Interactions of the Anesthetic Nitrous Oxide with Bovine Heart Cytochrome c Oxidase

EFFECTS ON PROTEIN STRUCTURE, OXIDASE ACTIVITY, AND OTHER PROPERTIES

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The interactions of nitrous oxide with cytochrome c oxidase isolated from bovine heart muscle have been investigated in search of an explanation for the inhibition of mitochondrial respiration by the inhalation anesthetic. Oxidase activity of the isolated enzyme is partially and reversibly reduced by nitrous oxide. N₂O molecules are shown by infrared spectroscopy to occupy sites within the oxidase. Occupancy of sites within the protein by N₂O has no observed effects on visible Soret spectra or on the O₂ reaction site; no evidence is found for N₂O serving as a ligand to a metal. The anesthetic does not substitute for O₂ as an oxygen atom donor in either the cytochrome c oxidase or carbon monoxide dioxygenase reactions catalyzed by the enzyme. N₂O appears to affect oxidase activity by reducing the rate of electron transfer from cytochrome c to the O₂ reaction site rather than by interfering directly with the reduction of O₂ to water. Cytochrome c oxidase represents a target site for nitrous oxide and possibly other anesthetics, and the inhibition of oxidase activity may contribute significantly to the anesthetic and/or toxic effects of these substances.

Nitrous oxide, N₂O, has been used as a general anesthetic for over a century. Low potency, low solubility, and rapid induction as well as rapid recovery account for the widespread acceptance of N₂O as one of the safest and least toxic of the inhaled anesthetics (1). However, it is now clear that N₂O does have toxic properties which include effects on respiration, circulation, brain activity, and neuromuscular transmission (1). N₂O was recently reported to reversibly inhibit oxygen utilization by bovine heart and bean mitochondria (2). It was suggested that respiration was depressed due to interactions of N₂O with cytochrome c oxidase. Therefore, the oxidase may be a site of anesthetic and/or toxic effects of N₂O in animals and may also represent the site of observed effects of anesthetics on seed respiration, dormancy, and germination (3).

The mechanism of anesthesia remains a matter of great debate (4, 5). Where and how the anesthetic molecules exert their effects is still unclear. General anesthesia can be induced by a wide variety of structurally dissimilar molecules which appear to have but one thing in common. Namely, they are all more soluble in hydrophobic (lipophilic) environments than in hydrophilic environments (4, 5). The mechanism by which these molecules produce their anesthetic effects is thought to involve nonspecific interactions at the target site. Since anesthetic potency can be correlated with lipid solubility (Meyer-Overton rule) (6, 7), the lipid bilayer of neural membranes has been suggested as a primary target site. However, a few studies indicate that proteins may also provide sites of interaction with small anesthetic molecules (4, 5). Therefore, anesthesia may result from effects on proteins as well as on lipids. The nature of the environment experienced by the anesthetic molecules in tissue, a crucial point for understanding the mechanism of general anesthesia, has been difficult to determine directly. It has been shown that the uptake, distribution, and loss of nitrous oxide in the brain of a dog under halothane-N₂O anesthesia can be followed by the use of infrared spectroscopy (8). Effects of solvent structures on the infrared antisymmetric band of N-N-O near 2220 cm⁻¹ have demonstrated that stronger polar interactions between solvent and the vibrating dipole of the N-N-O molecule increase infrared band frequency (υ₁) which varies from 2230.1 cm⁻¹ for the nonpolar CH₃ to 2215.3 cm⁻¹ for the nonpolar CS₂. Bandwidths (Δυ₁/2) were found to reflect the degree of uniformity of interactions of N₂O with surrounding solvent molecules. Bandwidth increased as the conformational flexibility of the adjacent solvent molecules increased. These correlations of solvent molecule structure with N₂O infrared spectra permit the characterization of sites occupied by the anesthetic in more complex systems in terms of the polarity and mobility of molecules immediately adjacent to the anesthetic molecule. The infrared spectra of nitrous oxide in proteins, lipids, vesicles, red blood cells, and brain tissue show multiple stretch bands, demonstrating that N₂O is distributed among sites of differing polarity in these systems (10). Furthermore, the protein that can offer sites to N₂O can be either a hydrophobic membrane protein or a hydrophilic globular protein such as myoglobin (11) or hemoglobin (10). We have presented a brief preliminary report that CcO can also provide sites for N₂O (12).

CcO is an integral protein of the inner mitochondrial membrane and plays a key role in energy production via the respiratory metabolism of nearly all aerobic organisms (13).

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The abbreviations and trivial names used are: υ₁, stretch band wavenumber at maximum absorbance; Δυ₁/2, stretch band width in wavenumber at half the maximum absorbance; CcO, cytochrome c oxidase; Co(OIV) and Co(O), represent cytochrome c oxidase at the fully oxidized and fully reduced oxidation states, respectively; Co(II)/Co, Co(II)CO, and Co(OI)CO, represent 1-, 2-, and 4-electron reduced carbonyl cytochrome c oxidase, respectively; Co(OIV)/N₂O, Co(O)/N₂O, and Co(O)/CO/N₂O, represent the fully oxidized, fully reduced, and fully reduced carbonyl N₂O-bound cytochrome c oxidase, respectively.
14). In addition to its key bioenergetic role of catalyzing the reduction of dioxygen to water, reaction 1,
\[ \text{O}_2 + 4 \text{cytochrome c}^{2+} + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O} + 4 \text{cytochrome c}^{3+} \] (1)

CcO also catalyzes the oxidation of carbon monoxide as in reaction 2
\[ \text{O}_2 + 2\text{CO} \rightarrow 2\text{CO}_2 \] (2)

(15). Thus, the protein is both a cytochrome c oxidase and a carbon monoxide dioxygenase. Studies by Orii (16) and Bicker et al. (17) have shown that CcO can catalyze the peroxidation of ferrocytochrome c under anaerobic conditions. Therefore CcO has wider catalytic capabilities than originally thought and could possibly catalyze similar reactions in which molecules other than O₂ and CO serve as donors and/or acceptors of oxygen atoms. Nitrous oxide represents a molecule that might serve as an oxygen donor as shown in reactions 3 and 4.

\[ \text{N}_2\text{O} + \text{CO} \rightarrow \text{CO}_2 + \text{N}_2 \] (3)

\[ 2 \text{cytochrome c}^{2+} + \text{N}_2\text{O} + 2\text{H}^+ \rightarrow 2 \text{cytochrome c}^{3+} + \text{N}_2 + \text{H}_2\text{O} \] (4)

The possibility that nitrous oxide can participate in CcO-catalyzed reactions receives support from its known reaction chemistry. N₂O is generally inert at physiological temperatures and pressures (1, 18), but numerous reactions have been described. With a thermodynamic potential for reduction to dinitrogen and water of +1.77 V versus the normal hydrogen electrode N₂O₃ is a potent oxidant. It has been used as an oxidant to study the activity of several binary metalloporphyrins as electrocatalysts (19) and can act as a mild oxidizing agent toward transition metal complexes (20, 21). It oxidizes vitamin B₁₂ in aqueous solution thereby converting cob(III)alamin to cob(II)alamin according to the stoichiometry shown in reaction 5 (20, 21).

\[ \text{N}_2\text{O} + 2\text{Co(I)} \rightarrow \text{N}_2 + 2\text{Co(II)} \] (5)

In the catalytic oxidation of Ph₃P to Ph₃PO by N₂O using [(Ph₃P)₅Co(H)]⁻ as a catalyst, N₂O oxidizes a ligand rather than the metal (21). In studies by Bottomley et al. (22) on the reaction of N₂O and O₂ with dicyclopentadienyltitanium carbonyl complexes, carbon monoxide is oxidized. The authors suggest that the mechanism involves insertion of an oxygen atom of N₂O into O₂ to form a Ti-O bond. The ability of N₂O to serve as a ligand has not been investigated extensively. N₂O, which is iso-electronic with NCO⁻, might be expected to act as a unidentate ligand, binding either through nitrogen or oxygen. Only one complex containing N₂O appears to have been characterized, [Ru(NH₄)₂(N₂O)]⁺, and it is N-bonded and readily replaced by other ligands (23).

N₂O is a common product and substrate of bacterial denitrification, the reduction of nitrate or nitrite to N₂. The nitrogen oxide reductases of this process serve as terminal carriers in anaerobic respiration which supports proton translocation, oxidative phosphorylation, and growth (24, 25). N₂O is an intermediate which is reduced to N₂ by nitrous oxide reductase (28). Participation of cytochromes in the electron transport mechanism of the reduction of N₂O to N₂ has been proposed (27). Copper has been shown to be indispensable for the biosynthesis of the enzyme and has been suggested to be an integral part of the reductase (28–30). The enzyme has also been shown to be inhibited by acetylene, CO, azide, and cyanide (24, 26, 28). Thus, N₂O reductase shows many similarities with CO, which contains Cu, Zn, and Mg as well as heme Fe (31). The reaction of N₂O with the reductase along with the demonstrated reactivity of N₂O toward the vitamin B₁₂ enzyme, suggests the possibility that N₂O may interact with other electron transport systems in the body, including CcO. Thus, several lines of evidence suggest that N₂O is not as inert as originally thought and may be a substrate for CcO in the CO and cytochrome c³ oxidation by O₂.

We report here studies of interactions between N₂O and bovine heart cytochrome c oxidase and effects of these interactions on the structure and function of the enzyme. The N₂O infrared spectra show that N₂O molecules are distributed among different sites within the protein. The occupancy of one or more of these sites by N₂O partially inhibits the oxidation of cytochrome c³⁺ by O₂. No evidence is obtained to suggest that N₂O can serve as a ligand to any of the metals or as an oxygen atom donor in reactions catalyzed by CcO.

**Materials and Methods**

Bovine heart CcO was isolated as described previously (31, 32). The final product in 10 mM sodium phosphate buffer, pH 7.4, was concentrated to 1–2 mM in terms of heme A and stored at -70°C. The Sweeter reagent used for removal of trace amounts of O₂ from N₂O and CO was made up of propylene, methylviologen, and EDTA (Sigma and Aldrich) and activated by illumination (33). N₂O, CO, and N₂O/O₂ were obtained from General Electric (99.99%) and used without further purification. N₂O/CO/O₂ mixtures were prepared by bubbling N₂O through a gas calibration cell containing CO and measurement of absorbance at wavelengths characteristic for CO₂. The infrared spectra of these solutions were obtained by scanning the concentration of CO₂ and HCO₃⁻ dissolved in the solution (30). A cyclindrical air-tight cell of pathlength 10 cm and volume 89.3 ml was used for gaseous infrared spectral measurements. The cell was equipped with a sidearm quartz cuvette which permitted measurements of visible Soret spectra of any solution present. The possible oxidation of CO by nitrous oxide was investigated in the same manner as was the oxidation by O₂ except for the use of N₂O in place of O₂. In control experiments N₂ was substituted for N₂O.

The oxidation of CO to CO₂ in the presence of O₂ or N₂O was followed by the intensification of the CO₂ bands at 2340–2360 cm⁻¹ in the infrared spectrum. Prior to injection of O₂ or N₂O, the 2400–2280 cm⁻¹ region was scanned several times until no further increase in absorbance at 2360 cm⁻¹ was observed. The number of moles of CO₂ produced was calculated from the increase in absorbance at 2360 cm⁻¹ using an extinction coefficient of 0.129 mM⁻¹ cm⁻¹ and correction for the amount of CO₂ and HCO₃⁻ dissolved in the solution (15). Partial reduction is necessary for activation of the enzyme. Exposure and incubation of the resting enzyme, CO/(IV), to excess CO (or N₂O) leads to a slow formation of the required one or two electron-reduced carbonyl species, CO/O/CO₂ or CO/CO₂, respectively (34). The redox state of the enzyme solution prior to injection of N₂O or O₂ was determined from the visible spectra.

**Possible Oxidation of Cytochrome c³⁺ by N₂O—Oxidation of cytochrome e²⁺ by N₂O was monitored by visible spectroscopy and the disappearance of N₂O by gaseous infrared spectroscopy. The oxidation of cytochrome c²⁺ was followed by absorbance changes at 550, 555, and 560 nm and the decrease in gaseous N₂O by comparing the absorbance in the N₂O asymmetric (ν₁) stretch region (2280–2130 cm⁻¹). The amount of residual oxygen present was determined from the extent of cytochrome c³ oxidation prior to injection of N₂O (N₂O in air present). The extent of autooxidation of cytochrome c²⁺ was determined similarly but without CO present.**

**Oxidase Activity Measurements**—The effect of N₂O on enzyme activity was investigated with N₂ and air as controls. CO solutions (1 mM in heme A) were flushed with N₂O (N₂ for control) for 1 h. A third sample was untreated (i.e., kept under vacuum). The reaction mixture (0.1 M phosphate buffer, pH 6.0) containing 15 mM reduced cytochrome c was under air at atmospheric pressure. Activities were measured spectrophotometrically by following the oxidation...
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of reduced cytochrome c at 550 nm (35) upon addition of 10 μl of oxidase solution (under air, N_2, or N_2O) to 3 ml of reaction mixture at 25 °C. Triplicate assays were performed for each of five enzyme preparations. The reversibility of the N_2O effects was shown by flushing the N_2O-treated CcO solutions with N_2 for 15 min. Rates are expressed in s⁻¹/mg of protein/3 ml. The rate constants (the slopes of the first order plots) were determined by logarithmic regression.

Recording of Spectra—The infrared spectra were recorded on either a Perkin-Elmer model 180 spectrometer interfaced to a Tektronix 4051 computer, or on a Perkin-Elmer model 580B spectrometer with a model 3500 Data Station. The solution infrared spectra were carried out at 25 °C in variable-temperature IR cells with CaF₂ windows and a pathlength of 0.05 mm. The spectra obtained on the Perkin-Elmer 580B spectrometer in transmittance mode 3 under dual-beam operation with 2.1 cm⁻¹ resolution were converted to absorbance values. The Perkin-Elmer 180 spectrometer was operated in an absorption mode at a scan rate of 6.11 cm⁻¹/min and time constant of 4, with resolution settings of 2.0 cm⁻¹ at 2280 cm⁻¹ and 2.0-2.5 cm⁻¹ at 2000 cm⁻¹ for the N_2O and CO regions, respectively. The spectra were collected at an interval of 0.1 cm⁻¹ from 2280 to 2180 cm⁻¹ for N_2O and from 1995 to 1945 cm⁻¹ for CO. Slope correction and analysis by deconvolution were carried out as previously described (9). A Gaussian distribution was used to analyze the CO spectra whereas a mixture of Gaussian/Lorentzian (0.3/0.7) was used to deconvolute the N_2O spectra. The gaseous CO infrared spectra were recorded between 2400-2280 cm⁻¹ on a Perkin-Elmer 180 spectrometer operated in a constant I₀ linear absorbance mode with scan time of 7.4 sec/min and a time constant of 4. The gaseous N_2O infrared spectra were recorded over the region 2280-2130 cm⁻¹ on a Perkin-Elmer 580B spectrometer in transmittance mode 2 with 2.8 cm⁻¹ resolution. The spectra were plotted in absorbance and the integrated absorbance, J_{abs}, determined as the area under the curve using a computer integration routine. The partial pressure, P, of N_2O present in the gas cell was determined from Beer’s law (J_{abs} = P (cm⁻¹ atm⁻¹) × S (cm⁻² atm⁻¹ cm⁻¹) using infrared absorption strength, S, for the ν₂ band system (ν = 2224 cm⁻¹) of 1421 ± 76 cm⁻² atm⁻¹ at 298 K (36). The number of moles of N_2O present was calculated using the ideal gas law (n = PV/RT). Visible Soret spectra were recorded on a Cary model 17 spectrometer.

RESULTS

Infrared Spectra of Nitrous Oxide in CcO—The infrared spectrum of N_2O in a solution of fully oxidized CcO is significantly different from the spectrum of N_2O in pure water (Fig. 1). In water the spectrum can be deconvoluted into two bands: a N_2O ν₂ antisymmetric stretching fundamental at 2231.1 cm⁻¹ (Δν/ν₂ = 11.5 ± 0.5 cm⁻¹) and a much weaker “hot band” at 2216 cm⁻¹ (Δν/ν₂ = 15 ± 1.0 cm⁻¹). Hot bands are present in all spectra of N_2O in solutions (9). The difference spectrum, N_2O in the CcO(IV) solution minus N_2O in water (Fig. 1, bottom panel), is clearly asymmetric. Deconvolution of this difference spectrum yields two major bands at 2226.4 and 2217.9 cm⁻¹, and a minor third band at ~2325 cm⁻¹. The spectrum observed for the CcO(IV) solution is represented by a minimum of four bands (Fig. 1, lower middle). The aqueous fundamental and hot bands are located at 2231.9 and 2217.9 cm⁻¹, respectively. The other two bands at 2226.4 and 2218.4 cm⁻¹ must arise from the presence of N_2O in two different environments within the protein. A minor band near 2255 cm⁻¹ is suggested by the curvature between 2230 and 2240 cm⁻¹ in the residual (the difference between the experimental curve and the sum of the four theoretical curves) (Fig. 1, lower middle). The weak hot bands associated with the fundamental bands other than the aqueous band are not readily detected.

The spectra for N_2O in solutions of the fully reduced enzyme, CcO(0)/N_2O, and its carbonyl, CcO(0)/CO/N_2O, are similar to the spectra observed for CcO(IV)/N_2O (Fig. 2). The infrared band parameters for the different N_2O complexes are listed in Table I. The visible Soret spectra of CcO(IV), CcO(0), and CcO(0)/CO solutions are unchanged by the presence of N_2O (Fig. 3).

Effect of N_2O on CO Binding—Nitrous oxide does not alter the CO infrared spectrum of the fully reduced carboxyl enzyme, CcO(0)/CO, as shown by a flat difference spectrum (Fig. 4). The frequencies, bandwidths, and relative intensities of the four carbonyl bands observed in the CO infrared spectrum are unchanged by the presence of N_2O. Adding CO to the CcO(0)/N_2O complex gives a visible spectrum identical to that of CcO(0)/CO (Fig. 3). Activity Measurements—The ability of CcO to catalyze the oxidation of cytochrome c⁺⁺ by O₂ is impaired by the presence of N_2O. The rate constants for the untreated, N_2, and N_2O-treated enzyme solutions for five different preparations are listed in Table II. The reaction follows first order kinetics with and without N_2O present (Fig. 5) but prior exposure to N_2O reduces the activity ~40% (Table II). The activity of the enzyme upon incubation under N_2 is the same within experi-

FIG. 1. Infrared spectra of N_2O in water and in a solution of fully oxidized cytochrome c oxidase (1.3 mM) in 10 mM sodium phosphate buffer at pH 7.4 and 25 °C. Top, spectrum of N_2O in water. Upper center, spectrum of N_2O in the oxidase solution. Lower center, spectrum of N_2O in the oxidase solution (upper center) spectrum deconvoluted into four theoretical curves of 30% Gaussian and 70% Lorentzian character with the band parameters listed in Table I. Bottom, difference spectrum of the N_2O within the enzyme obtained by subtracting the spectrum of N_2O in water (top) from the spectrum of N_2O in the oxidase solution (upper center). The normalization factor used to determine the amount of N_2O in water spectrum to be subtracted was estimated from the intensity of the fundamental band at 2231.9 cm⁻¹.
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Oxidation of Carbon Monoxide in the Presence of CcO—CcO catalyzes the oxidation of carbon monoxide to carbon dioxide in the presence of oxygen. Fig. 6 shows the initial and final gaseous CO infrared spectra following injection of 200 μl of air into the sample gas cell containing CO and a solution of CcO. Autoxidation of the enzyme solution was confirmed by visible Soret spectra. The stoichiometry observed was 2 mol of CO produced for 1.3 mol of O₂ added. These results are in agreement with results obtained earlier in our laboratory (15). When N₂O or N₂⁴O was injected in place of air, no unequivocal evidence for CO oxidation by N₂O was obtained. Preliminary experiments on the possible oxidation of CO by H₂O₂ suggest that much less than stoichiometric amounts of CO₂, if any, were produced.

DISCUSSION

Sites Occupied by N₂O in CcO—The N₂O infrared bands observed at 2231.9 and 2217.9 cm⁻¹ for N₂O in oxidase solutions can be assigned to the fundamental and hot bands of N₂O in buffer (9). Hot bands are uniformly observed at ~14 cm⁻¹ lower frequency than the respective fundamental. The intensity of the hot band in water represents only ~7% of the total intensity, whereas in other solvents the hot band intensity is about 13% of total intensity (9). For N₂O in protein solutions, fundamental and hot bands due to N₂O molecules in water (buffer) are expected near 2230 and 2216 cm⁻¹, respectively. However, the 2-cm⁻¹ shift of these bands to higher frequency in CcO compared to water, indicates a subtle change (increased polarity) of the buffer environment due to the presence of the protein. The bandwidths of 11.5 and 15 cm⁻¹ for these fundamental and hot bands, respectively, are similar to those observed for N₂O in pure water. The additional N₂O bands indicate that nitrous oxide molecules occupy nonaqueous sites of different polarity within the enzyme. The band at 2226.4 cm⁻¹ is assigned to the fundamental arising from N₂O in an environment that is less polar than water. N₂O in dimethylacetamide and acetone results in bands at 2225.8 and 2226.7 cm⁻¹, respectively (9). Therefore, the 2226.4 cm⁻¹ N₂O band in CcO represents an environment with a polarity analogous to that of a ketone or amide compounds with a carbonyl group.

TABLE I

| Band parameters from deconvolution of infrared spectra of N₂O in oxidized, reduced, and reduced carbonyl cytochrome c oxidase solutions |
|---|---|---|---|---|---|
| Species | Band 1 | Band 2 | Band 3 | Band 4 |
| | ν₁,cm⁻¹ | Δν₂,cm⁻¹ | %B | ν₁,cm⁻¹ | Δν₂,cm⁻¹ | %B | ν₁,cm⁻¹ | Δν₂,cm⁻¹ | %B | ν₁,cm⁻¹ | Δν₂,cm⁻¹ | %B |
| CcO(IV)/N₂O⁺ | 2231.9 | 11.5 | 57.3 | 2226.4 | 12.0 | 25.2 | 2218.4 | 12.5 | 15.1 | 2217.9 | 15.0 | 4.3 |
| CcO(IV)/N₂O⁻ | 2232.4 | 11.5 | 59.7 | 2227.1 | 12.0 | 24.1 | 2218.1 | 12.5 | 11.8 | 2218.3 | 15.0 | 4.4 |
| Cc(0).CO/N₂O | 2231.9 | 11.5 | 55.2 | 2225.9 | 11.0 | 25.0 | 2218.1 | 13.0 | 15.5 | 2217.9 | 15.0 | 4.2 |

* %B represents the percentage of the total band area.

A small band is observed at ~2335 cm⁻¹ (Δν₂ ~12 cm⁻¹) in the CcO(IV) → H₂O difference spectrum. This band comprises ~2.5% of the total absorbance of N₂O in CcO(IV) solution.
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FIG. 3. Visible Soret spectra of solutions of fully oxidized (top), fully reduced (center), and the carbonyl of fully reduced (bottom) species of cytochrome c oxidase in the presence and absence of N₂O. Conditions are as in Fig. 2.

FIG. 4. Infrared spectra of carbon monoxide liganded to fully reduced cytochrome c oxidase in the presence and absence of N₂O. Top spectrum, slope-corrected spectrum in the absence of N₂O. Center spectrum, spectrum in the presence of N₂O. Bottom spectrum, observed spectrum in the presence of N₂O (center spectrum) with four theoretical Gaussian curves from deconvolution superimposed. Upper residual, the difference between the observed spectrum for Co(O)(0)CO/N₂O and the sum of the Gaussian curves is flat which demonstrates the success of the curve-fitting procedure. Lower residual, difference spectrum between the spectra of Co(O)(0)CO in the presence and absence of N₂O. Conditions are as in Fig. 2.

The signal-to-noise ratio of the spectra (9). The bandwidths of the four fundamentals are similar, 11.5–12.5 cm⁻¹, indicating similar diversity in the different environments experienced by the N₂O molecules. The highly nonpolar site, corresponding to the fundamental at 2218.4 cm⁻¹, may represent sites within lipid portions of the oxidase molecule or associated detergent. Spectra for N₂O in cooking oils, cream, and phosphatidylycholine vesicles exhibit major N₂O bands at 2217–2218 cm⁻¹ (10). However, proteins contain similar nonpolar sites; for example, a band at 2220 cm⁻¹ is seen for N₂O within bovine myoglobin (11). The 2226.4 cm⁻¹ bands observed for N₂O in CcO probably arise from N₂O molecules.
TABLE II

<table>
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<th>Preparation</th>
<th>Treatment*</th>
<th>Rate constant, $k$</th>
<th>% Activity</th>
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<td>$s^{-1}/mg$ protein/$m^3$</td>
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* Air, and air$_4$ represent oxidase solutions assayed at the beginning and at the end (2-3 h later), respectively, of a given set of experiments. N$_2$O/N$_2$ represent N$_2$O-treated oxidase solutions which had been flushed with N$_2$ for 15 min.

$^a$ The protein amount is based upon iron analysis with 10 nmol of Fe/mg of protein (31).

Discussion:

The reversible effects of nitrous oxide on cytochrome c oxidase activity. The data are obtained with preparation I of Table II under conditions described in the text. $\bullet$, untreated (under air) oxidase solution, $\Box$, N$_2$O-treated oxidase solution, $\bigcirc$, N$_2$O-treated enzyme solution after flushing with N$_2$ for 15 min. The plots represent absorbance change at 550 nm with time during the oxidation of reduced cytochrome c by cytochrome c oxidase. The values for O.D. at a given time minus O.D. for the totally oxidized cytochrome c are on a logarithmic scale.

The N$_2$O molecules interact in a similar manner (occupy the same sites) with the reduced and carbonyl enzyme as with the fully oxidized enzyme. This is indicated by similar N$_2$O infrared spectra for the different oxidase-N$_2$O complexes (Fig. 2 and Table I). The changes observed among the three oxidase-N$_2$O complexes are reflected in their difference spectra which show a slight shift of band 2 for the fully reduced enzyme to higher frequency compared to the fully oxidized and the carbonyl enzyme (Table I). Neither the change in redox state nor the binding of CO has much effect on the environments experienced by the N$_2$O molecules. These results are consistent with lack of changes in the visible Soret spectra of CcO(IV), CcO(CO), and CcO(CO)CO in the presence of the anesthetic (Fig. 3).

Lack of Effect of N$_2$O on CO Binding to CcO—Nitrous oxide does not affect the binding of CO to the heme $a_1$ iron either electronically or stereochemically as indicated by lack of effect on the CO infrared and visible Soret spectra (Figs. 3 and 4). These findings indicate that N$_2$O molecules neither bind to the heme $a_1$ iron nor perturb the environment of liganded carbon monoxide.

Effects of N$_2$O on Oxidase Activity—The oxidase activity is decreased by exposure of CcO to N$_2$O (Fig. 5). In the activity assay, 1 $\mu$M enzyme solution is diluted 300-fold when added to the reaction mixture that is exposed only to air. Exposure of the enzyme solution to N$_2$O prior to injection into the reaction mixture results in about 40% lower activity. The activity is fully restored by passing N$_2$ over the N$_2$O-treated enzyme solution for only 15 min. The assay is carried out open to the air, i.e., no N$_2$O is added to the atmosphere above the assay reaction mixture. Thus, the prior exposure of the enzyme solution to N$_2$O results in a marked reduction in the rate of cytochrome c$^{-}$ oxidation even though the partial pressure of N$_2$O is lowered greatly upon 300-fold dilution of the enzyme solution in the assay reaction mixture. This

![Fig. 5. The reversible effects of nitrous oxide on cytochrome c oxidase activity. The data are obtained with preparation I of Table II under conditions described in the text. $\bullet$, untreated (under air) oxidase solution, $\Box$, N$_2$O-treated oxidase solution, $\bigcirc$, N$_2$O-treated enzyme solution after flushing with N$_2$ for 15 min. The plots represent absorbance change at 550 nm with time during the oxidation of reduced cytochrome c by cytochrome c oxidase. The values for O.D. at a given time minus O.D. for the totally oxidized cytochrome c are on a logarithmic scale.](image1)

![Fig. 6. Infrared spectra for gaseous CO$_2$ before and after the addition of air into a cell containing cytochrome c oxidase, carbon monoxide, and nitrogen. Conditions are described in the text. Top, the spectrum recorded prior to the addition of air. Bottom, the spectrum recorded 7 h after the addition of air.](image2)
marked inhibition of CO oxidation by N\textsubscript{2}O under the experimental conditions used for the assay of activity appears surprising in view of the low solubility and rapid equilibration of N\textsubscript{2}O in blood and tissue (1). Furthermore, a first order plot is maintained over the time course of the assay (60–120 s, Fig. 5). These findings suggest that N\textsubscript{2}O either diffuses slowly away from the rate controlling site(s) or that the inhibitory effect of N\textsubscript{2}O is retained over the period of the assay even though N\textsubscript{2}O is no longer occupying the site(s) within the protein. The latter possibility suggests that N\textsubscript{2}O causes a conformational change in the protein giving rise to the decreased activity. In this event the conformational change is not reversed within the time frame of the activity assay. However, exposing the enzyme solution to N\textsubscript{2}O for 15 min restores the original conformation to give full activity. Since N\textsubscript{2}O does not perturb visible Soret and infrared CO spectra (Figs. 3 and 4), it is concluded that the decrease in activity is caused by N\textsubscript{2}O interacting at sites away from the hemes. Therefore, N\textsubscript{2}O molecules may interfere with interactions between cytochrome c and CcO and/or with the intramolecular electron and hydrogen transfers required for dioxygen reduction.

**Lack of Evidence That N\textsubscript{2}O Can Serve as an Oxidizing Agent in CO-catalyzed Reactions**—The oxidation of CO by O2 catalyzed by CcO with a stoichiometry of 2 mol of CO2 produced/1 mol of O2 (reaction 2), is confirmed. However, no conclusive evidence was obtained for the CO-catalyzed oxidation of CO by N\textsubscript{2}O at high PCO/P\textsubscript{N2O} (reaction 3). The CO-catalyzed oxidation of carbon monoxide in the presence of O2 but not in the presence of N\textsubscript{2}O, may be due to greater inhibition by CO of the N\textsubscript{2}O reaction than the O2 reaction. N\textsubscript{2}O is a very poor ligand and would not compete effectively with CO for a metal site. Furthermore, the oxidation of cytochrome c\textsuperscript{2+} by N\textsubscript{2}O (reaction 4) in the presence of COc was not observed. The failure to observe reaction 4 is possibly due to N\textsubscript{2}O inhibition of intramolecular electron transfer. However, the most likely reason for the lack of oxidation of both CO and cytochrome c\textsuperscript{2+} by N\textsubscript{2}O is the inability of N\textsubscript{2}O to serve as a ligand to the heme a\textsubscript{3} iron or copper.

**Concluding Comments**—These findings support the suggestion made earlier that the component of the respiratory chain of bovine heart or bean mitochondria reversibly inhibited by N\textsubscript{2}O is COc (2). Three sites occupied by N\textsubscript{2}O molecules within the enzyme were detected by N\textsubscript{2}O infrared spectroscopy and were shown to differ greatly in polarity with one highly polar, one of intermediate polarity, and one of low polarity. No evidence for N\textsubscript{2}O binding as a ligand to metals nor for N\textsubscript{2}O interference with the O2 reaction site was observed. The occupancy of one or more sites within the enzyme resulted in a partial inhibition of the oxidase activity that was reversible. N\textsubscript{2}O was not observed to serve as an oxygen atom donor in the enzyme-catalyzed oxidation of either reduced cytochrome c or carbon monoxide. This suggests that oxygen donor reactions of N\textsubscript{2}O involving CcO are unlikely to contribute to the toxicity of the anesthetic. However, the inhibition of respiration by N\textsubscript{2}O could contribute to N\textsubscript{2}O toxicity generally. In neural tissue reduced respiration may contribute to anesthesia and analgesia. The precise locations of the sites occupied by N\textsubscript{2}O and the detailed mechanism whereby N\textsubscript{2}O interferes with O2 utilization are intriguing questions that remain to be answered. The findings reported here suggest that the anesthetic is unlikely to interfere directly with O2 reduction *per se* and therefore probably interferes with electron transfer from cytochrome c to the O2 reduction site.

**REFERENCES**