

3. Absorption and metabolic fate of nitrogen oxides

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The health effects of an air pollutant are determined by its absorption, transport and metabolism. As the respiratory organs first come into contact with NO_x , nitrogen oxides, a short introduction on basic data on the respiratory organs follows.

Basic data on the respiratory organs

Because of the irregular structure of the nose, the air breathed in through this organ is much more effectively heated and moistened than that inhaled through the mouth. The mucous membranes of the nose, trachea, and bronchi are provided with ciliated cells. Through ciliary action a thin layer of mucus is propelled backwards in the nose and upwards in the trachea and the bronchi. In addition, there are glands consisting of a single cell (goblet cell, Clara cell) and glands located beneath the mucous membrane (submucosal glands). The airways in the lung are surrounded by muscles that contract them when they are irritated.

The lung alveoli and the finest airways have no ciliated cells. The walls of the alveoli consist of two types of epithelial cells. One of them, known as alveolar type I cells, together with the cells in the capillaries, form the air-blood barrier. The alveolar type II cells line the inner surface of the alveoli and secrete surfactants. Through its property of lowering the surface tension, the surfactant layer stabilizes the alveoli and promotes normal gas exchange. When the type I cells are damaged, type II cells begin to divide and are converted to type I cells. The alveoli also contain macrophages, which are able to move freely in the fluid layer of the alveoli and to phagocytize any inhaled particles and cellular debris. Particles such as bacterial and fungi spores can be decomposed by macrophages.

The greater the solubility of a gas in water, the higher up in the airways the gas is absorbed. Sulfur dioxide, for example, which is very water soluble, is absorbed mainly in the nose and the upper airways. The less soluble nitrogen dioxide (NO_2) is taken up at all levels of the respiratory tract. Gases having a low solubility in water, such as nitric oxide (NO), descend to the alveoli, and, in contrast to NO_2 , they are not very reactive with the lung tissue, entering the blood stream and possibly giving rise to adverse systemic effects.

Absorption in the respiratory tract

Inhalation studies have been performed to determine the absorption of NO_2 in concentrations comparable to those in urban air. The total uptake of the respiratory tract has been measured in healthy and diseased humans. In healthy humans exposed to 564-13 550 $\mu\text{g}\cdot\text{m}^{-3}$, 81-90% of the NO_2 was absorbed during normal ventilation (1). When the inhaled and exhaled NO_2 concentrations were measured in adult asthmatics exposed to 13 550 $\mu\text{g}\cdot\text{m}^{-3}$, average uptake during rest was 73% (2). With maximal ventilation the absorption was 90% for both the healthy subjects and the asthmatics.

The distribution of $^{13}\text{NO}_2$ inhaled for 7 to 10 min by rhesus monkeys was investigated by Goldstein et al (3). They concluded that inhaled NO_2 was distributed throughout the lungs and that 31-50% was absorbed. Uptake has been measured in the upper respiratory tract of dogs, rabbits, and rats (4). The uptake values ranged from as low as 25% to as high as 85% depending on the study, species, airflow rate, and mode of breathing (nasal or oral).

Postlethwait & Mustafa (5) measured the uptake of NO_2 by an isolated ventilated perfused rat lung. The lung was exposed for 90 min to an NO_2 concentration of 9400 $\mu\text{g}\cdot\text{m}^{-3}$. Thirty-six percent of the ventilated NO_2 was retained in experiments carried out both with and without erythrocytes in the perfusate. In a later and similar experiment (6), with the exposure concentration ranging from 7520 to 37 600 $\mu\text{g}\cdot\text{m}^{-3}$, it was found that the quantity of NO_2 retained was linearly related to the inhaled quantity of NO_2 ; the percentage of uptake ranged from 60 to 72% with an average of 65%. This result differed considerably from that of the first experiment, and no reasons for the differences were given.

Yosida et al (7) found that <10% of the NO "inhaled" by isolated perfused rabbit lung was absorbed. However, the results obtained with a perfused lung would be expected to differ from those obtained with a living system. With regard to NO absorption into the living body, Wagner (1) reported that more than 80% of NO was absorbed in normal breathing and more than 90% was absorbed in deep breathing when concentrations of 620-9400 $\mu\text{g}\cdot\text{m}^{-3}$ were inhaled. Similar results have been reported (8) after the inhalation of an NO_2 concentration of 18 800 $\mu\text{g}\cdot\text{m}^{-3}$ (rats). Thus respiratory tract absorption of NO is

similar to that of NO_2 in spite of solubility differences. The lower solubility of NO may, however, result in greater amounts reaching the pulmonary region, where it then diffuses to blood and reacts with hemoglobin (8). In fact, exposures in vivo seem to indicate that NO has a faster rate of diffusion through tissue than does NO_2 (9).

Transformation in the lung

Once deposited, NO_2 dissolves in the lung fluids, and various chemical reactions occur that give rise to products found in the blood and other body fluids. In the study of Goldstein et al (3), in which rhesus monkeys inhaled $^{15}\text{NO}_2$, the authors suggested that the persistence of pulmonary ^{15}N radioactivity at virtually unchanged levels throughout the postexposure period confirms a binding of the pollutant or its chemical derivatives to pulmonary tissues. They proposed that such binding probably results from the interaction of inspired NO_2 with water in the vapor or liquid phase to form nitric (HNO_2) and nitrous (HNO) acid.

The chemical reactivity, and presumably cytotoxicity, of NO_2 is the result of its existence as a nitrogen-centered free radical. Studies on isolated perfused rat lungs have demonstrated that inhaled $^{15}\text{NO}_2$ is absorbed in a manner proportional to its delivery rate, and it is converted to nitrite (NO_2^-) as a consequence of its pulmonary uptake (5, 6). In isolated perfused lungs ventilated with NO_2 -air mixtures, NO_2 -nitrogen appears as NO_2^- in the vascular space (70% of absorbed NO_2) and tissue cytosol (30%). In addition, NO_2 -nitrogen weakly binds to insoluble tissue elements, does not form lipid-based stable addition products, and accounts for all absorbed NO_2 . The distribution and binding of NO_2 -nitrogen, as mimicked by the air-space instillation of NO_2^- , suggest that the univalent reduction of absorbed NO_2 to NO_2^- is quantitative and occurs near the air space surface (10). Thus this evidence links pulmonary absorption to both the inspired concentration and direct reaction product formation. Postlethwait & Bidani (11) showed that the rate of NO_2 removal from air spaces is, in part, limited by reaction(s) between NO_2 and epithelial surface components. NO_2 uptake was shown to be saturable and temperature-dependent. These characteristics argue against simple aqueous physical solubility as the predominate mechanism governing NO_2 removal from the air space phase. Because the initial site of primary absorption interactions involves the epithelial lining fluid, Postlethwait et al (12) investigated whether interactions between epithelial lining fluid (obtained from rats via bronchoalveolar lavage) and NO_2 could account for pulmonary NO_2 reactive absorption. They found that the rate of NO_2

uptake into the epithelial lining fluid of rats, like intact lung, is limited, in part, by chemical reaction(s).

In a later study, Postlethwait et al (13) showed that, in the presence of reactant solutes, unreacted NO_2 does not penetrate through the epithelial lining fluid. Reactive absorption must therefore occur primarily within the compartment of the epithelial lining fluid so that the reaction products that induce subsequent toxicity are generated as a result of the initial uptake interactions.

Meyer et al (14) determined the pulmonary diffusing capacity for NO in dogs. The unique features of NO for lung diffusion studies result from the extremely high affinity of NO for hemoglobin. Hence, uptake of NO in blood could be considered to be determined by diffusion alone, because the chemical reaction is not limiting (15). However, Meyer et al (14) found that factor(s) other than the uptake in pulmonary capillary blood was responsible for the loss of NO from the gas phase. One possible mechanism for the loss could be the transformation reaction of NO to NO_2 in the presence of oxygen. However, the authors concluded that literature data, as well as their own experiments, clearly showed that the transformation reaction in the gas phase is considerably slower than pulmonary NO uptake. The role of the absorption or chemical transformation of NO in lung tissues is more difficult to assess. The transformation rate of NO in Krebs buffer in contact with tissue has been determined in a vasodilator bioassay set-up consisting of rabbit aorta strips (16). The results indicated that tissue contact can be rather effective in removing NO through chemical reactions. However, no data are available for lung tissue.

Metabolites in blood and other body fluids or organs

Nitrogen dioxide

Once deposited, NO_2 dissolves in lung fluids, and various chemical reactions occur that give rise to products found in the blood and other body fluids.

Using estimates of radiolabeled nitrate (NO_3^-) and nitrite (NO_2^-) levels in the blood and urine of rabbits following exposure to NO_2 , Svorcova & Kaut (17) suggested that inhaled NO_2 enters the blood stream. When studying isolated perfused rat lungs Postlethwait & Bidani (10) found that 70% of absorbed $^{15}\text{NO}_2$ appeared in the perfusate, 30% appeared in the lung tissue, and that NO_2^- accounted for the ^{15}N -nitrogen.

Intratracheally instilled NO_2^- has been shown to be rapidly absorbed into blood from the lungs (18). Oda et al (19) noted a dose-dependent increase in both NO_2^- and NO_3^- levels in the blood of mice during exposures to NO_2 . The blood levels of NO_2^- and

NO_3^- declined rapidly after the exposures ended, with decay half-times of a few minutes for NO_2^- and about 1 h for NO_3^- . The shorter time for the former was ascribed to its rapid reaction (oxidation) with hemoglobin, producing NO_3^- and methemoglobin, although such measurements were not made.

Oda et al (20) exposed mice to an NO_2 concentration of $75\ 200\ \mu\text{g}\cdot\text{m}^{-3}$ and determined nitrosyl-hemoglobin (NOHb) and methemoglobin (MetHb). Only 0.2% of the NOHb was found, and MetHb did not increase. A linear relationship between the concentration of NO_2 and NOHb could be observed; MetHb did not increase at any concentration of NO_2 .

Interpreting the data for NO_2 exposure is difficult. An NO_2 concentration of $75\ 200\ \mu\text{g}\cdot\text{m}^{-3}$ or lower produced MetHb with hemolysate or red blood cells in vitro, while NOHb was not detected photometrically (21). As for NOHb, nitrite, which is known to be formed from NO_2 , induced the formation of NOHb in blood suspension, and the reaction was inhibited by the addition of monoiodoacetic acid (22). This phenomenon suggests that enzymatic processes are involved in the formation of NOHb.

Nitric oxide

The strong affinity of NO to hemoglobin has been studied in relation to air pollution. In vitro data indicate that the affinity of hemoglobin to NO is several thousand times higher than for carbon monoxide. The increasing affinity from oxygen to carbon monoxide and NO is due mainly to a decrease of the dissociation velocity constant, which varies about $2 \cdot 10^5$ times from oxygen to NO. Furthermore, NO constitutes the most rapidly binding ligand to hemoglobin so far discovered. The velocity constant of a combination of NO and hemoglobin is about 280 times faster than that for carbon monoxide, and also about five times higher than that for oxygen (15). It has also been shown that NO produces NOHb in the absence of oxygen, and MetHb in the presence of oxygen, and that NOHb is easily and rapidly oxidized to MetHb in the presence of oxygen (9, 23). In vitro measurement of oxygen dissociation curves for partially NO-liganded human hemoglobin have shown that NO binding tends to reduce the dissociation of bound oxygen on the molecule (23). In a study by Wennmalm et al (24), plasma or whole venous or arterialized blood from healthy human donors was incubated with NO, and the resulting formation of MetHb, NOHb, and plasma nitrite and nitrate was measured. In plasma, NO was converted to nitrite and nitrate in a ratio of 5:1. In arterial blood (oxygen saturation 94-99%), NO was almost quantitatively converted to MetHb and nitrate. No nitrite was detected and NOHb formation was low. In venous blood (oxygen saturation 36-85%), more NOHb and less nitrate was formed, in comparison with the corresponding formation in arterialized blood.

In an in vivo study, Oda et al (20) exposed mice

to 40 ppm of NO and found that NOHb became constant (0.7%) within 30 min and declined rapidly with a half-time of several minutes when the mice were removed to room air. An increase of MetHb (5%) was also caused by exposure to NO, and the time course was almost the same as that of NOHb. A linear relationship could be observed between the concentrations of the NO and NOHb. There was an exponential increase in MetHb after NO exposure and a much higher content of MetHb than that of NOHb, particularly at a high concentration of NO. The results showed that the amount of NOHb in the blood of animals exposed to NO was much smaller than expected from in vitro data.

Lifetime (23 to 29 months) exposures of mice to an NO concentration of $4500\ \mu\text{g}\cdot\text{m}^{-3}$ resulted in the blood content of NOHb remaining relatively steady at 0.01%, while the maximum amount of MetHb was 0.3% (25). Thus, although the results of various studies have shown that the final product of the NO reaction with hemoglobin is MetHb, with some persistent NOHb, this effect of NO is not generally lethal because of the rapid oxidation of NOHb into MetHb, and the subsequent reduction of MetHb into ferrous hemoglobin by MetHb reductase and other enzymes present in red blood cells (26). As long as the activity of MetHb reductase is maintained, the conversion of NOHb into MetHb should mitigate any potentially toxic effect on hemoglobin as a result of NO inhalation. In long-term exposure studies (25), no evidence of hypoxic damage was found in an examination of organs sensitive to oxygen depletion (eg, brain and heart).

Excretion pathways of nitrogen dioxide and nitric oxide

In a comprehensive study on the relationship between inhaled NO_2 and urinary NO_3^- , Saul & Archer (27) exposed rats for 24 h to NO_2 concentrations of 2300 – $31\ 800\ \mu\text{g}\cdot\text{m}^{-3}$. They showed that mostly NO_3^- , with very little NO_2^- , was excreted in the urine. The small amount of urinary NO_2^- appeared to be an artifact originating from an in vitro reaction with urine. The rate and linearity of the conversion of NO_2 to urinary NO_3^- suggested that NO_2 does not form NO_3^- by reacting with respiratory water, but reacts instead with oxidizable tissue to form NO_2^- . Nitrite is then further oxidized in the blood by oxyhemoglobin (28) to form NO_3^- , which is excreted in the urine. NO_2^- and NO_3^- was also found in the urine of guinea pigs exposed to an NO_x concentration of $1000\ \mu\text{g}\cdot\text{m}^{-3}$ (mainly NO_2 , ~0.5 ppm) 8 h a day for 120 d (29).

In their ^{15}N inhalation experiments on rats Yoshida et al (30) demonstrated that ^{15}N stemming from inhaled ^{15}NO was relatively quickly excreted into the urine, and it did not stay in the lung and

other organs. In the experiments, marked increases in the NO_3^- and NO_2^- contents of the serum and urine were noted that suggested that metabolites of NO are NO_3^- and NO_2^- . In a later study (31), it was shown that, of inhaled ^{15}NO , the ^{15}N recovery in urine after 48 h was 55%, 75% of which appeared as NO_3^- and 24% as urea. In the urine of rats injected with ^{15}N -nitrite, about 20% unidentified ^{15}N compounds, not discovered in the inhalation experiment, was found. When ^{15}N -nitrite was injected into mice, 60.7, 7.8 and 0.3% of the injected ^{15}N was found in urine, feces, and exhaled gas (NO , NO_2 and NH_3 in the gas were caught) respectively, up to 48 h after injection, and 1.6% was found in the body 48 h after the injection, but the remaining 30% of ^{15}N could not be recovered.

Sources of nitrite and the formation of N-nitroso compounds

In 1962, Druckrey & Preussman (32) proposed that the stomach could provide the acidic environment required for the formation of N-nitrosamines from NO_2^- and secondary amines. Studies that followed showed that the feeding of amines and NO_2^- led to tumor formation (33).

Humans are exposed to NO_2^- via the inhalation of nitrogen oxides in urban air, via tobacco smoking, and via ingestion. The estimated intake via different sources can be seen in table 3.1.

Ingested nitrate can be converted to nitrite by bacterial reduction in the saliva, the gastrointestinal tract, and the urinary bladder.

In addition to the production of NO_2^- by the bacterial reduction of NO_3^- , it was postulated by Tannen-

baum et al (37) that large amounts of NO_2^- and NO_3^- are endogenously formed in the human body. They found that humans and rats, when fed a low NO_3^- diet, excreted levels well above the ingested amount, and they postulated that NO_2^- and NO_3^- are formed in the intestine, possibly by heterotrophic nitrification. (See table 3.1.) When the same type of experiments were carried out, comparing conventional rats with germ-free rats, the same results were observed, namely, that excretion was greater than ingestion (38). This last finding is important in terms of ruling out gut microflora participation in the reaction.

It has later been shown that most of the endogenously produced NO_2^- and NO_3^- probably originates from the NO synthesized in endothelial cells and in macrophages from the amino acid L-arginine. (For references see reference 36 and chapter 6.)

N-nitroso compounds

Because of evidence that NO_2 can produce NO_2^- and NO_3^- in blood, and NO_2^- is known to react with amines to produce animal carcinogens (nitrosamines), the possibility that NO_2 contributes to the production of N-nitroso compounds has been investigated.

Iqbal et al (39) was the first to demonstrate a linear time-dependent and concentration-dependent relationship between the amount of N-nitroso morpholine (NMOR) found in whole-mouse homogenates after the mice were gavaged with 2 mg of morpholine and exposed to NO_2 .

An extension of the previous study was performed (40) using dimethylamine, an amine that is slowly nitrosated to dimethylnitrosamine. A concentration-related increase in the biosynthesis of dimethylnitrosamine was found to occur at NO_2 concentrations as low as $190 \mu\text{g}\cdot\text{m}^{-3}$.

In vivo nitrosation was also demonstrated by Norkus et al (41) after morpholine administration and a 2-h exposure to an NO_2 level of $84\ 600 \mu\text{g}\cdot\text{m}^{-3}$.

Postlethwait & Mustafa (42) examined the problem of the in vivo production of nitrosamines using isolated perfused rat lung. Rat lungs were ventilated with NO_2 , and the perfusion media was supplemented with 10 mmol of morpholine. An excess of NMOR was found in the NO_2 -exposed group when the lung tissue and perfusate were combined. Control experiments could not exclude the possibility that NMOR was produced in the perfusate.

Another study (43) that gavaged mice with 1 g morpholine $\cdot$ kg body weight $^{-1} \cdot$ day $^{-1}$ and then exposed them to NO_2 revealed that NMOR could be produced in vivo.

Garland et al (44) found results that indicated a positive relationship between the atmospheric NO_2 levels and the urinary excretion of N-nitrosomethylamine in normal human study objects. The urinary excretion of N-nitrosoproline was not correlated with the atmospheric concentration of NO_2 .

Table 3.1. Estimated intake of nitrite (NO_2^-) and nitrate (NO_3^-) from different sources. (NO=nitric oxide, NO_2 =nitrogen dioxide, NH_3 =ammonia)

Source	Nitrate ($\text{mg}\cdot\text{person}^{-1} \cdot \text{d}^{-1}$)	Nitrite ($\text{mg}\cdot\text{person}^{-1} \cdot \text{d}^{-1}$)
Inhalation of urban air (52-570 ppb NO; 25-113 ppb NO_2) (34)	1-9	1-7
Moderate smoker (35) 10 cigarettes a day		0.1-5
Foodstuff (34)	110-180	1-3
Formation of NO_2^- from NO_3^- in the saliva (34)		10-20
Formation of NO_2^- from NH_3 in the gastrointestinal tract (34) or in another way endogenously formed (36)		65-100

The question of whether endogenously derived NO_2^- can be responsible for the formation of N-nitroso compounds has been investigated by Mirwa et al (45). They showed that macrophages from mice treated with LPS (*Escherichia coli* lipopolysaccharide), known to produce NO_2^- and NO_3^- in a ratio of 3:2, and with added morpholine, synthesized NMOR. The levels produced depended on the conditions of the experiment and ranged from 100- to 1000-fold lower than the amount of NO_2^- and NO_3^- synthesized.

In summary, at present, studies on humans and animals have clearly demonstrated that endogenous nitrosation occurs intragastrically and at least at one other extragastric site. Several types of cells, including macrophages, endothelial cells, neutrophils, brain cells and bacteria, produce nitric oxide in vitro under conditions that can be duplicated for the whole animal. Nitrosation by macrophages and bacteria has been demonstrated in vitro. All of these cells, with the exception of bacteria, utilize nitrogen from L-arginine in the production of nitric oxide; see chapter 6. According to the known chemistry for nitrosation reactions, it is possible that NO produced endogenously can react with oxygen and, subsequently, nitrosate secondary amines to produce carcinogenic N-nitrosamines in vivo (46).

Summary and concluding remarks

Present knowledge about the absorption and metabolic fate of the nitrogen oxides NO_2 and NO can be summarized as follows.

Nitrogen dioxide

1. About 80-90% of inspired NO_2 is absorbed in humans.

2. Absorbed NO_2 does not penetrate through the epithelial lining fluid unreacted. Nitric acid is formed via hydrogen abstraction from organic substrate(s) and transformation into NO_2^- ions due to nitric acid dissociation.

3. Most of the absorbed NO_2 enters the blood as NO_2^- . In vitro NO_2 produces MetHb but not NOHb with hemolysates or red blood cells. NO_2^- induces the formation of NOHb in blood suspension, and this reaction is inhibited by moniodoacetic acid, a phenomenon suggesting the involvement of enzymatic processes in the formation of NOHb. In mice exposed to NO_2 , NOHb and MetHb have been determined. Only 0.2% NOHb and almost no MetHb was found. NOHb was linearly related to the concentration of NO_2 , while MetHb did not increase at any NO_2 concentration.

4. Inhaled NO_2 is excreted in the urine as NO_3^- .

When ^{15}N -nitrite was injected into mice, 61% was found in the urine, 8% in feces, 0.3% in the exhaled gas (NO , NO_2 , NH_3) and 1.6% in the body. The remaining 30% could not be recovered.

Nitrogen oxide

1. About 80-90% of inhaled NO is absorbed in humans and rats, and less than 10% is absorbed in isolated perfused rat lung.

2. Most of the absorbed NO enters the blood unreacted.

In vitro studies have shown that the affinity of hemoglobin increases from oxygen to carbon monoxide to NO. NO produces NOHb in vitro in the absence of oxygen and MetHb in the presence of oxygen. NOHb is oxidized to MetHb in the presence of oxygen. In plasma from healthy human donors incubated with NO, NO was converted to NO_2^- and NO_3^- at a ratio of 5:1. In arterial blood (oxygen saturation 94-99%), NO was converted to MetHb and NO_3^- . No NO_2^- was detected. The NOHb level was low. In venous blood (oxygen saturation 36-85%), more NOHb and less NO_3^- were formed.

In vivo data have shown that the amount of NOHb in the blood of animals exposed to NO is much smaller than expected from in vitro data and that, although the final product of NO reaction with hemoglobin is MetHb, this effect (reduction of oxygen transport) was not lethal because of the rapid oxidation of NOHb to MetHb and the subsequent reduction of MetHb to hemoglobin by MetHb reductase.

3. Absorbed NO is relatively quickly excreted in the urine of rats. After 48 h 55% of ^{15}NO -nitrogen is found in the urine, 75% of which appears as NO_3^- and 24% as urea.

N-nitroso compounds

The main source of NO_2^- in the body is formed endogenously. The contribution of NO_2^- from foodstuff is almost equal to that from inhaled urban air. However, foodstuff via the formation of NO_2^- from NO_3^- in the saliva contributes to a higher extent than exposure from urban air does. One study has shown an association between the urban air concentration of NO_2 and the formation of N-nitroso compounds. However, the relative importance of inhaled nitrogen oxides to nitrosamine formation has not been demonstrated.

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