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DONNER LABORATORY

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UPTAKE AND METABOLISM OF NITROGEN OXIDES IN BLOOD

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ABSTRACT

During the past forty years, numerous studies have examined the reactions of hemoglobin and whole blood with nitrogen oxides, generally using very high doses. This work describes the formation of nitroxyhemoglobin (Hb-NO) and its direct conversion to methemoglobin (met-Hb) in vitro, as well as the effects of human exposure to typical combustion effluents. Whole blood from mice was exposed to an NO atmosphere, resulting in the in vitro formation of Hb-NO, low-spin met-Hb, and high-spin met-Hb (measured by electron paramagnetic resonance spectroscopy). Hematocrit analysis revealed no hemolysis during the exposure. Subsequent exposure to clean air after purging results in the disappearance of Hb-NO with a 2-hour half-time. This is accompanied by the simultaneous and stoichiometric appearance of high-spin met-Hb. No low-spin met-Hb is produced from the decay of Hb-NO. In addition, human and rabbit blood specimens were obtained during in vivo exposure to 1-3 ppm NO, 0.3 ppm NO2, and up to 50 ppm CO. These pollutant levels (especially NO and NO2) are typical of the home environment during operation of combustion appliances. Blood levels of carboxyhemoglobin

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(Hb-CO) average about 5 per cent of the total hemoglobin during exposure, and decay upon relocation into clean air. No Hb-NO could be detected in this work, meaning that levels were less than 0.01%. Blood copper levels, in contrasts were easily detected and remained constant throughout. On the other hand, met-Hb levels in the blood rise linearly throughout the exposure, from 0.5 per cent beyond 2 per cent. Only high-spin met-Hb could be detected, suggesting its origin from Hb-NO. NO is therefore a toxic gas in its own right, at sub-occupational concentrations and without any need for its conversion to NO₂. If the present data are indicative, 3 ppm NO is comparable to 10-15 ppm CO in its effects on human health. NO at these levels is frequently encountered in the home regardless of the presence or absence of significant CO production.
INTRODUCTION

One of the most critical factors for understanding the health effects of air pollution from any source is an assessment of the uptake, transport, bio-transformation, and metabolism of air contaminants. The uptake and transport of pollutants within the body determines which organs will be targets and which physiological functions can be interrupted. Biotransformation of an air pollutant either in the bloodstream or in the target organ usually means that the physiological effects in these tissues will be different from effects in the respiratory tract and the lungs. The many metabolic reactions which are susceptible to attack by air pollution will therefore be different from organ to organ. Individual lesions in the structural and functional integrity of one tissue metabolic process should have cascading secondary and tertiary effects on other body processes, in addition to the direct effects of pollutant metabolites which manage to reach other parts of the body. All of these phenomena are highly dose-dependent.

The very high levels of gaseous air pollutants encountered in many of the indoor environments (1-4) have led us to consider the biochemical and physiological responses of individuals to these exposures. Since the lung and respiratory tract are obviously the primary targets for any exposure to air contaminants, these systems merit close scrutiny and are presently being investigated. However, if pollutant levels are sufficiently elevated (above current EPA standards), the bloodstream becomes a secondary target with the air contaminants and their metabolites being transported systematically. The present study assesses the impact of typical "indoor"-type concentrations of CO, NO, NO₂, SO₂, and particulate matter on the development of adverse biochemical changes in the blood of animals and humans.
Table I summarizes the typical reactions of gaseous pollutants with hemoglobin in whole blood. Numerous previous investigations have attempted to characterize the chemistry of hemoglobin in vitro and in vivo, in the presence of pure atmospheres or at least very high doses of the gases. Some of the most pertinent studies are cited in Table I, which also includes some data from the present report. Only two forms of hemoglobin, Hb- and Hb-O_2, are physiologically active and useful. The CO derivative of hemoglobin Hb-CO has been studied extensively, and its toxicology and therapy are well known (19). These investigations of blood Hb-CO levels form the basis of the EPA criteria document for CO (20).

Less is known about the interactions of oxidants and nitrogen oxides with hemoglobin. Recent studies (18, 21) have suggested that ozone may generate methemoglobin, as evidenced by Heinz body formation in whole blood in vitro. The complexity of this type of process is underscored by the likelihood that secondary reactions of O_3 or, for that matter NO_x (see ref. 16), may occur with the apoprotein of hemoglobin. Additional reactions of this sort have clearly been observed with ozone (21). Comparable in vivo experiments with O_3 are hampered by the reduced survival capacity of the subjects under the prevailing exposure conditions. In vivo methemoglobin chemistry under various O_3 exposure conditions is currently being investigated in this laboratory.

Interactions between hemoglobin and the oxides of nitrogen have enjoyed a more colorful history. Keilin and Hartree (11) first demonstrated the existence of both Hb-NO and met-Hb-NO as distinct entities in vitro in 1937. The very high affinity of (ferrous) hemoglobin for NO was graphically demonstrated by Gibson and Roughton (9), who observed similar binding kinetics.
<table>
<thead>
<tr>
<th>Gas</th>
<th>Hemoglobin Derivative Formed</th>
<th>Iron Valence</th>
<th>Spin State</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>Deoxyhemoglobin Hb-</td>
<td>+2</td>
<td>2</td>
<td>5-7</td>
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<tr>
<td>O₂</td>
<td>Oxyhemoglobin Hb-O₂</td>
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<tr>
<td>SO₂</td>
<td>no significant reaction</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>NO</td>
<td>Nitroxyhemoglobin Hb-NO</td>
<td>+2</td>
<td>1/2</td>
<td>5,9,11,12,13</td>
</tr>
<tr>
<td></td>
<td>Methemoglobin met-Hb</td>
<td>+3</td>
<td>5/2</td>
<td>14,15</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitroxyhemoglobin Hb-NO</td>
<td>+2</td>
<td>1/2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Methemoglobin met-Hb</td>
<td>+3</td>
<td>1/2;5/2</td>
<td>15,17</td>
</tr>
<tr>
<td></td>
<td>Nitroxymethemoglobin (unstable)</td>
<td>+3</td>
<td>&quot;0&quot;</td>
<td>11</td>
</tr>
<tr>
<td>O₃</td>
<td>Methemoglobin met-Hb</td>
<td>+3</td>
<td>1/2;5/2</td>
<td>18</td>
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</tbody>
</table>

Reduces Methemoglobin (Case, Dixon and Schooley, unpublished)
for NO, CO, and \( \text{O}_2 \) to Hb, but orders of magnitude longer dissociation times for Hb-NO than for the \( \text{O}_2 \) and CO complexes. In fact, the *in vitro* half-time for the dissociation of NO from Hb-NO is several hours (9,22). Nitroxyhemoglobin is much less stable than Hb-NO, and spontaneously dissociates within minutes even in a pure NO atmosphere (11).

The results of *in vivo* studies on the interactions of hemoglobin with NO tell an entirely different story. If the *in vitro* results of Gibson and Roughton (9) were applicable, atmospheric levels of NO in the 0.3 ppm (370 \( \mu \text{g/m}^3 \)) range should elicit the same response as 600 ppm (750 mg/m\(^3\)) CO. The latter figure is the median lethal dose observed for CO poisoning (20). Accordingly, NO levels as low as 0.04 ppm (50 \( \mu \text{g/m}^3 \)) should be sufficient for the appearance of acute pathological symptoms. This clearly is not observed for NO, even at levels as high as 3 ppm (3750 \( \mu \text{g/m}^3 \)). Examination of human and animal blood samples for Hb-NO under *in vivo* conditions originally failed to detect any of the derivative (22), and more recently only small traces of Hb-NO (23). This absence of significant Hb-NO accumulation in the blood of animals exposed to relatively high concentrations of NO formed the basis of the EPA decision *not* to recommend an ambient air quality standard for NO (24).

Whatever happened to nitroxyhemoglobin in the blood? One must consider that NO either is trapped before it can enter the bloodstream, or else is catalytically removed from Hb-NO by some biological detoxification mechanism. Recent observations of low steady-state levels of Hb-NO in the blood (13, 23) support the latter alternative. The results of the present investigation suggest that NO does get incorporated into the blood, with the resulting formation of methemoglobin from a nitric oxide derivative.
Increases in the methemoglobin content of blood following exposure to high doses of NO/NO₂ have been known for many years (14, 15, 17) and have resulted in the promulgation of standards for both NO (25 ppm or 31 mg/m³) and NO₂ (5 ppm or 9 mg/m³) under occupational exposure conditions (see refs. 17, 25). These reports have generally attributed the met-Hb formation to the action of nitrite ion generated by either NO or NO₂ in solution, and have actively discounted the direct uptake of NO by hemoglobin in the blood. The ability of nitrites in food and drink to elicit methemoglobin accumulation has been well documented (see ref. 26 for review), but its mechanism is presently unknown.

The present study examines the role of Hb-NO in the formation of met-Hb following exposure to nitrogen oxides. This report also describes a method for discriminating the origin of methemoglobin formed in vivo among the various possible sources, and also discusses the effects of human exposure to typical "indoor" air in view of the pollutant levels present (4).

EXPERIMENTAL

Blood samples were drawn from the cardiac left ventricle of mice, from the central artery of the ear in rabbits, or from the fingertips of human volunteers. Excess heparin was present in all cases to prevent clotting in the samples. All blood samples were then frozen in liquid N₂ and analyzed at low temperatures for nitroxyhemoglobin and methemoglobin by electron paramagnetic resonance (EPR) on a Japan Electron Optics Laboratory model VCX-2 spectrometer. Low temperatures used for the analyses were obtained by transferring liquid He through an Air Products model 110 Heli-Tran transfer line into the microwave resonance cavity, and were
monitored continuously with a calibrated carbon resistor. Total hemoglobin content and the concentration of carboxy-hemoglobin (Hb-CO) were determined spectrophotometrically on a Cary 17 UV-visible spectrophotometer (see ref. 27). Hb-CO could also be determined by flash photolysis at low temperatures according to the method of Yonetani et al (28). The methemoglobin standard was prepared by hemolyzing whole human blood in the presence of 23 mM K₃Fe(CN)₆, and dialysis followed by freezing in liquid N₂ (29).

Mouse blood was exposed in vitro to an atmosphere of pure NO (0.05 per cent NO₂) in a chamber which had been previously evacuated (experiments of Figures 1 and 2). The gas exposure/evacuation cycle was repeated at 5 minute intervals during the 30 minute total exposure period. Afterwards, the system was evacuated, relocated into clean air, and blood samples drawn periodically for analysis. Hematocrit analysis gave values of 43%, within normal range (17), indicating no hemolysis.

For the experiments of Figure 3 and Figure 4, human volunteers were stationed adjacent to the principal eastbound bore of Caldecott Tunnel approximately 100 m from the traffic exit. Through this tunnel in north-east Oakland passed about 900 motor vehicles per hour during the experiment, all traveling uphill through the bore. Pollutant gases and aerosols in the tunnel were sampled and analyzed either in the fan chamber immediately above the traffic bore, or else in the traffic bore itself. Concentrations of gases ranged between: 25-50 ppm (27-55 µg/m³) for CO; 2.5-4.0 ppm (3.1-5.0 mg/m³) for NO; 0.3 ± 0.05 ppm (0.55 mg/m³) for NO₂; and 0.14-0.20 ppm (0.37-0.50 mg/m³) for SO₂. CO was analyzed by non-dispersive infra-red spectroscopy; NO and NO₂ by chemiluminescence methods; and SO₂ by ultraviolet fluorescence (see ref. 4). Number and size distribution of the combustion
aerosols were determined with a nephelometer and a Whitby electrostatic mobility analyzer, and the chemical composition of the particulates was determined by X-ray fluorescence and by photoelectron spectroscopy (ESCA) in addition to gas phase chromatography-thermal conductivity methods. "Clean air" blood samples were obtained at sites at least 300 m from the tunnel, in which the traffic volume was no more than 3 vehicles per hour. Air quality in these situations was assumed to be typical of semi-urban ambient air: CO less than 1.5 ppm (1.7 mg/m$^3$); NO, NO$_2$, SO$_2$, and O$_3$ all less than 0.05 ppm (0.07 µg/m$^3$). O$_3$ levels never exceeded the outdoor background.

For the "indoor" air exposure experiments, blood samples were drawn from rabbits situated 1 meter away from gas stoves in kitchens. Sampling and analysis methods for both air quality and blood composition were identical to those used in the tunnel experiments above, and are described in detail in ref. (4). Gas levels in the kitchens ranged between: 6-8 ppm (7-9 mg/m$^3$) for CO with no pans sitting on the burners, but 13-30 ppm (15-33 mg/m$^3$) for CO with pans present; 0.8-2.0 ppm (1.0-2.5 mg/m$^3$) for NO in either case; 0.2-0.3 ppm (0.33-0.55 mg/m$^3$) for NO$_2$ in either case; and less than 0.01 ppm (25 µg/m$^3$) for SO$_2$ in all instances (4). Clean air blood samples were obtained prior to exposure in the kitchens, as above.
RESULTS

Figure 1 depicts a typical EPR spectrum of Hb-NO from mouse blood exposed to gaseous NO\textsubscript{x}. A strong resonance is observed at $g = 2$, with several poorly resolved fine structure peaks due to hyperfine coupling to the $^{14}\text{N}$ nucleus present. This spectrum is very similar to that reported by Kon (12) and confirmed by Yonetani and Yamamoto (30), but differs significantly from the spectra reported by Rowlands and Gause (16) and Oda et al (23). Differences in the measurement temperature cannot account for the discrepancies between the Figure 1 spectrum and the results of other workers (16, 23, but compare ref. 30). Neither can differences between the various \textit{in vitro} and \textit{in vivo} exposure conditions. Our Hb-NO spectra from the human and rabbit \textit{in vivo} exposure experiments appear indistinguishable from the Figure 1 spectrum. Rowlands and Gause (16), likewise, observed no differences in the Hb-NO spectra of blood exposed either \textit{in vitro} or \textit{in vivo} to cigarette smoke; however, their spectra exhibited much more fine structure in both cases than is evident in the present data or in the work of Kon (12) or Yonetani (30). In separate experiments, we were able to obtain a Hb-NO spectrum like those in Refs. (16, 23) by adding NaNO\textsubscript{2} and Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} to the blood.

The time course for the disappearance of Hb-NO is given in Figure 2 (top). The magnitude of the $g = 2$ EPR signal increases slightly in the initial stages, but then declines slowly and steadily with a half-time on the order of two hours. This decay rate is somewhat faster than the 12 hour half-time estimated by Gibson and Roughton (9) and the 5 hour half-time reported by Sancier \textit{et al} (22), but agrees reasonably well with the 100 minute \textit{in vitro} half-time observed by Anbar (Personal communication) using
a different method. This observation, in itself however, does not reveal any information regarding the fate of Hb-NO either in vitro or in vivo.

The middle and bottom portions of Figure 2 display the time courses for the low-spin and high-spin forms of methemoglobin, respectively, under identical conditions. Both forms exhibit an initial "rapid" rise in methemoglobin. However, the low-spin form of methemoglobin then decays to a basal level which is subsequently time-independent. In contrast, the level of high-spin methemoglobin (Figure 2, bottom) continues to increase with time. This indicates de novo met-Hb formation, and not conversion from low-spin to high-spin. If one compares the absolute signal amplitudes in Figure 2 with those obtained for pure met-Hb from human blood, the amount of high-spin met-Hb produced during the decay of Hb-NO is approximately 25 per cent of the total hemoglobin. Given the total met-Hb that must have been present immediately after the NO exposure, the maximum amount of Hb-NO which was formed initially could have been as much as 55-60 per cent of the remaining total hemoglobin. Decay of half this amount would imply that a maximum of 27-30 per cent of the total hemoglobin was converted from Hb-NO into a different form during the first two hours. Within experimental uncertainty, the present results strongly suggest that Hb-NO is converted directly and stoichiometrically into high-spin methemoglobin. This process entails the oxidation of the iron atom in Hb-NO from the 2+ valence state to the 3+ valence, in addition to the oxidation of the NO ligand to its solution product. Molecular O₂ is probably required, since other workers (5, 11, 12) have demonstrated that Hb-NO is stable for long periods of time in an inert atmosphere.

Experimental results indicating changes in the in vivo blood levels
of Hb-NO and met-Hb have been obtained for rabbits and humans (Figures 3
and 4). While these experiments were carried out inside an automobile
tunnel, the pollutant gas levels encountered during this exposure closely
resemble those found in many gas kitchens during meal preparation conditions
(see Experimental, and also ref. 4). Figure 3 demonstrates clearly the
ability of combustion effluents containing 3 ppm (3750 μg/m³) NO to raise
the steady-state level of methemoglobin in human blood in vivo. All of the
met-Hb produced as a result of the exposure is of the high-spin type; the
in vivo level of low-spin met-Hb remains indistinguishable from zero through
the exposure period. Since some 15-40 per cent of the total met-Hb formed
from the usual chemical oxidation of hemoglobin (by ferricyanide, for example)
is usually comprised by the low-spin component at 14°K, the absence of any
low-spin met-Hb production suggests the origin of the high-spin met-Hb which
arises during the exposure as a nitric oxide derivative. Furthermore, the
absence of any other oxidant species in the atmosphere, except for some
NO₂, supports the view that the increase in met-Hb is due to Hb-NO
metabolism.

The time courses for blood copper, met-Hb, and Hb-CO formation and
decay in human blood prior to, during, and following exposure to a tunnel
atmosphere are given in Figure 4. Copper levels on the order of 0.15 to
0.2 per cent of the total Hb are routinely observed in human blood (Figure 4,
top); No change in the steady-state concentrations of copper could
be detected at any time point in the experiment. Using the EPR tech-
nique, we were unable to detect any Hb-NO in any of the blood
samples. Hb-NO levels as low as 0.01 per cent of the total Hb should have
been visible. In contrast, Anbar et al (13), using a different analytical
method for Hb-NO, reported typical base levels of 0.25 per cent Hb-NO in whole human blood regardless of the smoking and exposure history of the subjects. The present results also differ from the recent report of Oda et al. (23) who detected an increase in the in vivo blood concentration of Hb-NO in mice from about 0.02 per cent to 0.12 per cent during a one-hour exposure to 8-10 ppm (10-12 mg/m³) NO. We simply have not observed any such effect. Neither of these investigators (16, 23) reported any measurements of methemoglobin in their experiments.

The upper half of Figure 4 also expresses the in vivo in changes high-spin met-Hb as a function of exposure to combustion gases in the tunnel. Human blood characteristically contains between 0.2 and 0.7 per cent met-Hb in the absence of high levels of nitrogen oxides (Compare Figure 4, top, with ref. 31). Exposure to an atmosphere containing combustion effluents steadily increases the met-Hb level to approximately 1.5 per cent of the total hemoglobin within a 3 hour exposure. Other experiments have suggested met-Hb levels in the blood in excess of 2 per cent as the result of exposure to typical "indoor" atmospheres in some cases. There is no obvious indication in the Figure 4 experiment that the blood level of met-Hb would plateau had the exposure period continued. Additional experiments to determine the maximum level of met-Hb which can be induced in animals as the result of exposure to NO are currently in progress.

As the bottom portion of Figure 4 indicates, the blood level of Hb-CO rises fairly rapidly during the tunnel exposure to a final level of about 5 per cent of the total hemoglobin. Because the CO concentration in the tunnel atmosphere was as high as 25-50 ppm (27-55 mg/m³), the elevated Hb-CO levels are expected. However, in many of the "indoor" studies,
kitchen levels of CO were much lower, while the NO and NO₂ levels remained nearly as high as in the tunnel experiment.

Separate experiments with rabbits exposed to a variety of indoor and outdoor environments give results which are essentially the same as in the upper section of Figure 4. Again, no Hb-NO could be detected. Levels of blood copper were low but measurable (≈ 0.2 per cent, compare with ref. 23), and remained constant. On the other hand, the concentration of high-spin met-Hb steadily and linearly rose throughout the exposure to gas stove exhausts, from about 0.5 per cent to 1.5-2.0 per cent within the first 30 minutes. Again, no sign that the met-Hb concentration might level off was evident.

DISCUSSION

The present results clearly indicate the metabolic reaction of hemoglobin with the oxides of nitrogen to give rise to methemoglobin in vivo. Several reports (14, 15, 33), using much higher concentrations of NO and NO₂ in the exposure conditions, have previously shown that met-Hb formation can occur in response. More important, we have observed the formation of significant blood levels of methemoglobin from atmospheric NO concentrations which are frequently encountered by most individuals in their own homes. In a subsequent report, we shall show the interactions of ambient level ozone with whole blood, in which O₃ completely reduces met-Hb, and subsequently gives rise to an unidentified organic free radical in the blood (Case, Dixon, Schooley, unpublished). To place these observations in the proper perspective, however, one must consider the relative
impact of \( \text{NO}_x \) exposure in terms of all known environmental sources of methemoglobin, and the relative importance of methemoglobin in the assessment of health risks imposed on the public by all environmental contaminants.

For example, the formation of methemoglobin from nitrates and nitrites in foods and drinking water has been extensively documented (see ref. 26). Several national and state government agencies have imposed stringent limitations on the nitrate/nitrite content of cured meats, baby foods, and drinking water as a direct result of information in the toxicological literature, much of which implicates food and water sources in cases of acute poisoning. If the reaction between nitrite and hemoglobin is stoichiometric within a factor of two and if conversion of \( \text{NO}_3^- \) to \( \text{NO}_2^- \) is 75 per cent complete (31), then the amount of \( \text{NO}_3^-/\text{NO}_2^- \) required for the initial conversion of 2 per cent of the total Hb to met-Hb (equivalent to the met-Hb formed from indoor-type \( \text{NO}_x \) exposure) is approximately 55 mg for an average adult (17, 26, 31). In the same adult individual, an additional 11 mg/hr of \( \text{NO}_3^-/\text{NO}_2^- \) would be required to maintain the blood level of met-Hb at 2 per cent, since the normal adult half-life of met-Hb is approximately 2.5 hours (31). This corresponds to an initial dose of five hot dogs followed by one additional hot dog every hour thereafter, or 1-2 ounces of most kinds of vegetables (26). One hot dog every 3 hours is sufficient to maintain a blood met-Hb level of 0.7 per cent, which is the normal adult human base level of met-Hb (Figure 4; also refs. 26, 31). Because the prevailing form of hemoglobin in infants and unborn children is different from the adult type, the sensitivity of infants to \( \text{NO}_3^-/\text{NO}_2^- \) is at least 10-fold greater than for adults (26, 31).

The implication of the present work is that nitrogen oxides
generated by household combustion appliances can account for a substantial fraction of the total met-Hb which is present in the blood of most humans. Typical concentrations of these gases can elevate the met-Hb content of blood to potentially dangerous levels, and represent a dose comparable to the five hot dogs. The physiological effect of an accumulation of methemoglobin in the blood is nearly identical to the buildup of carboxyhemoglobin (31). Like Hb-CO, met-Hb is totally ineffective as an oxygen carrier, but nevertheless occupies blood volume. The increased probability of tissue hypoxia or the increased load on the heart would be the same in either case. Both blood contaminants are effectively "removed from circulation" slowly, (CO from the lungs; met-Hb by the spleen and liver) with respective half-times on the order of 30 minutes for Hb-CO (5, 9, 20) and 2.5 hours for met-Hb (31). Consequently, 2 per cent met-Hb should elicit the same long-term effects as 2 per cent Hb-CO (see ref. 32 for review), and so on.

One should now note that the EPA air quality standard for CO (20) is 9 ppm (10 mg/m\(^3\)) for an average 8-hour exposure period. This standard is based on evidence that Hb-CO concentrations in excess of 1.5-2.0 per cent elicit measurable pathological symptoms in humans and animals (20, 32). The same situation is necessarily true for met-Hb generated by NO\(_2\) and NO, the latter for which no ambient air quality standard presently exists. If the present data are indicative, 3 ppm (3.75 mg/m\(^3\)) NO is physiologically comparable to CO levels on the order of 10-15 ppm (11-17 mg/m\(^3\)). On this basis, the existing occupational air quality standards for NO (25 ppm or 31 mg/m\(^3\)) and CO (50 ppm or 55 mg/m\(^3\)) are self-consistent (17, 25).

Whether any of these standards are realistic is a different question altogether. Since the CO standards were formulated on the basis of blood
levels of Hb-CO (20), the problem of personal dose measurements does not apply. The original investigators were able to determine directly the true personal doses of CO (see ref. 32 for review) although no attempt was made in these investigations to remove possible met-Hb interferences from the Hb-CO analyses in blood. On the other hand, most of the epidemiological studies which correlated specific pathological responses to the blood Hb-CO concentration neglected to carry out parallel measurements of met-Hb (20, 32). Since the combustion processes which emit CO usually give off prodigious amounts of NO as well, one can probably assume that the effects on human health responses derive from the combined action of CO and NO, and not from reactions involving only one of these species. The major pitfall and a source of error in these studies arises as the result of human exposure to effluents from the combustion of "clean" fuels. Recent work in this laboratory has shown that the combustion of natural gas ("clean" from the standpoint that virtually no CO is emitted in the flame itself) nevertheless gives off substantial amounts of NO and NO₂ (4). It is now abundantly clear that future studies of the health effects of air pollution should consider the impact of indoor as well as outdoor sources of pollution, and should examine human populations for the presence of met-Hb as well as Hb-CO.
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Lawrence Berkeley Laboratory, University of California, Berkeley, California.


Figure 1. EPR spectrum of nitroxyhemoglobin. Mouse blood, total heme content = 14 mM. EPR conditions: midscale magnetic field, 3300 gauss; modulation amplitude, 10 gauss; modulation frequency, 100 KHz; microwave frequency, 9.21 GHz; receiver gain, 1; temperature, 14°K.
Figure 2. Time course for the conversion of Hb-NO into met-Hb. Mouse blood. Conditions as in Figure 1. Top: Nitroxyhemoglobin, $g = 2$ signal. Midscale magnetic field, 3300 gauss; range, 500 gauss. Middle: Low-spin methemoglobin, $g = 3$ region. Midscale magnetic field, 2200 gauss; range, 500 gauss. Bottom: High-spin methemoglobin, $g = 6$ signal. Midscale magnetic field, 1100 gauss; range, 500 gauss.
Figure 3. In vivo concentrations of methemoglobin in blood as a function of exposure to combustion exhausts in Caldecott Tunnel. EPR spectra of high-spin methemoglobin, g = 6 region. Midscale magnetic field, 1100 gauss; range, 500 gauss. Other EPR conditions as in Figure 1, except that receiver gain of 100 = x1. Upper Spectrum: Taken after 2-hour exposure to tunnel atmosphere (see Experimental). Lower spectrum: Taken in an "outdoor" environment in Berkeley away from vehicular traffic.
Figure 4. Uptake and reactions of air pollutants in blood. In vivo exposure included atmospheres containing combustion effluents, and subsequently to clean air. Blood samples drawn and frozen for analysis. Circles and triangles represent data from two organisms. Upper Figure: High-spin methemoglobin and copper (II) in the Blood detected by EPR spectroscopy. Lower Figure: Carboxyhemoglobin (Hb-CO) under identical conditions, measured optically at two different visible wavelengths (open and solid symbols). No nitroxyhemoglobin detected.
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