DETECTION AND CHARACTERIZATION OF NITROUS OXIDE SITES IN THE BRAIN OF A DOG UNDER HALOTHANE—N_2O ANESTHESIA BY INFRARED SPECTROSCOPY

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Summary: Infrared spectra of N_2O in a variety of solvents and in the brain of a dog under typical conditions of halothane—N_2O anesthesia have been determined. The appearance or disappearance of N_2O in the brain was readily followed as N_2O was administered or withdrawn. The sites in brain were of two major types; one, with ν_3 = 2229.8 ± 0.4 cm^{-1} and $\Delta\nu_{\frac{1}{2}}$ = 13.0 ± 0.6 cm^{-1}, is rather like the polar site in water and the other, with ν_3 = 2216.8 ± 0.8 cm^{-1} and $\Delta\nu_{\frac{1}{2}}$ = 9.6 ± 1.0 cm^{-1}, is non-polar and is probably associated with membrane lipid. The significant variations in the antisymmetric stretch (ν_3) of N_2O as the polarity and other properties of the medium (solvent) vary make possible the characterization of in tissue sites occupied by this anesthetic.

Introduction: The probing of small molecules in intact tissue has been difficult technically. In the case of ligands that bind to hemeproteins, infrared spectroscopy has recently proven very useful. For example, CO bound to hemoglobin, myoglobin, and cytochrome c oxidase in heart muscle (1) and O_2, as well as CO, bound to hemoglobin within the red blood cell (2,3) have been studied by infrared techniques. These vibrational spectra are useful in qualitative discrimination among sites because the frequency and the shape of infrared bands are sensitive to variations in structure of the binding site. Furthermore, band intensities are useful in quantitating the amount of ligand at a given site (3,4). Since there is little scattering of the long-wavelength infrared radiation, the samples need not be homogenous; tissues may be examined directly even though they are opaque to visible light.

The successful studies of gaseous and other ligands at metal protein sites by infrared spectroscopy suggested that an analogous approach might be useful for the elucidation of the distribution of anesthetics in tissue—a poorly understood aspect of anesthetic function. In this paper, we report the study of the antisymmetric stretch frequency (ν_3) of the anesthetic nitrous

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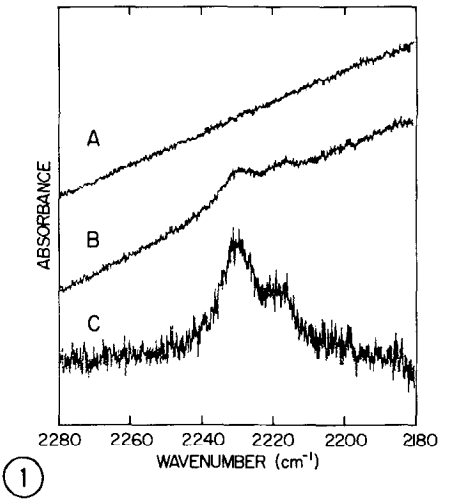
oxide (N_2O) near 2220 cm^{-1} in the brain of a dog under anesthesia and in a variety of polar and non-polar solvents that may mimic the sites in which N_2O is located in brain tissue.

Experimental: A healthy mongrel dog was given thiamylal sodium (8 ml) and placed under halothane-O_2 anesthesia. A section of the skull was removed to expose a portion of the brain. White brain matter (ca 0.2 ml) was taken from the general area of the post central gyrus by withdrawal into a 3 ml syringe (without a needle) that had been inserted under the surface. The sample was quickly forced from the syringe into an infrared cell with CaF_2 windows of pathlength 0.05 mm. Care was taken to plug the orifice of the syringe during the transfer from brain to cell. The uniform distribution of white matter over the cell window could be ascertained by visual inspection. The cell was placed in a Beckman RIIX cell holder cooled with an ice-water mixture and the spectrum recorded on a Perkin Elmer Model 180 spectrometer interfaced with a Tektronix computer graphics system (No. 4051). Absorbance measurements made at intervals of 0.1 cm^{-1}, resolution of 1.2 at 2280 cm^{-1}, over the range from 2280 to 2180 cm^{-1} were stored on tape; scanning time was 5 minutes. No reference cell was used. Samples were taken at approximately ten-minute intervals over a five-hour period with and without introduction of N_2O into the gases breathed.

The spectra of N_2O dissolved in pure solvents at 25°C were obtained under instrumental conditions similar to those described above over the range 2290 to 2140 cm^{-1} with a 30 minute scanning time. A difference spectrum was obtained by subtracting the pure solvent spectrum from the N_2O plus solvent spectrum by a computer routine.

Results: The spectra for the first few samples of brain, which were obtained when only halothane was mixed with O_2, were essentially linear scans as shown typically in Fig. 1A. After about 10 min of N_2O administration (N_2O:O_2, 60:40) along with halothane, a spectrum as shown in Fig. 1B was obtained; 16 such spectra were obtained under these conditions. Two major bands in the region expected for the ν_3 band of N_2O are clearly evident. Use of the computer to subtract a line of the same slope as the background from the spectrum of Fig. 1B leaves the bands due to N_2O, as shown in Fig. 1C. When administration of N_2O was stopped, these bands disappeared; readministration of N_2O resulted in the gradual reappearance of these bands in confirmation of N_2O as the origin of these bands.

To increase the signal-to-noise ratio, 16 scans such as Fig. 1B were each slope-corrected as described above and the slope-corrected spectra averaged to provide the spectrum of Fig. 2A. The band frequency and half-band width of the larger band of Fig. 2A were measured to be 2229.8 ± 0.4 cm^{-1} and 13.0 ± 0.6



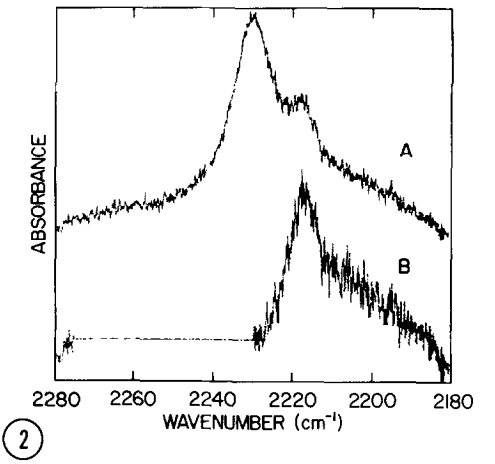


Figure 1. Infrared absorbtion spectra of dog brain in the region 2280 to 2180 cm^{-1}. Curve (A): a single scan during administration of halothane—O_2. Curve (B): a single scan during administration of N_2O—halothane—O_2. Curve (C): the scan shown in B after slope-correction and ordinate scale expansion.

Figure 2. Infrared spectra of dog brain during administration of N_2O—halothane—O_2. Curve (A): an average of 16 scans, such as Fig. 1B, each slope-corrected. Curve (B): the spectrum of the smaller peak left after subtraction of the larger peak (by taking the mirror image of the left side).

cm^{-1}, respectively. The larger band, assumed to be symmetrical, was then subtracted from the spectrum of Fig. 2A by a mirror-image technique to leave the smaller band, as shown in Fig. 3B, with frequency of 2216.8 ± 0.8 cm^{-1} and $\Delta\nu_{1/2}$ of 9.6 ± 1.0 cm^{-1}.

Examples of spectra obtained for pure solvents are in Fig. 3. A shoulder is more or less evident on the low-frequency side, depending upon the band width. This shoulder can be ascribed to a "hot" band (5). It may be noted that the band causing a shoulder for the larger band of the brain spectrum would be located at the same position as that of a smaller band. However, the smaller brain band is much too intense to represent a shoulder or "hot" band corresponding to the larger peak when compared with the intensities of band

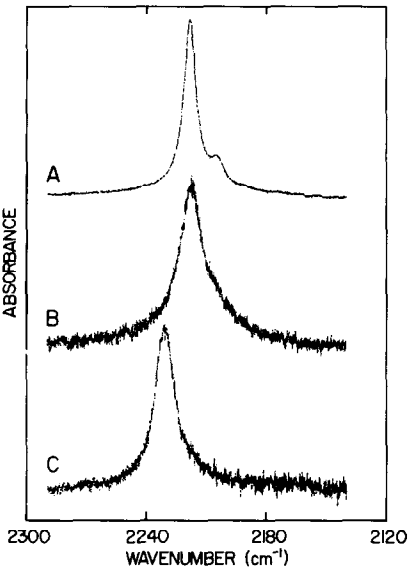


Figure 3. Infrared absorption spectra of N_2O in different solvents in the region 2290 to 2140 cm^{-1}. In each case the absorption spectrum of the solvent has been subtracted by computer from the absorption spectrum of N_2O in the solvent; the solvents were nearly saturated in N_2O at 25° C. Curve (A): N_2O in CCl_4. Curve (B): N_2O in n-hexane. Curve (C): N_2O in H_2O.

shoulders in all other solvents examined. Indeed, we assume that the contribution of the shoulder of the larger peak is negligible since the shoulder becomes smaller as the solvent becomes more polar; the larger band is located at a frequency which corresponds to that of N_2O in water, the spectrum of which exhibits a scarcely detectable shoulder (Fig. 3C).

Discussion: The molecular properties of N_2O have been thoroughly analyzed, and its three fundamental infrared absorption bands, as well as several harmonics, have been well characterized (5). The three fundamental bands correspond to the three vibrations of the linear N_2O molecule, with ν_1 near 1300 cm^{-1} being the symmetric stretch, ν_2 near 600 cm^{-1} the bend, and ν_3 near 2200 cm^{-1} the anti-symmetric stretch. The ν_3 band is the most convenient to study in biological tissues because it is found in a region of low absorbance by both

TABLE 1

Infrared Parameters for the Antisymmetric Stretch of Nitrous Oxide in Different Solvents and in Brain

Solvent	ν_3 (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1})
CCl_4	2218.2 ± 0.2	7.4 ± 0.3
$CHCl_3$	2221.0 ± 0.2	9.8 ± 0.3
CH_2Cl_2	2222.0 ± 0.2	9.4 ± 0.3
cyclohexane	2216.4 ± 0.2	11.0 ± 0.3
n-hexane	2217.3 ± 0.3	14.9 ± 0.4
olive oil	2219.3 ± 0.3	12.7 ± 0.4
water	2230.7 ± 0.4	11.5 ± 0.6
water with protein[a]	2230.2 ± 0.4	14.2 ± 0.6
glycerol	2227.5 ± 0.4	13.3 ± 0.6
methanol	2225.5 ± 0.2	12.0 ± 0.3
brain	2229.8 ± 0.4	13.0 ± 0.6
	2216.8 ± 0.8	9.6 ± 1.0

[a]Concentrated solution of bovine serum albumin in water.

water and protein; consequently, there is little interference from water and protein absorption (6).

Useful parameters for studying infrared bands are the frequency, or wavenumber $\tilde{\nu}$, at maximum absorption and the band width at half of the peak height in absorbance, $\Delta\nu_{1/2}$. In the case of an unbound molecule, such as N_2O, the frequency depends upon the ability of the environment to enhance or restrict the molecular vibration, while $\Delta\nu_{1/2}$ depends upon the stability or uniformity of

the interactions between the vibrator and its environment. The nature of changes in these parameters of the ν_3 band of N_2O in a large number of polar and non-polar environments will be described in detail in a future paper. Figure 1 and Table 1 provide examples of observed variations in ν_3 and $\Delta\nu_{\frac{1}{2}}$ for several solvents. The value of ν_3 is high (2230.7 cm^{-1}) for the very polar and highly hydrogen-bonded solvent water while it is low (2217.3 cm^{-1}) for the non-polar solvent n-hexane. The band is broad ($\Delta\nu_{\frac{1}{2}}$, 14.9 ± 0.4 cm^{-1}) in the non-uniform environment of n-hexane, while it is narrow ($\Delta\nu_{\frac{1}{2}}$, 7.4 ± 0.3 cm^{-1}) in the highly specific environment of CCl_4.

When one looks at an inhomogeneous sample containing N_2O, such as brain tissue, N_2O molecules distributed between two or more environments may be seen. The natures of these environments can be characterized from the band frequencies and widths. It is of interest that, because the infrared time scale is very fast (on the order of 10^{-13} seconds), the absorbance from the population of N_2O molecules in each environment is seen, within sensitivity limitations. In contrast, with NMR spectroscopic measurements a time-average of environments may be seen, as in the study of halothane in model membranes (7), because the exchange between environments may be more rapid than the time scale of the NMR method.

The frequency of the larger peak in brain (2229.8 cm^{-1}) is near that for N_2O in water (2230.7 cm^{-1}), whereas the smaller peak (2216.8 cm^{-1}) is between those found for cyclohexane (2216.4 cm^{-1}) and n-hexane (2217.3 cm^{-1}) (Table 1). Thus, based on frequency, the larger band is assigned to N_2O in an aqueous environment and the smaller band to N_2O in a non-polar environment such as that expected for membrane lipids. The larger band is somewhat broader than the band of N_2O in pure water (13.0 vs 11.5 cm^{-1}). The smaller band, on the other hand, is quite narrow, at 9.6 cm^{-1}, much narrower than the widths found for n-hexane (14.9 cm^{-1}), olive oil (12.7 cm^{-1}) and cyclohexane (11.0 cm^{-1}) and is nearer those for $CHCl_3$ (9.8 cm^{-1}) and CH_2Cl_2 (9.4 cm^{-1}).

In conclusion, nitrous oxide is found in two basic environments in brain,

one polar and the other highly non-polar, consistent with the widely predicted distribution of anesthetics between the aqueous and lipid phases (8-10). The larger (polar) band is broader than that found in pure water, consistent with the presence of protein and other solutes in the water (Table 1). The narrowness of the smaller band indicates a very limited range of environments occupied by N_2O in non-polar material despite the variety of lipids present in white brain matter. As we will discuss in greater detail elsewhere, the narrow width and low wavenumber probably indicate restrictions on the molecule's rotation without the restrictions on vibration that are found in hydrogen-bonding solvents such as water. No single solvent yet examined mimics both the ν_3 and $\Delta\nu_{\frac{1}{2}}$ values found for the non-polar band. This narrow, low frequency band might be ascribed to N_2O molecules which are consistently oriented in a specific way with respect to the membrane lipids, which are themselves more ordered and restricted in movement than are pure solvent molecules.

The preliminary experiments reported here suggest that these infrared approaches can be extended to locate N_2O in other carefully isolated sections of brain and other tissues, to improve sensitivity and resolution for study of the bands reported here and possible as yet unseen bands, to elucidate more precisely the chemical nature of the sites occupied, to follow rates of N_2O appearance and disappearance from these sites, and to probe anesthetics other than N_2O as well as other compounds in a variety of tissues.

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