Review

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Nitrous oxide abuse direct measurement for diagnosis and follow-up: update on kinetics and impact on metabolic pathways

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Abstract: Recreational use of nitrous oxide (N_2O) has become a major health issue worldwide, with a high number of clinical events, especially in neurology and cardiology. It is essential to be able to detect and monitor N_2O abuse to provide effective care and follow-up to these patients. Current recommendations for detecting N_2O in cases of recreational misuse and consumption markers are lacking. We aimed to update current knowledge through a review of the literature on N_2O measurement and kinetics. We reviewed the outcomes of experiments, whether in preclinical models (*in vitro* or *in vivo*), or in humans, with the aim to identify biomarkers of intoxication as well as biomarkers of clinical severity, for laboratory use. Because N_2O is eliminated 5 min after inhalation, measuring it in exhaled air is of no value. Many studies have found that urine and blood matrices concentrations are connected to ambient concentrations, but there is no similar data for direct exposure. There have been no studies on N_2O measurement in direct consumers. Currently, patients actively abusing N_2O are monitored using effect biomarkers (biomarkers related to the effects of N_2O on metabolism), such as vitamin B12, homocysteine and methylmalonic acid.

Keywords: nitrous oxide; vitamin B12; vitamin B9; homocysteine; methylmalonic acid; laughing gas

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Introduction

Nitrous oxide (N_2O), since its discovery in 1772, has been mainly used for anesthesia and analgesia. As the least potent inhalational anesthetic [1], N_2O is frequently combined with other agents such as oxygen. It can also be found in supermarkets for use as an agent in whipped cream dispensers. However, this form can also be used recreationally as 'laughing gas'. Its wide availability in many countries explains why recreational use of N_2O is becoming a major health issue. The Global Drug Survey (n=32,022) listed N_2O as the 14th most commonly used recreational drug worldwide in 2021 [2]. The widespread recreational consumption of N_2O globally, particularly attributed to its legal status (with notable exceptions like the United Kingdom and the Netherlands), and its accessibility, presents a unique public health concern. Unlike many psychoactive substances, N_2O remains legal in numerous jurisdictions, largely due to its established medical applications and commercial uses in food preparation. This legality contributes to its widespread availability, making it easily accessible to the general public. Furthermore, the relatively low cost and ease of procurement, often via online platforms or in commercial outlets, in the form of whipped cream chargers, facilitate its misuse. The perception of N_2O as a 'legal high' diminishes awareness of its potential health risks, leading to its increased popularity among recreational users. This situation is compounded by the challenge of regulating a substance that has a role in various legitimate industries, creating a complex scenario for public health authorities in balancing the substance's legitimate uses against its potential for misuse.

According to the European Monitoring Centre for Drugs and Drug Addiction, recreational use of N_2O is increasingly observed in the general population [3]. Data from different countries was gathered in a review that was released in 2022 in order to assess the growth of N_2O use. In the Western world, N_2O use is becoming increasingly common [4]. Epidemiological data show that it is used by mainly young people aged 18–25 years old. A survey by the French Monitoring Center for Addiction network showed that N_2O is consumed more by men, which is consistent with an American study [5, 6]. Until recently whipped cream cartridges were used recreationally, but now it is consumed in much larger quantities via canisters (Figure 1).

The clinical effects of recreational N_2O consumption have been widely reported for both the acute and chronic effects. Short-term adverse events may include digestive problems such as nausea or vomiting, dizziness, psychomotor retardation and ataxia, as well as cold burns if the skin comes into prolonged contact with the canisters. In the long term, neurological damage has been described, with subacute combined degeneration of the spinal cord and/or peripheral sensory and motor neuropathy, leading to sensory loss, limb weakness, ataxia, bladder and bowel disturbance and sexual dysfunction [7–9]. Central nervous disorders have also been reported, notably encephalopathy [10]. Several cases of thrombosis have also been reported, likely attributable to elevation of homocysteine level [11–13].

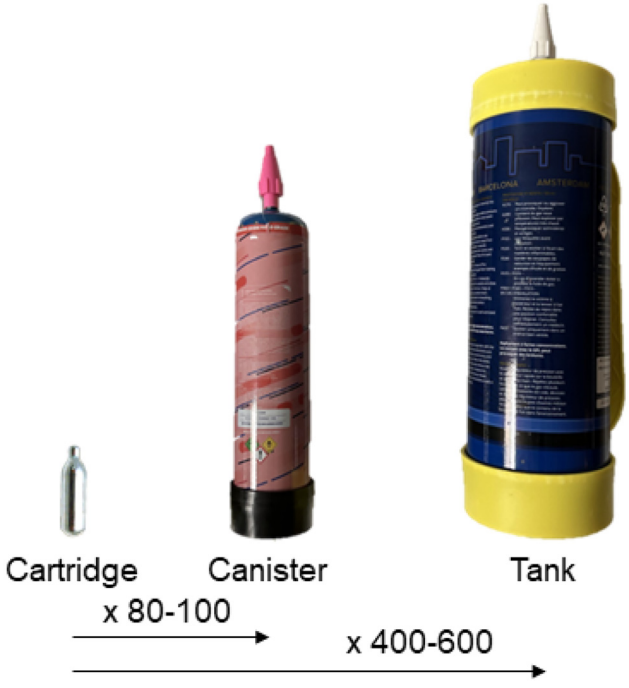

Figure 1: Consumption of nitrous oxide. The containers used for nitrous oxide recreational use have changed throughout time. Originally used for whipped cream siphons, small cartridges (on the left) have been gradually supplanted by canisters (middle), which hold 80 to 100 cartridges each. There are also tanks (on the right), which hold 400–600 cartridges each.

With regards to the risk of neurological harm and to the limited understanding of long-term outcomes, further research and standardized clinical care are needed [14]. In addition, N_2O has been increasingly implicated in some medico-legal matters, such as car accidents, especially if consumed while driving, and healthcare workers who develop symptoms after exposure [3]. It is therefore important to be able to biologically monitor N_2O consumption in selected individuals.

The kinetic properties of N_2O have been explored. The physical features of distribution between different media and tissues have been studied *in vitro* and in animal models. Because past exposure was primarily environmental, the main aim of clinical studies was to find a method of measuring this exposure. As a result, several investigations have focused on sampling urine. We first aimed to review the various technologies available for N_2O measurement, to establish whether these methods are suitable for routine use in medical laboratories. Then, we reviewed available toxicokinetic data in the literature regarding absorption, distribution, metabolism, and elimination in order to evaluate biological diagnosis and monitoring of recreational use of N_2O.

The analytical methods currently able to measure N_2O will be discussed in order to validate analytical measurement of nitrous oxide. Secondly, we will discuss the biological kinetics of N_2O after consumption, to determine the optimum choice of biological medium for its measurement. Finally, we will focus on the consequences on metabolism, so as to be able to propose possible biological markers of interest. To carry out this literature review, we used the PubMed and Medline databases, using keywords related to N_2O and measurement methods. We excluded most studies related to the environment (non-biological studies), but retained studies involving animal exposure, given the small number of human studies available.

Pharmacological effects

N_2O is known for its analgesic, anesthetic, anxiolytic, and antidepressant properties. These pharmacological effects depend on N_2O concentration [15]. N_2O is the least potent among the inhaled anesthetic and its effect involves the non-competitive inhibition of the NMDA receptors [16]. This blockage also partly explains N_2O's analgesic properties. The analgesic effect depends on the activation of opioid receptors, especially kappa opioid receptor [17], and to a

lesser extent adrenoreceptors (α1 and α2B) activation. The anxiolytic effect of N_2O entails activation of the GABAergic system, in particular the GABA type A receptor, which is also involved in the action of benzodiazepines, major anxiolytics. The antidepressant potential of N_2O is much less known and more recently described. It is comparable to ketamine as similarly brief, and mediated via non-competitive inhibition of NMDA receptors [18, 19].

Measurements methods

There are several methods for measuring N_2O, but most are used for environmental measurements to estimate global warming and ozone depletion, making them difficult to transpose to the medical laboratory. There are few publications focusing on measurement in biological matrices from organisms such as humans. Here, we summarize the methods that may be of potential interest to the medical laboratory, and have not focused on methods that cannot be applied to clinical chemistry, such as chamber methods for measuring gas flow [20], air flow measurement methods [21–23] and optical methods [24, 25], which are better suited for environmental applications. Methods of potential interest to laboratory medicine are chromatography and infrared spectroscopy.

Gas chromatography-mass spectrometry

Most N_2O analytical methods rely on gas chromatographic techniques. Environmental studies have shown that gas chromatography-isotope ratio mass spectrometry [26] and gas chromatography-mass spectrometry (GC-MS) [27–29] are used. These mass spectrometry-based approaches have also been used in other fields of study, such as investigation of biological pathways [30, 31]. GC coupled with a thermal conductivity detector (TC) and a micro-ionization cross-section detector (MICS) are alternative methods for measuring N_2O [32]. Methodological shortcomings include limited sensitivity and the use of internal standards that are not optimally selective at the molecular level for N_2O measurement. Due to the lack of acceptable internal standards, external calibration is required, which is not the best strategy for gas analysis given the potential risk of leaks during sampling, extraction, and analysis [33, 34]. Even though previous published GC-MS techniques have improved sensitivity, the use of pre-loaded hermetic gas syringes should alleviate any quantification difficulties caused by probable leakage.

A variant of GC-MS called Headspace-GC-MS (HS-GC-MS) is a method also used to measure N_2O. The sample is placed in a hermetically sealed container for HS-GCMS analysis. To create a headspace, the sample is heated to boiling point. The GC injection needle draws the gaseous substance into the headspace and injects it into the chromatographic column. In 2009, Poli et al. used HS-GC-MS to measure N_2O in numerous tissues on patients who had been deceased for several days [35]. During autopsy, blood, liver, bile, kidney, fat, and brain samples were collected; a urine sample was also taken from the eighth deceased patient [35]. All of the tissue samples were immediately weighed and then transferred to 10-mL headspace (HS) vials with airtight plugs. The gaseous samples from the sample bags and syringe were also transferred to HS vials, which were completely filled [35]. In, 2015, Giuliani et al. suggest a new HS-GC-MS method for the quantification of N_2O by using hydrogen sulfide as internal standard [36]. Although the authors validated this method, no independent study incorporated HS-GC-MS as their method, and its implementation in medical laboratories appears challenging due to its preparation difficulty and lack of reproducibility. Hence, use of HS-GC-MS for N_2O measurement could be promising but requires further technical development and clinical validation studies for use in laboratory medicine. A few medical laboratories already employ HS-GC-MS for measurement of alcohols and glycols, so these laboratories that have the equipment and expertise could certainly investigate measuring and optimizing N_2O by HS-GC-MS without significant cost [37].

Infrared spectroscopy

Infrared spectroscopy (IR), based on the molecule's ability to absorb light at a specific wavelength, is another technique for measuring N_2O. As described by Heusler et al., IR techniques are ideal to measure in air, but not in biological fluids such as blood or urine, where GC-MS is the preferred choice [38]. Closed path methods are preferred with greater sensitivity. The advantage of open path systems is that the concentration can be measured continuously, which can be useful when using N_2O for anesthesia to control flow rate or to prevent occupational exposure [39–41]. However, interest in using IR technology for measuring direct unique or recreational N_2O consumption is more limited [42].

Fourier transform infrared spectrometers (FTIR) are well described. This technique uses broadband IR radiation covering the entire IR spectrum, enabling many gases to be analysed at the same time with high precision, which explains why this technique is so popular for atmospheric

measurements [25, 43, 44]. Another IR technique that can be used to measure N_2O is infrared laser-absorption spectroscopy [45]. In contrast to FTIR spectroscopy, which measures the complete spectrum, laser spectroscopy uses a single wavelength to boost sensitivity. Specificity can be enhanced by working at low pressure, which results in narrower lines [42].

We have not found data on the use of IR methods for the determination of N_2O in biological fluids other than exhaled air.

Exposure monitoring: toxicokinetic of N_2O

N_2O assay methods are available. However, identifying it in biological matrices necessitates a thorough understanding of its toxicokinetic properties in order to know which matrices to look for and when to look for it after intake. The four phases of toxicokinetic are studied: absorption, distribution, metabolism and elimination. In order to study N_2O, it is necessary to know the quantities absorbed by the body in relation to the quantities taken in by consumers. The study of distribution is essential in order to know in which tissue and/or fluid N_2O can be easily found, and in a quantity large enough to be measured. As an exogenous substance can be transformed in the body by detoxifying metabolic systems, knowing the metabolites of N_2O may be of interest in finding markers of exposure other than N_2O itself. Finally, an understanding of the elimination pathways is crucial to find the right fluids for N_2O assay, and the timescales within which we can carry out the assay.

Absorption

Consumption-dependent quantity

There are two types of N_2O exposure: therapeutic and recreational. In cases of analgesia without loss of consciousness, an equimolar mixture of oxygen and N_2O (EMONO) is utilized or diluted in oxygen at concentrations of 50–70 % [46]. The anesthesiologist adapts the flow rate to the patient ventilatory rate, but this practice is waning.

The average flow rate recommended according to an SFAR study (French Society of Anesthesiologists and Reanimators) was 1 L/min or less. Thus, patient receives N_2O at a rate of about 500 mL/min [47], or at 0.9 g/min of N_2O since the gaseous form has a density of 0.0018 g/mL (Figure 2). EMONO is still frequently used in dental surgery, particularly on young patients. The majority of treatments run no longer than 40 min, and the N_2O concentration used ranges from 20 to 50 %, depending on the complexity of the intervention [48–50].

The levels of N_2O used for recreational purposes are difficult to quantify since they differ depending on the consumer and the mode of consumption (Figure 1). A canister holds 8 g of N_2O, while a cylinder holds 640 g. Several sources claim that casual N_2O users only consume a few dozen canisters per occasion (equal to about 100 g of N_2O). However, recreational use of N_2O is on the rise. Many people now use N_2O in cylinders rather than canisters, with some people consuming numerous cylinders per day (Figure 1). Some claim to have consumed up to 25 cylinders per day (equal to 16 kg of N_2O) (Figure 2). As a result, recreational exposures can be substantially higher than medical doses.

Uptake

The absorption of a gas is the result of the balance between the concentration inhaled and concentration in the alveoli, blood and tissues. Because N_2O has relatively low solubility in blood (and other tissues), it equilibrates very quickly through tissue saturation. Due to its poor solubility in blood and tissues, the increase in alveolar concentration upon exposure is very quick, resulting in rapid saturation of the body. After 5 min of treatment, the alveolar fraction of N_2O equals 90 % of the fraction inhaled [51]. The rate of increase in alveolar concentration is influenced by inhaled concentration. The greater the inhaled concentration, the faster the alveolar concentration rises [52]. Uptake is altered in cases of pulmonary disease such as chronic obstructive pulmonary disease (COPD) [53]. This has been demonstrated in patients undergoing surgery and receiving N_2O. They were classified into three groups according to the severity of their respiratory impairment, on the basis of the Tiffeneau ratio. When comparing patients with COPD to healthy patients, concentration measurements in inspired and exhaled air revealed significative differences, especially with regard to equilibration time [53].

Distribution

In order to determine the quantity of N_2O in blood, for consumer screening for example, it is essential to study the distribution of the gas in the body. It is important to understand the distribution kinetics in the various tissues to determine the best timing for N_2O detection into the blood.

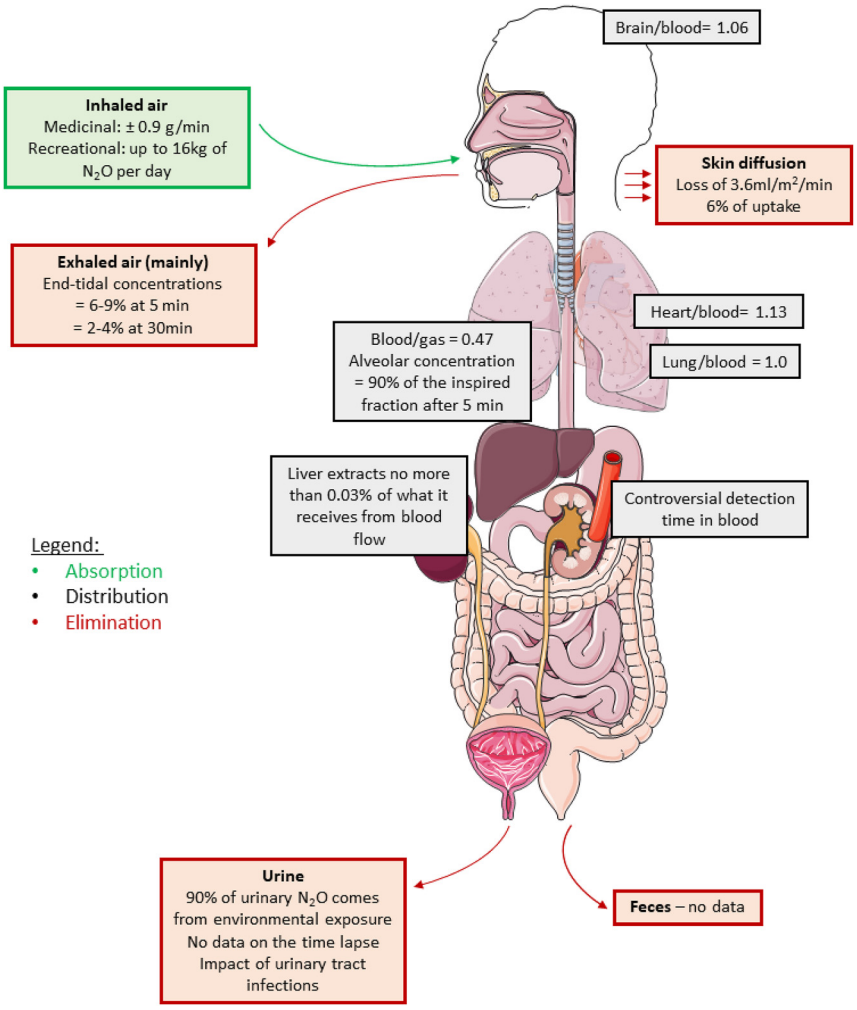

Figure 2: Summary of global toxicokinetic of nitrous oxide. Nitrous oxide is absorbed by the respiratory tract alone. It is then distributed to all tissues. Due to its low solubility, the body is rapidly saturated. It is not metabolized, and is rapidly eliminated in unchanged form, mainly in exhaled air. A fraction is eliminated via the urine, and a small proportion via the transdermal route.

Partition coefficients

Because studying human tissue is highly challenging, extrapolations are frequently made from findings made during pre-clinical studies on animals or *in vitro* models. Tissue/gas distribution coefficients of N_2O and cyclopropane were determined in rabbits and compared to the distribution coefficients of these agents in homogenates of identical *in vitro* tissues [54]. Rabbits anesthetized by parenteral injection of sodium pentobarbitone were used and received N_2O mixed with oxygen (70/30). In order to maintain a constant arterial partial pressure, exhaled gas samples were evaluated by GC during the experiment, and the inhaled concentration was adjusted accordingly. Blood, brain, heart, kidney, liver and skeletal muscle were extracted. Equilibrating homogenates of the same tissues (unused *in vivo* samples where any remained) with a known partial pressure of N_2O yielded the *in vitro* coefficients. A discrepancy of 6 % was detected between than *in vitro*, higher, and *in vivo* distribution coefficients. For the six animals used, a three-way cross classification analysis of variance supported the fact that this difference is statistically significant, but the authors pointed out that this difference, although real, still allows us to have confidence in the *in vitro* distribution coefficients in order to extrapolate to humans.

Kety et al. conducted *in vitro* and *in vivo* experiments in dogs to evaluate the solubility of N_2O in whole blood and brain as well as the partition coefficient between the two. Human and dogs appear to have equivalent values [55]. In human whole blood, they found a solubility of 0.412. This solubility explains how quickly the alveolar concentration rises to the inspired concentration. The solubility in human

entire brain is 0.437, giving a brain/blood partition coefficient of 1.06 (Figure 2).

As a result of lower tissue/blood partition coefficients, the equilibration of N_20 in most tissues occurs rapidly.

Blood distribution

N_2O does not bind to any protein and circulates freely in the blood. Anesthetists, surgeons and nurses were subjected to an occupational exposure study to determine their blood levels of N_2O. For all processes, the N_2O flow rate was standardized at 6 L/min. Except for surgeons, from whom collections were taken at the end of the operation, blood samples (blood gas syringes) were collected every hour. Simultaneously, an air sample was taken from the subject's breathing zone for 60 s. A correlation was showed between blood and breathing zone atmosphere concentrations (n=116) but for some samples (32 %, n=37), blood concentration could not be accurately matched to atmospheric concentration [56]. Nonetheless, this study holds additional significance because they checked the N_2O blood levels of subjects before exposure, which was undetectable in any of the groups. It leads to the conclusion that the N_2O blood level following exposure and its correlation to the atmospheric concentration are solely due to current exposure. A quantitative study comparing variance in blood and atmospheric concentrations revealed more stable blood concentrations. This shows that this is the result of blood N_2O saturation rather than a single exposure [56].

In subjects receiving N_2O directly for dental analgesia, N_2O was detected in the blood after 10 min of inhalation at concentrations 2 times lower than those measured in the nasal mask and up to 5 times lower than the concentration set on the machine [57]. After 5 min of 100 % oxygen inhalation, venous concentration divided by 2.

In 2010, after several patients' death from hospital poisoning with N_2O, a team of researchers showed that N_2O was still present in their blood one month later [35]. These patients had mistakenly received N_2O instead of oxygen for periods ranging from 25 to 125 min. During the autopsy (one month after death), various samples were taken, including blood, and N_2O concentration measured by HS-GC-MS. Large quantities of N_2O were still measurable 1 month after death (11.29–2,152.04 mg/L). However, measurements taken so long after death are questionable since the tissues could have released N_2O stored in the blood due to post-mortem autolysis and falsely elevated the measurements.

Involvement for laboratory medicine

Data on N_2O blood distribution are lacking in direct consumers of recreational dose(s). Recreationally absorbed doses are not equivalent to those absorbed by professionals exposed in hospitals. Chronic consumption of large doses has not been studied. The absence of detection in professionals just prior to experimental exposure suggests that, with regular exposure to low environmental doses, N_2O is rapidly distributed and eliminated. Measuring it in the context of a diagnosis would therefore appear to be uninformative, due to the possibility of false negatives related to rapid disappearance from the blood. Again, these data are related to occupational exposure and the findings could be different for recreational consumption (Figure 2).

Metabolism

Xenobiotics are generally metabolized, mainly in the liver, to produce metabolites. If the substance is rapidly metabolized, it is challenging to detect it. On the other hand, if the substance is completely metabolized, the products could be of interest for exposure study.

Metabolic transformations

The CNESST (Commission on standards, equity, health and safety at work) sheet on N_2O states that it is poorly metabolized in the liver, as the liver extracts only a tiny fraction of quantity received (Figure 2).

Because of its redox characteristics, N_2O can be reduced by a variety of substances found in the body. This was demonstrated in 1952 when bacteria were exposed to an anaerobic, N_2O-containing environment [58]. Pressure changes have been reported as a result of the transition of N_2O into N_2. The enzymes involved are adaptable and bacteria that have evolved to nitrates and nitrites have also adapted to N_2O. As a result, N_2O is a common intermediate in denitrification. In 1985, another experiment studied the denitrification by bacteria. The catalyzed equation is: $N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$ [59].

Because bacteria may change N_2O, a small amount of N_2O can be digested by the microbiota. Rats (male and female) were sacrificed to recover a portion of gut and large intestine. The intestinal contents and wall were separated, and two of them received antibiotics (tetracycline, neomycin

and bacitracin) for three days prior to sacrifice. Human intestinal contents were also studied at the same time by collecting faeces samples from one of the researchers. To avoid contamination from the intestinal bolus, the wall was cleaned. After diluting, samples were incubated with radiolabeled pure N_2O or a mixture of N_2O/O_2 at various doses. The intestinal wall was incubated for 3 h and the contents for 16 h at 37 °C. The radiolabeled N_2 generated by the reaction was analysed by mass spectrometry and quantified using an internal N_2 standard. These findings demonstrated that human and rat intestinal contents metabolize N_2O, as labelled N_2 concentrations were measured in incubated samples [60]. At concentration of 10 and 20 %, and to a lesser extent at 5 %, oxygen inhibits this process. The intestinal wall samples had a very low quantity of N_2, supporting the fact that metabolism is carried out by the intestinal microbiota [60].

Elimination

Data on N_2O elimination may permit determining best matrix for consumption detection. Active substances could be eliminated from the body unchanged, inactivated, or in both forms, and in variable proportions. Regarding its metabolism, N_2O is eliminated unchanged, as a tiny fraction seems to be metabolized. Elimination studies have focused primarily on exhaled air, urine and to a lesser extent skin.

Pulmonary elimination

As described in the absorption/distribution section, elimination of gases depends on solubility and partition coefficient. The blood/gas and tissue/gas coefficients reflect how readily some tissues can store xenobiotics. On the other hand, N_2O is eliminated extremely rapidly. When N_2O administration is stopped, the significant difference in concentration between the alveoli and the blood results in a rapid decrease in N_2O concentration. Salanitre et al. demonstrated in 1962 that the uptake and elimination curves are comparable [61]. The aim of their study was to validate or refute the equivalence between N_2O uptake and excretion. N_2O was administered to five subjects at subanesthetic doses (10 % N_2O/90 % O_2), and the amount absorbed and eliminated was measured. A good correlation between N_2O uptake and excretion was found, although for some patient curves not (but it could be attributable to the method) [61]. The excretion curves data were compared to a previous study with anesthetized patients. The only difference was an upward displacement of the curve in the anesthetized group, which might be explained by anatomic and physiological differences between the two groups, as well as a higher saturation in N_2O in the anesthetized group.

The concept of diffusion hypoxia or diffusion anoxia should not be underestimated. The diffusion hypoxia is caused by the fact that N_2O returns to the alveoli faster than nitrogen leaves them leading to a dilution of the oxygen concentration in the alveoli. To simulate gas exchange in the pulmonary alveolus, an *in vitro* experiment was performed. Because the solubility of N_2O in blood is similar to that of water, water could represent blood. Air represents the pulmonary alveolus. A flask containing 50 % water and 50 % air is saturated with N_2O. N_2O is 35 times more soluble in water than nitrogen, and 20 times more soluble than oxygen. At equilibrium, N_2O partial pressures in water and air are equivalent. When the flask is brought into contact with air, the equilibrium of partial pressures is disturbed. The partial pressure in air is lower than that in water. As a result, N_2O diffuses from the water into the air, diluting the oxygen and nitrogen in the air [62]. This explains the hypoxia that may result from N_2O inhalation during the reoxygenation period.

In 1990, an experimental investigation was conducted to evaluate biological exposure to N_2O in occupational exposed subjects (surgeons, anesthetists and nurses). Exhaled air and urine were obtained from 20 participants, working in five operating rooms, as well as samples of the ambient air in the operating room. A correlation (r=0.911) was found between concentrations in air and exhaled air (r=0.911) [63]. These subjects were not directly exposed to N_2O. Einarsson et al., investigated the lung clearance rate of N_2O in surgical patients in 1993 [64]. Thirty-six subjects were divided into six groups based on the duration of their N_2O exposure (30, 60 and 120 min) and the type of ventilation after this exposure (normo- or hypoventilation). End-tidal N_2O concentrations were evaluated using infrared spectroscopy and inhaled concentrations of 30 % oxygen and 70 % N_2O. During normal ventilation, the concentration of N_2O dropped rapidly after the end of the exposure, from 66–70 % to 6–9 % at 5 min and 2–4 % at 30 min [64] (Table 1) (Figure 2). It decreased a bit more slowly during hypoventilation (16–23 % at 5 min and 5–9 % at 30 min).

Furthermore, there was no effect of exposure duration on the rate of removal, indicating that the organism had reached maximum saturation after 30 min. There is no evidence of difference in the decline in N_2O rate according to age [65]. After 30 min of N_2O inhalation, the levels and rates of exhalation are not significantly different between elderly males (63–69 yo) and young adult males (22–30 yo). The rate of elimination therefore appears to be similar in all subjects, independent of age.

Table 1: Nitrous oxide determination in biological fluids after exposure.

Study	Number of subjects	Exposure dose	Exposure time	Expired air	Blood (time)	Urine (time)
Einnarson et al. [64], 1993	18	68–70 %	0.5, 1 or 2 h	66–70 % (0 h) 6–9 % (5 min) 2–4 % (30 min)	NA	NA
Brugnone et al. [66], 1996	Urine=76/blood=80	49 ± 45 µg/L	NA	NA	23 ± 18 µg/L (0 h)	26 ± 19 µg/L (0 h)
	Urine=23/blood=27		NA	NA	0.9 µg/L (18 h)	1.5 µg/L (18 h)
Zaffina et al. [68], 2019	21	24.17 ppm	Minimum 4 h	NA	NA	9.04 µg/L (0 h)
Henderson et al. [113], 2003	46	313 ± 358 ppm	4 h	NA	NA	114 ± 191 µg/L (0 h)
Imbriani et al. [114], 1995	835	75.5 ± 3.9 ppm	4 h	NA	NA	45.18 ± 3.44 µg/L (0 h)
Accorsi et al. [115], 2003	121	6.5 ppm	NA	NA	NA	9 µg/L (0 h)
Krapez et al. [56], 1980	34	57 ppm	NA	NA	83.6 µg/L (0 h)	NA

Urinary excretion

Numerous studies have evaluated urinary N_2O content following exposure, particularly in occupationally-exposed patients, because urine is an easy-to-recover matrix in medical staff. In 1995, urine and blood samples of patients working in nine hospital operating rooms were collected at the end of a professional exposure session. Personal passive samplers were used to monitor atmospheric N_2O in the operating room (n=80) and then compared to urine and blood levels in relevant participants (Table 1). A control group of urine (n=25) and blood (n=27) morning samples of blood donors were used to assess exposure due to atmospheric N_2O levels. This data revealed a significant correlation between both urinary and blood levels with environmental N_2O concentrations [66]. Finally, when compared to blood donors, the occupationally exposed group had significantly higher values. Particularly high and unexpected values were found in some patients (n=10). In this study, those extreme values were associated to participants who had a bacterial urine infection, even though they were asymptomatic. Another study found that urinary tract infections had an effect on N_2O levels in urine [67]. To study the kinetics of N_2O production, a healthy donor's urine was inoculated with *Enterobacter aerogenes*. Six vials were infected and incubated at 37 °C. The level of N_2O was measured at 0, 2, 5, 8, 24 and 48 h after inoculation. Gram negative bacilli produce high amounts of N_2O (78.5–464.7 µg/L) compared to others bacteria (0.1–4.2 µg/L) [67]. The production is time dependent and follows exponential kinetics (it is low for the first 5 h, increases exponentially up to 24–36 h, and the equilibration is reaching at 48 h) [67]. N_2O concentrations measured in urine infected with Gram-positive cocci and yeasts were not significantly different from the control sample. To study the correlation between N_2O concentration and the different bacterial strain, 16 urine samples from patients were collected. After acidification and storage of urine at 4 °C, N_2O concentration was measured within 24 h by headspace GC. There was a strong correlation between the bacteria involved and the level of N_2O in urine (high for Gram negative bacteria, lower for Gram positive cocci and almost non-existent for sterile samples). Because N_2O is most likely produced in the bladder, four urine samples were analysed immediately after collection and varying quantities of N_2O were detected, which could be linked to the time of infection onset [67]. This means that the amount of N_2O measured in the urine may be greater the longer the infection has been present. The levels produced by bacteria in cases of urinary tract infection are well above those found in cases of occupational exposure to N_2O. The average urinary N_2O concentration in subjects working in the operating theatre was 26 µg/L [66]. As a comparison, in 2018, N_2O level was measured in urine of a cohort of dentists and dental assistants exposed to N_2O. Concentrations ranged from 2.54 to 25.4 µg/L [68]. It is therefore difficult to differentiate between exposure and urinary tract infection when N_2O is detected in urine (Figure 2).

In 1983, volunteers were exposed to N_2O (850 ± 25 ppm) in a 20 m^3 chamber [69]. The exposure was intermittent, lasting 5–15 min and totaling 20–60 min. The last exposure was followed by a 30 min in N_2O-free air. Participants were equipped with in a gastight container. At the end of the experiment, urine was discharged into a syringe and was equilibrated with an equivalent volume of N_2O-free air. The syringe was shaken in a water bath at 38 °C for 20 min to obtain equilibration. N_2O concentration was measured by infrared spectrophotometer and GC. A strong correlation was discovered between the bag and urine concentrations, suggesting that 90 % of urinary N_2O is caused by environmental exposure [69]. As a result, the urine matrix may be a useful indicator of N_2O environmental exposure. Another cohort confirmed these results in 1985. The subjects were outfitted with pump-bag sampling to monitor the N_2O

content, which they wore all day long (98 days of work in total). N_2O was analysed by IR or GC. They collected urine before starting anesthetic work and at the end (Table 1). They also investigated the effects of ventilation, close scavenging and types of anesthetics (mask, intubation, epidural) in several departments (pediatric, gynecological, general, orthopaedical and thoracic surgery). There was a positive correlation between N_2O in bags and urine headspace N_2O, this correlation being influenced by intra-individual factors and handling of urine specimens [70]. Duplicate samples were stored in a refrigerator or at room temperature and analysed up 24 h after (correlation coefficient between pump bag concentration and urine headspace gas measurement=0.94) [70]. The investigators concluded that urine specimens can be used in a practical way.

Similar studies on occupational exposure have been carried out, in several countries [68, 71]. These studies showed that a quantifiable concentration of N_2O was found in human urine after exposure, but the kinetics were not studied. There is no data on the time lapse after which it can be recovered. However, a correlation was found by Trevisan et al. between the concentration in exhaled air just after a 6-h exposure and the urinary concentration 2–3 min after the end of the exposure [63]. As previously mentioned, the concentration drops in exhaled air 5 min after exposure [64]. It may be hypothesized that urinary concentration falls in the same way after exposure ceases. This is consistent with an occupational exposure study which showed that urinary N_2O concentrations decrease highly after exposure ends. Urine and blood samples were collected the next morning from 25 exposed participants, approximately 18 h after the exposure ended. The mean N_2O level found was 1.52 µg/L compared to 40 µg/L at the end of operating session (Table 1). Moreover, this concentration so long after exposure is no different from the concentration of 1 µg/L found in control subjects (blood donors) [66].

However, these data are not derived from patients who were directly exposed, so they should be interpreted with caution. Furthermore, urine matrix is frequently difficult to obtain, and exposed patients may not want to provide. Moreover, N_2O doses differ from occupational to abuse. Hence, urine may be a complicated matrix for laboratory medicine and it may be more interesting to focus on blood or exhaled air.

Transdermal elimination

Cullen et al. used an *in vitro* test to demonstrate how N_2O diffused through the skin [72]. Healthy skin samples were recovered from amputated limbs. Each specimen (n=7) was immersed in a water bath in a diffusion chamber (37 °C or/and 24°). A steady flow of humidified N_2O (85.1 %) was applied at a constant rate of 1 L per minute. The gas spread through the skin to the other side of the nitrogen-filled glass box. Gas samples were analysed by GC. The N_2O rate diffusion through skin at 37 °C was 10.38 mL/min/m^2 with a mean difference of 2.47 mL/min/m^2 between 37 and 24 °C.

In 1968, the percutaneous loss in surgical patients anesthetized with N_2O was measured. Patients were subjected to a flow of 80 % N_2O, with 20 % O_2. A gas sample was collected at the start and then every 10 min and analysed by GC. The N_2O percutaneous loss was measured at 3.6 mL/m^2/min or 214 mL/m^2/h in an average sized adult after 100 min of anesthesia (plateau between 60 and 100 min) [73]. This is much lower than the rates found *in vitro* [72]. This may be due to the fact that the amount of N_2O reaching the skin in patients does not represent the total intake, and that it diffuses and is distributed elsewhere in the body. To describe the influence of temperature on this process, ice and heating patches were applied and the desired temperature was maintained for 30 min. There was a linear relationship between temperature (20–40 °C) and skin losses. The increase of skin temperature opening capillaries nearer the skin surface which decrease the path length for diffusion who increasing percutaneous loss. The conclusion was drawn that diffusion is the most important limiting factor for percutaneous loss [73].

Maternal milk

There is no information available about excretion in breast milk. According to Hale et al., N_2O is unlikely to be expelled in breast milk or ingested by newborns (albeit no experiments have been performed) [74].

Involvement for laboratory medicine

In the context of driving under the influence of N_2O and because N_2O elimination is primarily pulmonary, it may be useful to detect it with a technique based on a breathalyzer test kit. Elimination is however too rapid, considering that 5 min after the last inhalation, it has nearly entirely disappeared. Regarding the use of urine samples, in addition to the fact that patients are not always compliant for this type of sampling, data is lacking from patients who are direct consumers. Urine measurement seems possible even a few days after inhalation, but in addition to the above limitations, predicting pathological cut-off is complicated and there is a lack of studies in subjects chronically consuming

large quantities of N_2O. Also, patients with urinary tract infections may have false positive results.

As direct detection of N_2O is complicated to implement routinely due to the specific methods and difficult to interpret due to the lack of kinetics data, it is necessary to look at the effects of N_2O on metabolism in order to find indirect markers.

Effects monitoring: impact of N_2O on metabolic pathways

The most commonly metabolic target of N_2O in the literature is vitamin B12 (or cobalamin) [75]. Vitamin B12's structure includes a cobalt ion. This ion is the target of N_2O, which changes its oxidation state [76]. N_2O is a powerful oxidizing agent. *In vitro* studies evaluated reactions with various transition-metal complexes including cobalamins. The component of the gas mixture (N_2O, N_2 and H_2) was measured by mass spectrometry after N_2O was mixed with the transition-metal complex. N_2O reacted quickly with transition-metal complexes acting as a two-electron oxidizing agent with the cobalt ion of cobalamins [77]. N_2O was reduced into N_2 by the following reaction $2Co(I) + N_2O \rightarrow 2Co(II) + N_2$ [78]. When N_2O was added to a vitamin B12 solution, the amount of N_2 produced by the reaction was too small to be quantified. In a second experiment, the cobalamins were reduced using borohydride to get solely the Cob(I) forms before being exposed to N_2O. As a result, the amount of N_2 responding was high. N_2O targets cob(I) forms, whose oxidation disrupts the physiological metabolic cycle [77].

Cobalamin(I) normally interacts with 5-methyl-tetrahydrofolate (THF) to regenerate the methylcobalamin which is essential for methionine synthase (MS) function [79] (Figure 3). In this case, a metabolic adaptation prevents the cycle from functioning following oxidation of the reduced forms, because the cobalamin(II) form has to go through a reactivation cycle before it can be methylated back into the cobalamin(III) form [80]. Because this reactivation reaction combines methylation, utilizing S-adenosyl-methionine (SAM) as the methyl donor, with the reduction of cob(II) to cob(I), it is known as reductive methylation [81]. The chemical mechanics of this reaction are not fully understood [82, 83]. The reaction equation is as follows: 2 cob(II)alamin-[methionine synthase] + NADPH + 2 S-adenosyl-L-methionine=H^+ + 2 methylcob(III)alamin-[methionine synthase] + $NADP^+$ + 2 S-adenosyl-L-homocysteine (Figure 3). As a result, the concentration of various metabolites varies compared to physiological levels. There is a redistribution of the various folate derivatives detected. Following N_2O exposure, the cytosolic quantity of THF decreases, benefiting the 5-methyl-THF form, which is twice as abundant as in an unexposed rat population [84]. A significant variation is also observed in the measurement of the SAM/SAH ratio, due to the significant rise in SAM levels caused by the increased activity of methionine synthase reductase (MTRR) [85] (Figure 3).

Cobalamins are not produced by human cells, they are obtained solely from food from animal origin after intestinal absorption. The CD320 receptor transports vitamin B12 to cells in transcobalamin II-bound form, allowing for endocytosis [86] (Figure 4). Cobalamin is an important enzymatic cofactor for methylmalonyl-coA mutase (MM-CoAM) and methionine synthase (MS) respectively adenosylcobalamin and methylcobalamin [81]. MS is involved in the one-carbon metabolism [87], and in multiple regulatory pathways, including cell signaling and epigenetics via methylation of DNA, RNA and various proteins.

N_2O interferes with this metabolic cycle by inactivating the functions of vitamin B12 cofactors [88]. In this pathway, methionine is transformed into homocysteine to provide a methyl group for other compounds. MS converts homocysteine into methionine by transferring the 5-methyl-THF group via methylcobalamin (Figure 4). This folate-dependent re-methylation pathway is largely dependent on appropriate and sufficient intake of vitamin B9, but also of vitamins B6 and B2, cofactors of the enzymes involved in this cycle. A parallel, folate-independent re-methylation mechanism involving betaine also re-methylates homocysteine to produce methionine, via its conversion to dimethylglycine (Figure 4). Finally, homocysteine can be coupled with a serine molecule via cystathione β-synthase (CBS) to produce cystathione (transsulfuration pathway), which allows for the synthesis of glutathione, an anti-oxidant metabolite [89]. Adenosylcobalamin (another active form of vitamin B12) is engaged in mitochondrial metabolic pathways. It is the cofactor of MM-CoAM, that transforms methylmalonyl-CoA to succinyl-CoA (Figure 4). Metabolism of methylmalonyl-CoA to methylmalonic acid (MMA) is a minority metabolic pathway in case of MM-CoAM enzymatic blockage.

N_2O effect on the activity of enzymes co-factored with cobalamins has been demonstrated *in vitro* and in animal models [90–97]. In 1978, MS activity was evaluated using the method of Kamely et al. in rats under normal conditions and after exposure to 50 % N_2O/50 % oxygen (30 min and 6 h groups). Simultaneously, urine was collected from the same rats to determine the concentration of urine MMA. MS activity was reduced by 30 min of exposure to N_2O. No significant difference in the MMA was found between the groups [96]. However, it cannot be proven that urine MMA accurately reflects MM-CoAM activity. In 1980, methionine

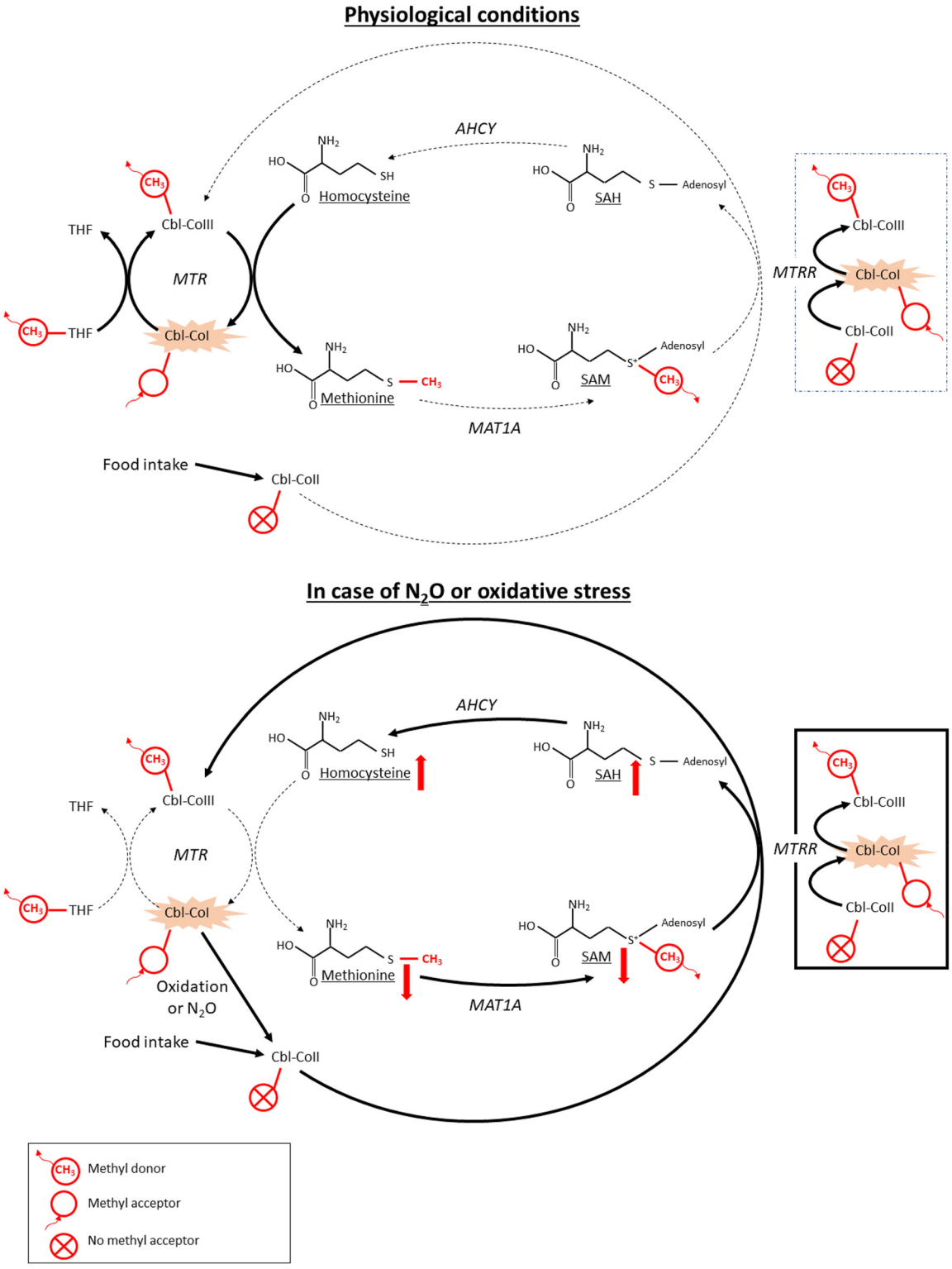

Figure 3: Consequences of oxidation of cobalamins. Cbl, cobalamin; Co, cobalt; THF, tetrahydrofolate; MTR, methionine synthase; MTRR, methionine synthase reductase; MAT1A, methionine adenosyltransferase 1A; AHCY, S-adenosyl-L-homocysteine hydrolase-MTR (=MS) uses methyl cobalamin(III) as a cofactor to supply a methyl group to homocysteine, thus forming methionine. This transmethylation forms a highly unstable, excited cobalamin(I) which, under physiological conditions, is mostly remethylated via folates. In the presence of N_2O and/or oxidative stress, the cobalamin(I) form is highly oxidized to cobalamin(II), thus blocking MTR activity, as the latter can no longer receive a methyl group. MTRR comes into play here, enabling electron transfer and cobalamin remethylation, this time using the methyl group of SAM.

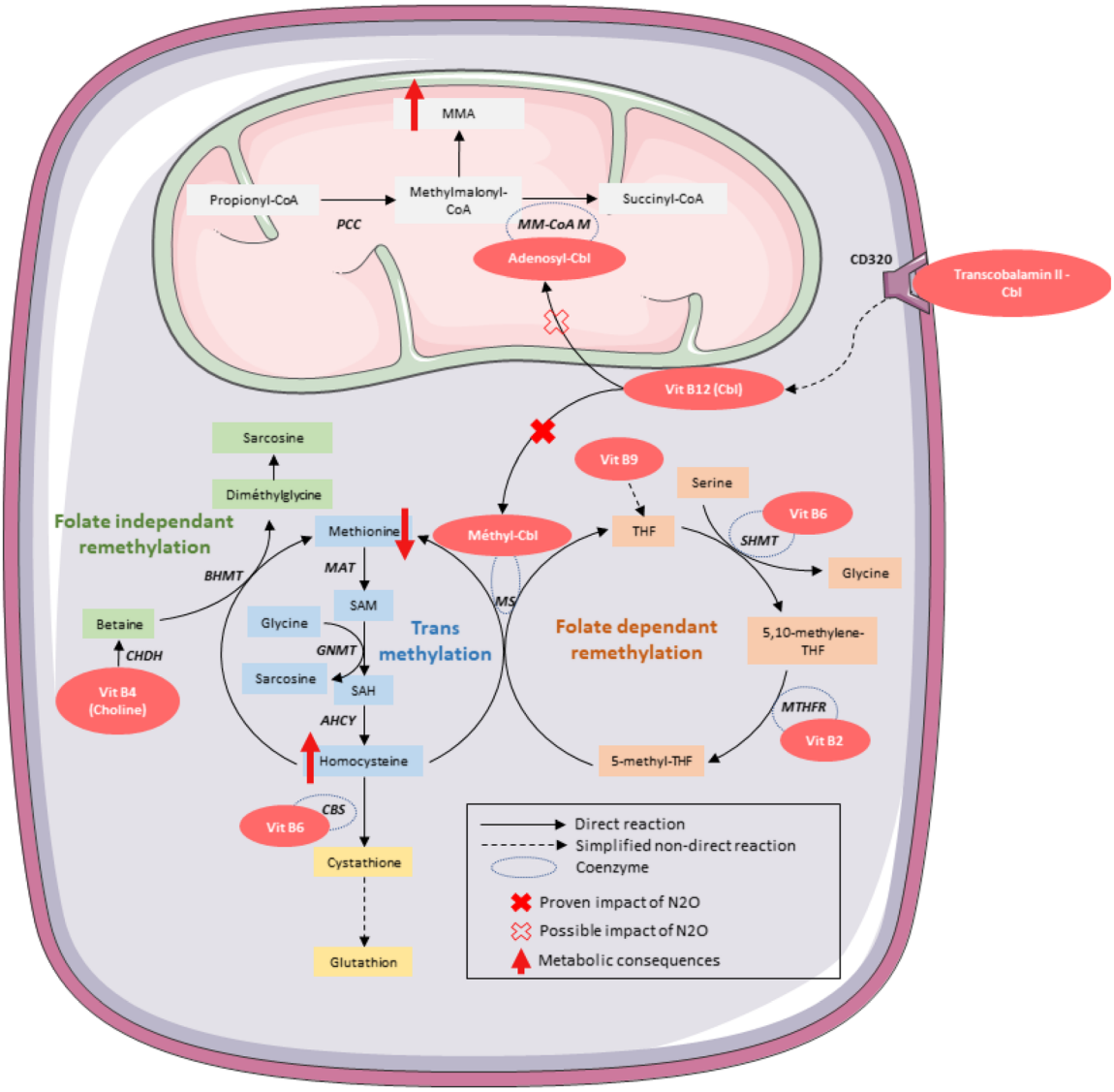

Figure 4: Impact of nitrous oxide on the one carbon metabolism. Cbl, cobalamin; THF, tetrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; PCC, propionyl-CoA carboxylase; MM-CoA mutase, methylmalonyl-CoA-mutase; MS, methionine synthase; MAT, methionine adenosyl-transferase; GNMT, glycine N-methyltransferase; AHCY, S-adenosyl-L-homocysteine hydrolase; CBS, cystathione β-synthase; BHMT, betaine-homocysteine S-methyl-transferase; CHDH, choline deshydrogenase; SHMT, serine hydroxymethyl-transférase; MTHFR, methylenetetrahydrofolate reductase.

production was evaluated to understand the observed decline in MS activity. Methionine levels in the liver decreased after 30 min of exposure to 50 % N_2O/50 % oxygen and were undetectable after 6 h. Half of the animals remained abnormal after 84 h, supporting the fact that recovery could be prolonged [98]. In rats exposed to N_2O, MS activity in the brain become undetectable. A deoxyuridine suppression test [99] was also performed, which is a sensitive index for the presence of megaloblastic anemia due to vitamin B12 or folate deficiency. It was normal in animals exposed for 30 min but became abnormal after 60 min and gradually declined until 6 h [98]. MS activity is decreased not only in the liver, but also in other tissues [100]. Rats were housed in a chamber with 80/20 combination of N_2O and oxygen and were euthanized after 18 h to obtain bone marrow, kidney, brain, and liver samples in which MS activity was determined. The percentages of inhibition of MS in the liver were the lowest (83.3 %), while the kidney was the most impacted (93.3 %) [100]. The loss of MS activity is closely linked to the decrease in concentration of its cofactor, methylcobalamin [91]. This correlation is also found for the recovery of enzymatic activity. The blocking of the MS is

irreversible and has been demonstrated in cell cultures subjected to N_2O. When exposure was stopped, some cultures were supplemented with cycloheximide (a protein synthesis blocking agent). Under these conditions, no recovery of activity was observed [91]. The blocking action of N_2O on MS activity is therefore irreversible.

Human patients anesthetized with N_2O have significantly lower hepatic MS activity [101]. When exposed to 66 % N_2O for 3 h, enzyme blockage reduces MS activity by 25 %. Plasma methionine (synthesis product of MS) correlates with patients' clinical severity, assessed by the PND (peripheral neuropathy Disability) score [102]. However, plasma concentrations remain within the physiological range, but the greater the clinical severity, the lower the level. Neurological damage is only partly explained by enzyme damage, and other hypotheses such as oxidative stress need to be explored [103]. On the other hand, analysis of biological data from a cohort of N_2O consumers showed an increase in plasma (plasma homocysteine increase was defined as >15 µmol/L) and MMA (plasma MMA increase was defined as >0.4 µmol/L) in these patients [104]. The increase in MMA is less significant because it has not been systematically found, but MMA levels correlate with the patient's clinical severity, which explain why this biomarker represents the extent of metabolic alteration. Because N_2O interferes with the action of cobalamin cofactors, studies have investigated total vitamin B12 dosage for the diagnosis of N_2O intoxication and it was not found to be a good marker. The alteration in metabolism is qualitative rather than quantitative. N_2O is responsible for a functional alteration of the cycle. Hence, a decrease in vitamin B12 levels is not systematically observed [104, 105] and basically represents a quantitative associated deficit. Furthermore, many N_2O users may supplement with vitamin B12, either by self-medicating due to unsubstantiated advice available on the internet and social networks, or after consulting with a medical practitioner. Thus, vitamin B12 consumption is unrelated to N_2O consumption.

Involvement for laboratory medicine

Consuming N_2O has metabolic side effects that are starting to be well understood, at least for the cytoplasmic pathway. It is still not well understood how N_2O enters the mitochondria, or at least how it affects this metabolic pathway. Vitamin B12, homocysteine and MMA assays are already available in medical laboratories. The techniques used are those traditionally employed, namely immunochemistry, liquid chromatography and mass spectrometry [106–110]. The combined use of these markers would appear to be relevant in assessing the metabolic damage attributed to N_2O consumption [104]. Mass spectrometry panels for homocysteine, methionine and MMA may be used to provide a complete interpretation of the patient's profile.

Summary and critical view

N_2O has generally been investigated in subjects with occupational or environmental exposure, but direct consumption has been less studied. Monitoring N_2O in the context of professional use is critical, but even more so in the context of recreational use, which is currently on the rise. Exposure monitoring is complicated to set up because it is therefore difficult to establish detection times and values to diagnose N_2O intake and its timing. Despite the fact that the pulmonary route is the predominant means of elimination, measurement in exhaled air is unusable, because of its rapid rate of elimination. On the other hand, blood and urinary concentrations are too unpredictable, due to a lack of data on direct consumers. In view of all the data described, determination of N_2O in biological fluids as an exposure biomarker does not appear to be simple and practical for routine use at this time.

On the other hand, effects biomarkers are biological parameters that can predict a clinically relevant endpoint or intermediate outcome difficult to observe clinically [111]. Some biomarkers could have an interest in the case of N_2O consumption. Importantly, a biomarker used to diagnose N_2O intoxication is not necessarily the same biomarker to determine clinical severity or to monitor patient. To identify one or more biomarkers, it is essential to understand the pathophysiology of intoxication [112]. For this reason, a detailed understanding of related metabolism and impact of N_2O on enzymes and metabolites in the cytoplasmic and mitochondrial pathways is essential.

Currently, there are no biomarkers specific to N_2O intoxication. Use of biomarkers reflecting metabolic alterations, such as homocysteine and MMA, seems more efficient and easier to interpret and are also available by routine analytical techniques in laboratory medicine (LC-MS, immunochemistry, enzymatic method).

Homocysteine appears to be the most sensitive biological marker of N_2O consumption with high plasma levels observed in most consumers. However, there is no correlation between the quantity consumed and homocysteine levels. Homocysteine rises very rapidly after the first consumption, but also decreases within a few days. A normal or slightly elevated level can therefore be attributed to a significant delay between sampling and last consumption; it is therefore a marker of recent consumption. However, this marker lacks specificity for recent N_2O consumption. Indeed,

its increase may also reflect common pathologies (renal failure, hypothyroidism, vitamin B12, B6 or B9 deficiency) as well as hereditary metabolic diseases such as methylenetetrahydrolate reductase (MTHFR), methionine synthase (MS) or cystathionine beta synthase (CBS) deficiency. Its utility in the clinical setting is also limited by its needing to be transported on ice and separated within a short time following collection.

MMA assays are useful, as they are more specific. On the other hand, an increase in MMA is not systematically observed. This biomarker is implicated in a separate metabolic pathway, which is mitochondrial. The effect of N_2O on methylcobalamin has been widely demonstrated in preclinical models, but not on adenosylcobalamin. However, in several cases reported in the literature, MMA increases were observed in the most severe cases of intoxication. A correlation has been demonstrated between elevated MMA levels and clinical severity [30], reflecting deeper metabolic alteration.

Today, none of these described markers are strictly related to N_2O consumption or the duration of its consumption [30], and further studies are needed to define the precise kinetics of those markers. Optimal patient management therefore relies on monitoring a combination of markers. Plasma homocysteine and MMA combination reflects metabolic saturation by N_2O. A concomitant assay of vitamins B12, B9 or even B6 is also necessary to detect any associated deficiencies and supplement the patient in case of associated deficiency. This is essential for clinical management, after definitive cessation of consumption, but also aids with the interpretation of the above-mentioned markers. Liver and kidney function tests, as well as a full blood count, should also be carried out to rule out other secondary causes of increases in these metabolic markers of interest.

Conclusions

Routine and direct measurement of N_2O in biological fluids is complicated and difficult to interpret. Literature show that measurement techniques are reserved for highly specialized laboratories, and nitrous oxide pharmacokinetics data show that this molecule is difficult to quantify in the usual matrices used in medical laboratories. Indirect metabolic markers such as homocysteine and MMA are therefore of major interest, but there is a lack of data on the kinetics of these markers in the context of nitrous oxide use, and these markers still lack specificity, so there is a need to have a better understanding of consequences and related pathophysiology identify new markers such as oxidative stress markers or other metabolites.

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