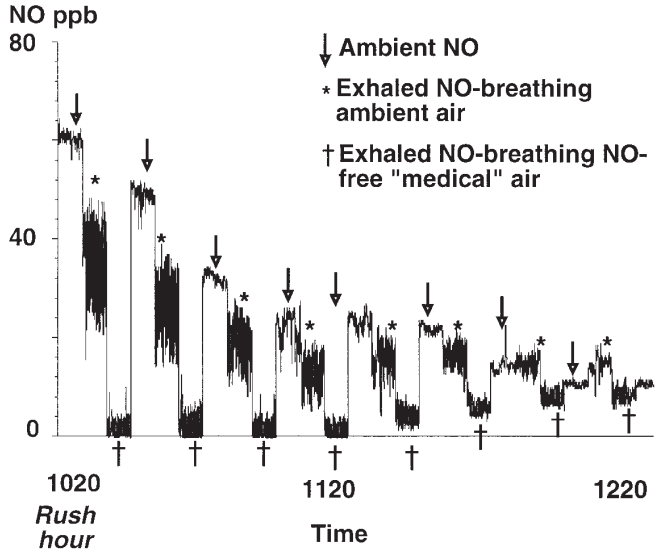


Figure 1. Exhaled NO measurements are more reproducible when the subject breathes NO-free air

In this subject, lower airway NO levels were measured repeatedly while the subject inhaled either room air or medial air, that contained < 3 ppb NO. The subject wore a nonrebreathing mask, which prevented contamination of the sample with nasal air. During the course of the experiment, the NO concentration in the ambient air fell significantly. It is evident that the exhaled NO concentrations obtained when the subject was breathing room air are falsely elevated and reflect primarily the atmospheric NO concentration in the room air. In contrast, NO concentrations measured whilst the subject inspired NO-free air are quite independent of environmental NO levels. The need for using NO-free air is further documented in a larger series of subjects in figure 4.

Need for measuring NO

It is clear that NO is essential in a significant number of normal physiological processes. However, the scope of its importance also spans pathophysiology of disease, such as in cardiogenic shock [14,15]. Inhaled NO is used for diagnostic purposes, and has therapeutic value, in diseases such as pulmonary hypertension [16-18], asthma and chronic obstructive lung diseases [12,19-21]. NO may even promote regression of diseases, such as atherosclerosis [22,23].

In view of the broad biological importance of this molecule, it is not surprising that basic and clinical research pertaining to NO has been so prolific. However, a computerised search of the past five years' literature found NO measurements reported in only 1089 of 26123 articles dealing with NO. Since many scientists don't measure NO, perhaps we should make the case for measurement explicit.

There are four major reasons why one should consider measuring NO:

Non specificity of NO inhibitors

Pharmacological experiments are useful in studying NO and its effects. Nevertheless, the non-specificity of NO inhibitors [24-26] impairs one's ability to draw sound mechanistic conclusions. For instance, N^G- monomethyl–L–arginine (L-NMMA), a NOS antagonist, displays hemodynamic effects that are not solely mediated by its ability to inhibit NO synthesis [27]. L-NMMA can also be metabolized to yield NO. Furthermore, even high doses of NOS inhibitors do not completely prevent NO synthesis [28]. Without measuring NO one cannot be assured that the observed effects resulting from administration of a NOS inhibitor reflect the complete loss of NO, nor can one exclude non-specific effects of these inhibitors.

The existence of multiple endothelium-derived vasodilators

It is known that vascular tone is mediated through the synthesis of various agents [29-33]. Agents such as acetylcholine, alter vascular tone through their ability to stimulate synthesis of the at least three vasodilatory agents, prostacylin, NO and endothelium-derived hyperpolarizing factor (EDHF) [29-33]. Therefore, it becomes important to quantify NO in the experimental medium, rather than simply assuming that it is the sole mediator involved.

Variability of the mechanism of endothelial-dependent vasodilatation among vascular beds

The hemodynamic effects and the mechanisms of action of endothelium dependent vasodilators, for instance acetylcholine and bradykinin, vary in different vascular beds and amongst species [34-37]. The contribution of NO to the effect of endothelium dependent vasodilators may vary within a vascular bed. For example, acetylcholine induced relaxation is not primarily due to NO in the rat pulmonary artery, whereas bradykinin and A23187 do cause NO-mediated relaxation in the same preparations [35]. The contribution of NO to acetylcholine-induced relaxation can even vary within segments of a single blood vessel [38]. Recently, Traverse *et al.* contrasted the mechanism of coronary vasodilatation induced by shear stress *versus* endothelium dependent vasodilators, both mechanisms reputed to involve NO [39]. Measuring coronary NO(*x*) at rest and during graded exercise testing, using the chemiluminescence assay, they found that no coronary NO production was detectable at rest or during the first 2 stages of exercise. Only at the maximal exercise was a small increase in coronary NO*x* production measured. In contrast, coronary production of NO*x* was significantly increased in response to endothelium-dependent agonists, acetylcholine and bradykinin. Thus they concluded that coronary NO production is greater in response to endothelium-dependent agonists than to the exercise-induced shear stress [39].

The need for quantification

Like many biologically active molecules, NO's effects may be beneficial or harmful to the organism, depending on the amount of NO produced and the redox environment in which it is produced (*i.e.* ambient levels of superoxide anion, hydrogen peroxide, etc.) [40-42]. It is therefore important to be able to measure the quantity of this labile substance.

An example of a type of investigation in which the need for measurement is crucial is the search for the identity of EDHF. EDHF is defined as an endothelial factor, other than NO or prostacyclin, which activates K+ channels on vascular smooth muscle cells, thereby hyperpolarizing and relaxing them [29]. From this definition it is clear one must exclude the presence of NO before confidently identifying "EDHF-activity". The fact that both NO and EDHF activate K+ channels [9] further underscores the need to utilise an accurate form of measurement to ensure that all NO has been removed from the system.

The chemistry of NO

It is impossible to discuss the chemiluminescence assay without first considering the chemical nature of NO (for more details see [43,44]). NO is a free radical which readily reacts with O_2, peroxides, O_2-radicals and metals [45-47] which explains its instability in biological models. Its small size and lack of hydration reaction allow NO to pass freely across cell boundaries. NO can be either an oxidant or a reducing agent, depending on its surrounding redox environment [43]. NO has a very high partition coefficient (≈ 20 molecules per unit volume gas/molecules per unit volume solution) and therefore preferentially exists in the gas phase. NO produces a toxic brown gas, nitrogen dioxide, when mixed with O_2 in the gas phase. Although nitrogen dioxide itself is quite toxic (causing a delayed onset hemorrhagic pneumonitis) toxicity is not a problem with sustained exposures to NO at levels below 25 ppm [48]. However, as NO becomes a therapeutic tool and longer exposures become likely, additional toxicology studies are indicated.

In low concentrations, and in the absence of O_2, NO is quite stable [48], although there is some loss of NO due to a slow thermodynamic disproportionation reaction [43] (Eq. [1]). NO can be stored in gas sample bags for hours without deterioration of the signal. This facilitates transporting breath NO samples and storing calibration gases [49]. The stability of breath NO in storage bags has recently been confirmed [50]

$$4NO \rightarrow N_2O_3 + N_2O \qquad [1]$$

The many fates of NO

The reaction of NO with O_2 will yield different products depending on the phase of the reaction (*i.e.* gas or liquid) and whether or not additional oxidizing agents are present. For example, in the gas phase NO will combine with O_2 to produce NO_2^- (Eq. [2]), which can either dimerize to N_2O_4 [44] (Eq. [3]) or react further with NO to produce N_2O_3 [45] (Eq. [4]).

$$2NO + O_2 \rightarrow 2NO_2^- \qquad [2]$$

$$2NO_2^- \rightarrow N_2O_4 \qquad [3]$$

$$NO + NO_2^- \rightarrow N_2O_3 \qquad [4]$$

In the liquid phase, provided that oxidizing agents such as oxyhemoproteins and superoxide anions are absent, NO reacts with O_2 to yield NO_2^- [47] (Eq. [5]). Indeed, when authentic NO is added to a closed system containing oxygenated water and a headspace of 20 % oxygen and 80 % nitrogen, the NO is largely converted to nitrite within one minute. Because of its high partition coefficient, most of the NO which escapes oxidation is found in the gas head space [44].

$$4NO + O_2 + 2H_2O \rightarrow 4HNO_2 \rightarrow 4NO_2^- + 4H^+ \qquad [5]$$

However, in most biological systems, the presence of additional oxidising agents yields different NO oxidation products. Oxyhemoproteins cause NO to be oxidised to nitrate (NO_3^-) [47], while the presence of superoxide anion causes NO to be oxidized to peroxynitrite ($ONOO^-$) [46,51]. This latter reaction is exceptionally rapid, exceeding the rate for dismutation of superoxide anion by superoxide dismutase. Additionally, NO_3^- can also be formed by oxidation of NO_2^- in solution. As a result, NO_3^- is the dominant species of the collective of NO oxidation products (NO_x) *in vivo*.

The chemiluminescence reaction

In gas medium, NO reacts with ozone to form an excited NO_2^* species that emits light as it returns to ground state (Eq. [6]). Because light emission is linearly related to the NO content and ozone is supplied in excess quantities, the chemiluminescence assay, pioneered by Fontijin in 1970 [52], can measure NO by quantifying the light emission. Light emission accompanying the reaction of O_3 or singlet oxygen with gases such as NO, NO_2, CO, and SO_2 was described in the early sixties. It was found that singlet oxygen reacts with all of these gases, while O_3 reacts most readily with NO. The chemiluminescence assay for NO depends on the gas phase reaction of NO with ozone which generates photons that can be detected [52,53] (Eq. [6]). Some of the NO_2 that is generated from oxidation of NO by ozone is in the excited state, which can return to the ground state by either emitting a photon (hv) or by mechanical quenching (collision with other gas molecules and transfer of kinetic energy) [54].

$$NO + O_3 \rightarrow NO_2^* + O_2,\ NO_2^* \rightarrow NO_2 + hv \qquad [6]$$

The light emitted by the reaction of NO with ozone is in the red and infrared region of the spectrum (wavelength ~ 600-3000 nm), with a peak intensity at ~ 1100 nm [54]. The amount of light emitted is linearly related to the concentration of NO in the sample [1,44] and increases substantially if the reaction chamber pressure is reduced to below 0.3 atmospheres [54]. By exposing the reaction chamber to a strong vacuum N_2 and CO_2 are removed, thereby decreasing the quenching that occurs when these inert gases interact with NO_2*. The amount of chemiluminescence is also strongly dependent on temperature [54]. The reaction is very fast, with a rate constant at room temperature [55] of 10^{-7} L mol^{-1} s^{-1}. It is therefore possible to measure NO concentrations with the chemiluminescence assay in "real time". In addition, the amount of light emitted by the reaction of NO with ozone makes the chemiluminescence assay for NO one of the most sensitive available [43]. It can detect ~ 1 ppb in gas phase and concentrations between 20 and 50 pmol/L in liquids [6,56,57].

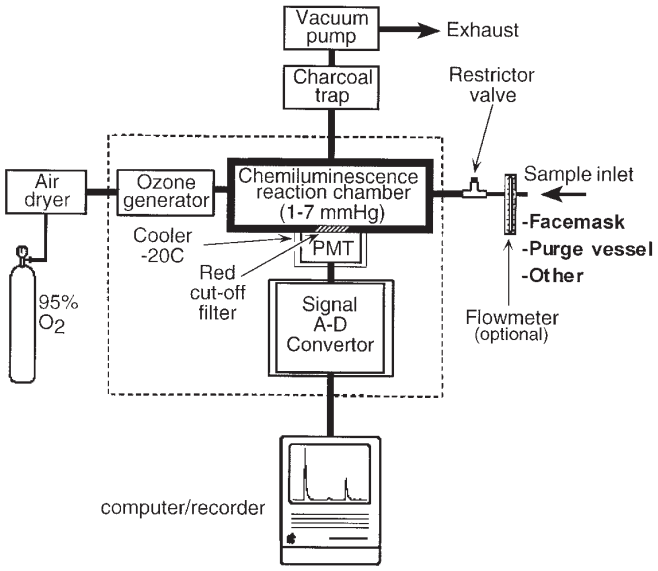
The specificity of the chemiluminescence assay for NO depends on the low activation energy of the reaction between NO and ozone (10.5 kJ) [58]. This specificity of the assay for NO depends on the chemical properties of NO. In order to be detected, a molecule must first enter the head space gas. NO has a high partition coefficient, while nitrates, nitrites and nitrosothiols remain in solution. Once in the gas phase, the molecule must be capable of undergoing a chemical reaction with ozone that emits photons. This reaction requires the presence of unsaturated or strongly polar groups (*e.g.* sulfides, amines) and these moieties are rare in small molecules [1]. Dimethylsulfoxide (DMSO), a common solvent vehicle, can cause a chemiluminescence signal in high doses [43]. It therefore should be used in small amounts. The more general problem of potentially decreased specificity caused by control solvents and vehicles can be addressed by verifying the lack of signal from these agents alone. It can be determined whether a particular signal is derived from NO chemiluminescence by placing an in-line "scrubber" (containing a 1M Fe^{2+} solution) between the purge vessel and the reaction chamber. The scrubber will remove NO within the headspace gas but not sulfoxides. This will significantly decrease the signal if it is in fact due to NO. A second method, which can be used with liquid samples, entails adding a substance to the reflux chamber that will rapidly inactivate NO. For example, the addition of reduced hemoglobin extinguishes NO within one minute [44].

Other oxides such as nitrogen dioxide have much higher energies of activation, and therefore react with ozone much more slowly. As a result, the presence of substances such as NO_2, CO_2, CO, C_2H_4, NH_3, H_2O and SO_2 in the reaction chamber do not significantly detract from the specificity of the assay for NO [52]. However, H_2S, as might be detected in the fermentation process, can be detected by chemiluminescence quite readily, potentially complicating interpretation of the assay in certain industrial applications. Indeed, a chemiluminescence assay similar to that for NO is used by beer manufacturers to monitor sulfur compounds in their products that give beer an unpleasant after-taste. However, in measuring sulfur compounds a stainless steel burner is used to convert them to sulfur monoxide (SO). The PMT then detects the light produced by the subsequent chemiluminescence reaction of SO with ozone.

The chemiluminescence reaction of NO and ozone occurs only in the gas phase. However, NO can detected in aqueous samples because of the tendency for NO to leave the aqueous phase and enter the gaseous headspace over the aqueous sample. The head space gas, over an aqueous sample, thus can be sampled to estimate the amount of NO that was in the liquid (described in detail in [44]).

The NO chemiluminescence analyser

In 1970, Fontijn, Sabadell and Ronco built the first NO chemiluminescence analyser [52]. Many brands of NO chemiluminescence analysers are commercially available, but all share fundamental components in common and all are basically luminometers. The essential structure includes an ozone generator, a reaction chamber (where ozone and the sample are admixed), and a sensitive, cooled photomultiplier, for light detection (Fig. 2).



Eq 1: $NO + O_3 \rightarrow NO_2^* + O_2$

Eq 2: $NO_2^* \rightarrow NO_2 + h\nu$

Figure 2. Design of a typical NO chemiluminescence analyzer.

A vacuum pump draws ozone and sample into chemiluminescence reaction chamber. In the reaction chamber, NO from the sample reacts with ozone and the emitted light is detected by cooled PMT and recorded. Ozone is created by electrical discharge in the ozone generator. The inflow rate of the sample gas is regulated by the needle valve with the aid of an optional flowmeter. A red cut-off filter increases specificity of NO detection.

The light signal is converted to an electrical signal that is then converted to a digital signal and displayed on a computer. We use the MacLab analog-digital recorder (A&D Instruments Milford, MA) and a Macintosh computer (Apple Computer, Cupertino, CA) (Fig. 2). Although many analysers are effective, the Sievers NOA 280 (Sievers Instruments Boulder, Colorado) has a solid record for sensitivity and reliability.

The reaction chamber typically has two inputs. The first carries ozone into the reaction chamber. Ozone is generated within the NO analyser by high-voltage electrostatic discharge into 95 % oxygen or compressed air which is supplied to the machine at ~ 7 psi. The oxygen supply to the ozone generator should be constant to ensure reproducible and predictable results. In general, the amount of O_3 produced greatly exceeds that required by the assay and it is not a limiting factor. The second input to the reaction chamber is the sample, which is entrained into the reaction chamber under vacuum (1-15 mmHg) *via* a gas-impermeable tube. An external pump connected to the exhaust line leading out of the reaction chamber creates the vacuum. In addition to drawing the sample into the chamber, the vacuum enhances the chemiluminescence signal by removing molecular quenchers from the chamber. A second effect of the vacuum is to stabilise NO by removing oxygen. This is desirable because O_2 very rapidly converts NO to NO_2, which does not produce measurable light upon contact with ozone.

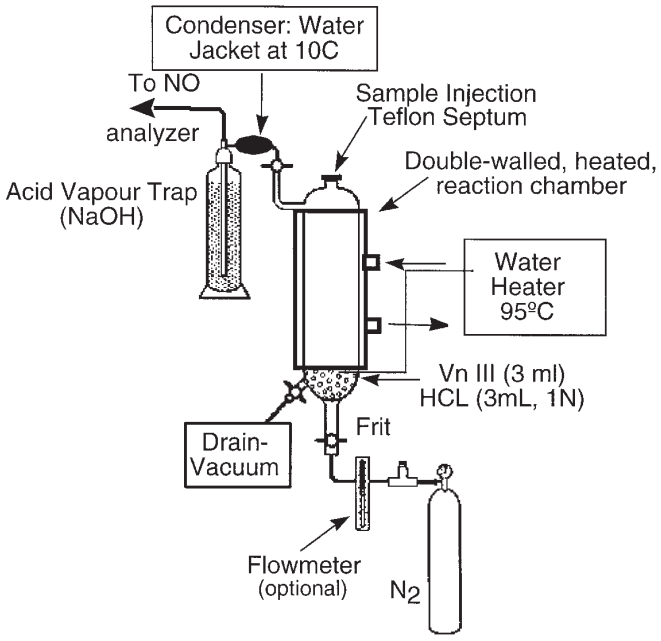
The sample flow rate is governed by a valve, which should be adjusted so that injections of known concentrations of NO yield rapidly rising peaks without slurring of the signal. The sample flow rate should also be kept constant to ensure consistent and predictable results; an inline flow meter may facilitate achieving consistent flow rates. When measuring NO from liquid samples, flow is also governed to a degree by a reducer frit in the inflow line, which acts to prevent excessive flows due to the powerful vacuum. If this frit should become clogged with particulate matter, it profoundly slows the response time to NO (manifest by slurred signals).

NO_2^* emits relatively weak red and infrared light. The light detector is typically a photomultiplier tube (PMT), chosen because it is sensitive to low levels of light at the red end of the spectrum. In the photomultiplier, photons strike a photosensitive surface and the impact releases electrons that are accelerated toward an electron-sensitive surface (the first dynode) by an electrical field. Each electron's impact causes emission of several electrons from the first dynode, and these are accelerated towards a second dynode. This step is repeated several times and finally the electrons are attracted to the terminal electrically charged element, the anode. The resulting current is measured. The amplification of this cascade is in the order of millions of electrons at anode for each electron emitted from the photosensitive surface.

Besides its high sensitivity, another advantage of the photomultiplier over alternative light detection devices is the stability of dark current (the background output of the photomultiplier). Several factors can affect dark current, including temperature and light and voltage history. For this reason it is desirable not to expose the analyser to excessive amounts of NO, as this leads to enormous light emissions and increased dark current. Disturbances of dark current may take days to stabilise [59]. A red cut-off optical filter is typically placed in front of the photomultiplier tube to improve specificity by eliminating photons from extraneous luminescent reactions.

The PMT's dark current doubles with every 100 °C increase in temperature [59]. To reduce noise, most NO chemiluminescence analyzers are equipped with a cooler that keeps the photomultiplier temperature low (usually at about –20 °C). Coolers typically maintain the photomultiplier temperature at a fixed level below the ambient temperature. For this reason the NO chemiluminescence analyser should be used in a temperature controlled room. Otherwise not only will the background noise increase with a rise in ambient temperature, but also the analyser sensitivity may be altered, as an increase in dark current may be associated with reduced photomultiplier sensitivity [59]. In the event that assay sensitivity decreases over time, it is important to establish that the PMT temperature is stable. If poor sensitivity persists, despite a stable and low temperature, one can open and clean the reaction chamber. This is rarely necessary unless the system has been flooded with humid gases or overflow of liquid specimens. In this scenario the light transfer to the PMT, which is separated from the reaction chamber by a filter, can be diminished. Exhaust from the reaction chamber (ozone, NO, NO_2) is externally vented after being passed through a charcoal filter. The filter eliminates most of these species, protecting the operator from exposure.

While measurement of NO from gaseous samples simply involves direct injection of the sample into the reaction chamber, measurement of NO from liquid samples requires the additional step of "stripping" the NO from the liquid sample. The liquid sample is placed into a glass purge vessel, whose bottom contains the reducer frit (Fig. 3). An inert gas such as N_2 is passed through the frit and bubbled through the liquid sample. Optimal flow rate for the inert gas is ~ 8-10 ml/min, and this can be judged by adding an in-line flow meter to the circuit. The stripping, together with NO's high partition coefficient, ensure that most NO in the liquid sample rapidly enters the head space gas above the liquid. This head space gas is then drawn into the reaction chamber by the vacuum. Complications of the stripping include formation of foam that can be drawn into the reaction chamber and coat the surface of the window to the photomultiplier tube, decreasing the sensitivity of the assay. Proteinaceous fluids are particularly problematic and an antifoaming agent may be required. We use the very inexpensive Dow compound (FG-10; Dow Corning, Midland MI). Additional components may include an acid vapor trap placed in series between the purge vessel and the reaction chamber. This trap eliminates acid vapor from the head space gas generated by adding acids and reducing agents to



$$NO_2^- + 2H^+ + e \rightarrow NO + H_2O$$
$$NO_3^- + 4H^+ + 3e \rightarrow NO + 2H_2O$$

Figure 3. A purge vessel designed to reduce NOx to NO and to allow stripping of NO from liquid to gas phase.

The double-walled, glass purge vessel is perfused by a recirculating water bath, which can be heated. The sample to be analysed is injected through a gas-tight, Teflon® septum. Inert gas (typically N_2) from a tank flows into the purge vessel through a frit in the vessel's bottom, stripping NO from the sample into the headspace and into the analyser. A valve, optionally monitored by a flowmeter, is used to set and maintain appropriate flow rate of the stripping gas. The same purge vessel is used to convert nitrite and nitrate into NO. When measuring NO_2^-, acid and a reducing agent, such as KI or vanadium (III), is added to the vessel prior to the sample. An acid-vapour trap should be used to prevent NO analyser damage. If the reduction is performed using weak acids or at room temperature, only nitrite is measured. If one uses vanadium chloride at 95C, all NOx species are reduced and measured. An external vacuum accomplishes removal of the reaction mixture after the analysis.

the sample liquid in order to facilitate measurement of NO_2^- and NO_3^- in the liquid (discussed below). An in-line drier to remove humidity may improve signal levels by 10-15 % [43].

Assay calibration, sensitivity and specificity

Gaseous or aqueous solutions of NO can be used to calibrate the PMT, and the methods for preparation of such standard solutions are described in detail elsewhere [44]. A calibration curve is generated by plotting the detected signal from the PMT versus the dose of NO standard delivered. Doses of NO used are typically in the ranges of 20-1000 pmol. The curve is then used to estimate the amount of NO in an experimental sample. The advantage to using a gas standard is that there is no need for complicated apparatus necessary to generate saturated NO solutions and no error introduced by incomplete stripping of NO into the head space gas. A second advantage of gas calibration over calibration using acidification of nitrite is that, with gas calibration, there is no error due to incomplete reduction of NO_2^- to NO. Since the chemiluminescence reaction is quite linear, most modern analysers require a simple two-point calibration. When calibrating to measure low levels of NO we use N_2 (0 ppb NO) and an NO standard (100 ppb NO). For applications, such as monitoring therapeutic administration of NO we use N_2 and a more relevant NO standard (1-10 ppm).

When constructing the calibration curve, one can either choose the peak height or area under the curve ($mV \cdot s$) as a measure of chemiluminescence signal intensity. The advantage of peak height is simplicity, but this technique is more prone to variability, particularly if sample flow rates vary. If the area under the curve is used, the integration time, or the time during which photons are counted before a value is presented and counting resumes, can be varied to optimise the assay for weak versus strong signals.

It is important that each laboratory establish standard curves to establish the sensitivity of their instrument to NO, both for reporting in the literature and for comparisons made over time. Demonstration of a reproducible calibration curve excludes most of the common NO measurement problems, including loss of PMT sensitivity, contamination of the optical pathway, inadequate cooling of the PMT and impaired ozone generation. Most investigators report the detection threshold of the chemiluminescence assay for NO is 20-50 pmol, in liquids [6,56,57] and 1 ppb in gas. Some authors report that the chemiluminescence/NO curve is linear over the range 300-3000 pmol [60,61], while others have found a degree of nonlinearity with increasing levels of NO [43].

Measurements of NOx in plasma

To measure NOx in plasma one should acquire and centrifuge blood to remove the serum and then ideally remove all proteins by precipitation (with alcohol or another denaturing agent). However, measurements can be obtained with either serum or plasma. In a recent study we found that normal volunteers, on nonrestricted diets, had plasma NOx levels of $6 \pm 1 \times 10^{-5}$ M [62].

Measurement of NO_2^-

In the presence of acid (*e.g.* acetic or hydrochloric acid) and a reducing agent (*e.g.* sodium or potassium iodide, NaI or KI), NO_2^- can be completely reduced to NO. The chemiluminescence assay for NO can therefore serve as an assay for NO_2^- if the liquid sample is first reduced in a reflux chamber (Fig. 3). For NO_2^- measurement, samples (0.1-0.2 ml) are added to the purge vessel which contains 3 ml of a stock

KI solution (3M) and 3 ml of acidic or hydrochloric acid (1N). KI and acid should be replenished, though, before the pH of the reagent mixture rises more than by ~1 unit [63]. Since these solutions cause a transient chemiluminescence signal, a period of time, during which the reagents are gently refluxed, must elapse for the signals to decay prior to measurements being made. Because of this transient signal, it is more efficient to add an excess of these agents to the reflux chamber, allowing one to make several measurements before needing to replenish the reagents. The HCl and KI do not cause chemiluminescence on their own, but do cause a large, reproducible spike that decays over 1 minute when mixed together. The mechanism of this signal is unknown. To avoid this, we recommend purging the solution without running the headspace gas through the NO analyser for 5-10 minutes. Since virtually all water contains NO_2^-, the signal from background NO_2^- levels in the solvents should always be accounted for by background subtraction from the experimental value. This problem can be avoided by using special relatively NO_2^--free water.

The chemiluminescence assay for NO_2^- is typically 10-fold less sensitive than the assay for NO [44,60], perhaps reflecting incomplete reduction of NO_2^- to NO. Acids other than HCl (*e.g.* acetic acid, sulfuric acid, and phosphoric acid) have been used [61] but HCl seems to offer slightly increased sensitivity [44]. Several factors must be optimised to ensure reproducible measurements, including the amount of acid and KI used and the rate of reflux. The chemiluminescence of NO_2^- increases in proportion to the concentration of KI up to 0.5 mol/l, and with sulfuric acid concentrations up to 0.18 mol/l, at which point the curves plateau [61]. The use of at least 1 mol/l of KI and 0.18 mol/l of sulfuric or hydrochloric acid optimises the assay from nitrite detection [61]. Other biological NO adducts (*e.g.* RSNOs and nitrosamines) are also reduced to NO under the same conditions. The presence of these compounds may therefore decrease the specificity of the assay for nitrite.

Measurement of NO_3^-

The predominant NOx species in human blood is NO_3^-, and it can me measured by a variety of assays, including the Griess reaction (modified so that nitrate reductase enzyme is used to convert any NO_3 to NO_2 during the assay. The chemiluminescence assay is more sensitive for nitrate than the Griess reaction when performed with careful attention to the strength of the reducing environment. The addition of stronger reducing agents to the solution (*e.g.* 1M Vanadium III) will reduce NO_3^- to NO, and allow the measurement of NO_3^- levels via chemiluminescence [63]. To fully reduce NO_3^-, it is necessary to heat the purge vessel to 80-95 ºC (Fig. 3). Under these conditions, both NO_3^- and NO_2^- in the sample will be reduced to NO and measured. Thus the signal is often referred to as NO*x*, the sum of all nitrogen oxides in the sample.

Vanadium (III)-HCL mixture itself creates a chemiluminescence signal. To avoid this confounding signal, one should reflux 3 ml of each reagent in the purge vessel until stable baseline signal is achieved, usually within 15-30 minutes. If experimental samples are small (10-100 µL), 20-30 measurements can be used before the vanadium/HCl must be replenished. Since the heated HCL can damage the reaction chamber, it is imperative that an acid-vapor trap (a saturated solution of sodium hydroxide) be placed proximal to the reaction chamber (Fig. 3).

Normal values for NOx

Tanaka *et al.* reported normal plasma NOx values of $4.9 \pm 2.3 \times 10^{-5}$ M (49 µmol/l) [64] and also noted that NOx levels were lower for patients with hypercholesterolemia. They also found temporal variability in NOx levels, which were most reproducible and lowest at 0600 [64]. This confirmed the work of Nakashima, who found that treatment of hypercholesterolemic adults with the cholesterol lowering agent, Simvastatin, increases plasma NO*x* (from 8 ± 17 µmol/l to 57 ± 32 µmol/l), within 12 weeks, in a manner that correlated with the rise in high density lipoprotein [65].

Dietary ingestion of nitrogen oxides

Certain food are particularly enriched in nitrogen oxides (nitrate) and thus it is ideal to standardize the diet of a research subject for several days (at least four), prior to measuring plasma or urine nitrogen oxides [66]. By excluding the following foods dietary nitrate ingestion can be minimised: cured meats, fish, cheese, vegetables, melons, strawberries, herb or black teas, beer, wine, malted beverages, commercial sauces, and certain types of water (well water, spring water, and mineral water) [66,67].

Breath NO

Interest in measuring exhaled NO as a marker of airway inflammation has increased dramatically since it was demonstrated that NO levels are increased in the exhaled breath of asthmatic patients [13,68]. Exhaled NO levels have been reported to be increased in both chronic obstructive pulmonary disease (COPD) [21] and bronchiectasis [69] as well. Exhaled NO measurement is an appealing technique in the management of inflammatory lung diseases for two main reasons. First, exhaled NO measurement directly measures a surrogate for airway inflammation, which conventional pulmonary function testing, peak flow measurement, or symptom diaries do not [70,71]. Perhaps more importantly from a practical point of view, exhaled NO measurements are non-invasive and can be made much more reproducibly than pulmonary function testing in children and infants [72,73]. Because nasopharynx and paranasal sinuses account for a large portion of exhaled NO [49], it is important to use standardised techniques when collecting the samples. Examples of techniques to exclude nasal NO include exhaling slowly against a resistance to close the soft palate, and the use of a "non-rebreathing mask" that occludes the nose. A restricted exhaled breath technique is one approach to overcome potential variable contamination of lower airway breath by nasal or sinus air [74]. The technique uses

expiratory resistance to elevate mouth pressure and close the vellum to eliminate nasal contamination. Furthermore, by monitoring and displaying the mouth pressure, the subject can maintain a constant pressure resulting in a constant expiratory flow rate. This technique may improve reproducibility, although in our experience most adult subjects produce similar results simply using a two-way, non-rebreathing facemask that excludes nasal air (Hans Rudolph Inc, Kansas City, Missouri). Standard techniques for collection of samples reflecting the lower respiratory tract and the upper respiratory tract (nasopharynx), including methods involving bag storage of the sample and offline measurement, have been described elsewhere [49,75-78].

One of the largest pitfalls of measuring exhaled NO is that ambient air may contain a variable amount of NO. Human subjects in clinical studies breathing ambient air will thus inhale varying amounts of NO. These levels usually are in the 1-100 ppb range and ambient NO levels may exceed endogenous levels. Since NO, like carbon monoxide is rapidly taken up by the capillary network in the lung, the subject is consuming NO. Under such conditions it is difficult to measure NO production accurately and moreover, reproducibility suffers (Fig. 1). Thus the subject should breathe a medical air which is free of NO for at least a minute prior to the measurements. If ambient NO levels in a laboratory are consistently low (less than 20 ppb) the correlation between lung NO production (minute ventilation X NO concentration) measured during respiration of ambient air correlates well with that measured while the subject breathes NO-free air. However, above 50 ppb, this relationship progressively deteriorates (Fig. 4). Ideally, all NO measurements should occur with the subject breathing NO-free air.

To improve reproducibility it is ideal to report lung NO production, rather than NO concentration. This is done using the following formula for VNO at standard temperature and pressure, where V_E is the minute ventilation, 0.826 is a conversion factor to adjust volumes to standard temperature and pressure, and $[NO]_{exh}$ is the exhaled NO concentration (in ppb).

$$V_{NO} = 0.826\ V_E \cdot [NO]_{exh} \qquad [7]$$

Normal values for breath NO

In a recent study we found the V_{NO} to be 40 ± 10 nL/min with breath NO concentrations of 7 ± 2 ppb in normal individuals [62]. Riley *et al.* reported a normal value of 101 ± 68 nL/min for V_{NO} in normal volunteers [79]. The NO concentrations and V_{NO} vary amongst species [80]. VNO levels are also reduced in humans with congestive heart failure. Adachi *et al.* reported V_{NO} was 45.3 ± 24.3 nl/min in CHF patients versus 117.5 ± 60.1 nl/min in normal subjects [81]. Furthermore, while V_{NO} did increase during exercise, as expected, it did not increase in CHF patients as much as in normal subjects (75.3 ± 43.4 nl/min *vs.* 512.9 ± 253.6 nl/min, respectively [81]. Bernareggi *et al.* reported that rabbits exhibited the highest exhaled NO concentrations and V_{NO} (12.9 ± 1.0 ppb, VNO 9.0 ± 0.7 nl/min), followed by guinea pigs (6.2 ± 0.70 ppb, V_{NO} 1.7 ± 0.19 nl/min) and rats (0.9 ± 0.01 ppb, VNO 0.25 ± 0.00 nl/min) [80].

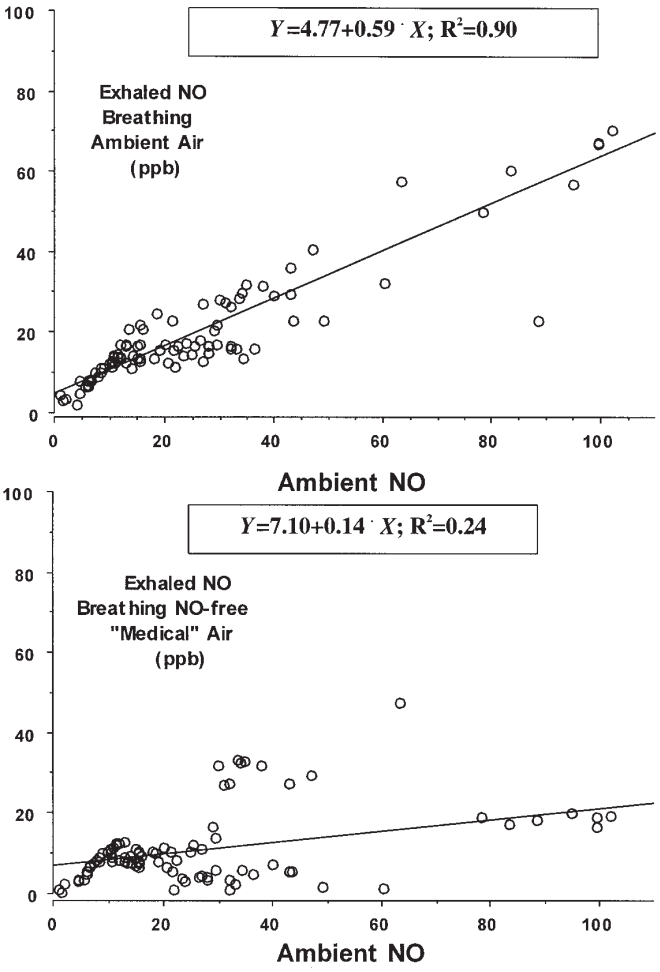


Figure 4. Variation of atmospheric NO levels, in Paris, France, complicate measurement of breath NO.

This figure shows how ambient or atmospheric NO levels vary over time and by day in the Paris air. Measurements were made at L'Hôpital Cochin (Paris, France) in 1997. All measurements were made in normal, nonsmoking volunteers. A non-rebreathing mask designed to exclude nasal air was used. Note that that exhaled NO measurements vary in direct proportion to ambient NO content when subjects breathe room air. This does not provide a true reflection of lung NO production. However, when the subject breathes medical air (NO-free air), the true expired lung NO concentrations can be measured without artifact caused by atmospheric conditions.

Nasal NO

The nose [49] and paranasal sinuses [82] are the most abundant sources of NO in the human airway. Thomas *et al.* recently reported nasal NO concentrations of 987 ppb (95 % CI, 959-1,015) [82]. However, there have been

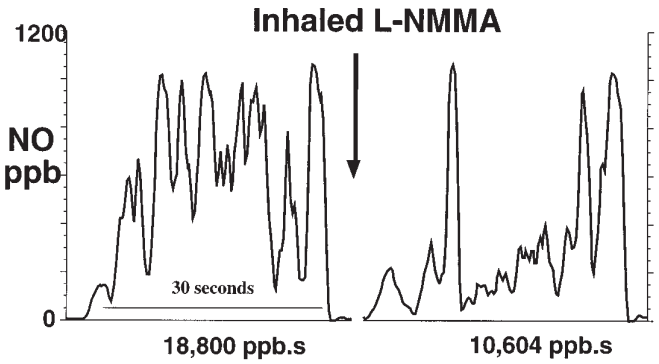


Figure 5. Inhaled L-NMMA reduces nasal NO production.

This is a representative trace showing the effects of inhalation of a NO synthase (NOS) inhibitor, NG-monomethyl-L-arginine (L-NMMA dose chose based on [85], ~490 mg). Note that L-NMMA decreases nasal NO within 5 minutes of administration. Also note the typical variability in the size of the signal during a 30-second apnea. The cause of this variability is uncertain, but does not appear to be related to obstruction of the sampling tube or variability in flow rate.

reports, consistent with our experience, that nasal NO measurements are quite variable within a subject and thus large differences must be observed (> 25 %) to be confident that the results are not simply measurement error [83]. Baraldi *et al.* have reported that the normal values for nasal NO concentrations was 216 ppb in children (95 % C.I., 204-228 ppb) [84]. In figure 5 one can see the immediate inhibitory effect of inhaled L-NMMA on a subject's nasal NO levels. We have previously shown that nasal NO levels are unrelated to bacteria in the nose, although they can be reduced somewhat by inhaled steroids, suggesting a contribution of the inducible isoform of NO synthase [49].

Monitoring of NO therapy (ICU)

The levels of NO used therapeutically 1-80 ppm, are an order of magnitude greater than those produced in the lung. These levels can be monitored accurately by less expensive electrochemical analysers.

Conclusion

The direct measurement of NO not only satisfies the scientific desire for precision, but it also allows for better research and therapeutic possibilities. As we further elucidate the complex interactions between NO and other physiologic agents, it will become increasingly important to identify the presence and to quantify NO. It is clear that no one method for measuring NO is optimal for all settings. Chemiluminescence is a very sensitive and specific method for measuring NO. It is likely the method of choice for the measurement of NO in the gaseous phase. Nevertheless, the chemiluminescence does not readily allow real-time, measurements of NO production from small arteries, a role better served by NO microelectrodes (Fig. 6).

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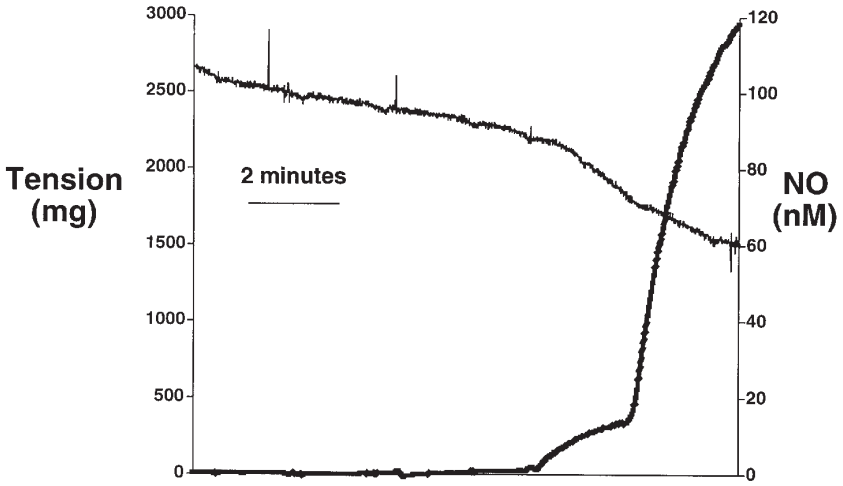

Figure 6. Real-time correlation of NO and vascular tone in a human internal mammary arterial ring using a microelectrode.

A NO microelectrode was used to measure the amount of NO produced when a known NO donor, diethylamine NO [86,87] was added to a tissue bath containing a human internal mammary arterial ring. Note the release of NO slightly precedes the onset of relaxation. This is an example of the type of application for which chemiluminescence methodology, though possible [35], is suboptimal. Measurements were made using a NO electrode ISO-NO Mark 2 (World Precision Instruments, Sarasota, Florida) using a 30 µm electrode tip.

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