# THE UTILITY OF INFRARED SPECTROSCOPY AS A PROBE OF INTACT TISSUE: DETERMINATION OF CARBON MONOXIDE AND HEMEPROTEINS IN BLOOD AND HEART MUSCLE

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Summary. Infrared methods permit detection of CO within tissue under nearly physiological conditions. The CO stretch bands exhibit frequencies, band widths and intensities characteristic of the particular binding site with areas related to concentrations. For small volumes (< 1 ml) of whole blood the % HbCO as well as certain abnormal Hbs are rapidly determined. In heart muscle, CO bound to cytochrome oxidase, hemoglobin and myoglobin is observed at 1963, 1951 and 1944 $cm^{-1}$ respectively, frequencies characteristic of the isolated proteins. Infrared methods discriminate among possible CO binding sites (hemeprotein or other) within any intact tissue. Many other infrared active molecules or groups could also be studied in tissue by infrared spectroscopy.

Introduction. Carbon monoxide (CO), a competitive inhibitor of dioxygen binding in hemoglobin (Hb) and other proteins, is an increasingly serious pollutant of our environment (1,2,3). A normal component of human tissues, CO is a product of heme catabolism (4). Thus it is normal for about 1% of the Hb in blood of nonsmoking humans to be present as HbCO (1,2,5). Significantly higher levels of HbCO are found after exposure to other sources such as atmospheric CO and tobacco smoke (3). The blood of heavy cigarette smokers in urban areas may contain well over 10% of the Hb as HbCO (1,2,5,6). Recent studies (7,8) have shown that changes in, or damage to, the cardiovascular system can occur at levels as low as 8-9% HbCO. However the exact pathological mechanism(s) for these changes and for the central nervous system symptoms of CO toxicity remain unclear. The lack of a probe capable of differentiation among the several CO binding sites within intact tissues under *in vivo* conditions and of measuring the level of CO bound to each site has been a critical deterrent to progress in the study of the effects of CO on living systems. We report here evidence that infrared spectroscopy can become such an uniquely useful probe. A technically simple infrared method

for the accurate determination of HbCO in whole blood has been developed and spectra are shown which allow rough quantitation of the CO binding heme proteins in heart tissue.

Materials and Methods. Approximately 0.5 ml of heparinized blood is needed for the HbCO determinations. EDTA and citrate also serve as satisfactory anticoagulants. The blood is centrifuged at low speed and most of the plasma removed. The packed erythrocytes are resuspended in the remaining plasma, drawn into a syringe and a portion of the sample injected from the syringe into a 0.05 mm pathlength infrared cell with $CaF_2$ windows. CO is then drawn into a syringe and the remaining blood incubated at $4^{o}C$ for 30 minutes to completely saturate the blood with CO.

$CaF_2$ windows are water insoluble and transmit sufficient energy through both the visible-uv region and the infrared region of interest to allow both types of spectra to be recorded on the same aqueous sample in the same cell. In the infrared region between 1900 and 2000 $cm^{-1}$ the major absorption of energy is due to water. Hb and other blood proteins contribute no sharp or intense broad bands except for bound CO at ca. 1951 $cm^{-1}$ (9). This allows water to be used as a reference material for the infrared difference spectrum.

The spectrum is recorded by placing the $CaF_2$ cell containing the packed erythrocytes in the sample compartment and a variable pathlength $CaF_2$ cell containing water in the reference compartment. The pathlength of the reference cell is adjusted to compensate for the water in the sample and the spectrum recorded (Fig 1, A).

Upon completion of this spectrum the CO saturated blood is injected into the same sample cell and the infrared spectrum of the CO saturated blood recorded in the same manner as for the original blood sample (Fig 1, C).

The spectrum of CO treated mouse heart tissue (Fig 2) is obtained by excising a mouse heart and placing it in 2-3 ml of physiological buffered saline (pH 7.2) containing a trace of sodium dithionite to reduce any metmyo-

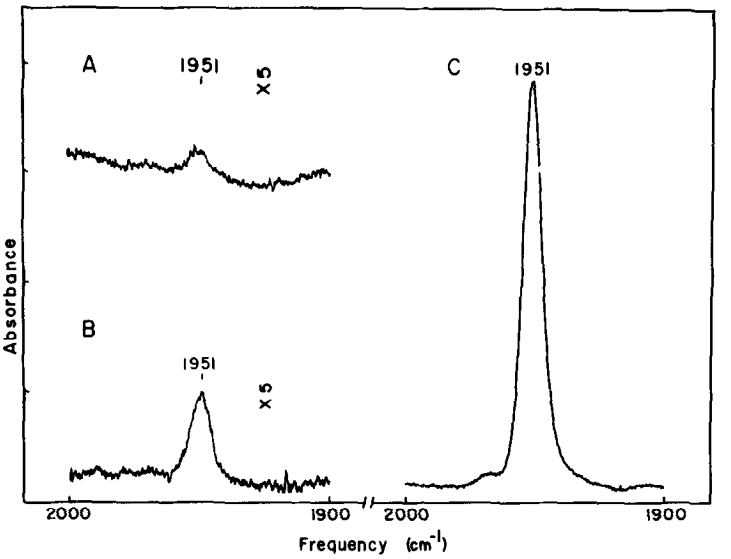


Figure 1. Infrared difference spectra of human whole blood vs. water. (A) CO stretching band of untreated blood from a nonsmoker with 1.5% of hemoglobin as carbonyl hemoglobin. (B) CO stretching band of untreated blood from a moderate cigarette smoker with 4.5% of hemoglobin as carbonyl hemoglobin. (C) Spectrum obtained upon saturation of the blood from (B) with CO. The intensity of the CO band in (B) relative to that in (C) gives directly the fraction of the hemoglobin present as carbonyl hemoglobin in the untreated blood of (B). The ordinates for spectra (A) and (B) are expanded 5 fold compared to spectrum C.

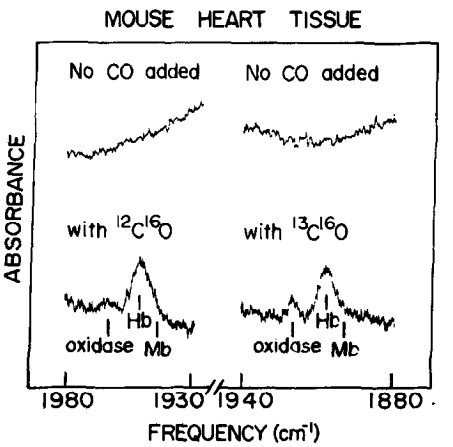


Figure 2. Infrared difference spectra of mouse heart tissue vs. water: Upper left and upper right: Spectra of tissue which was not treated with carbon monoxide. There are no infrared bands in the region 1980 $cm^{-1}$ to 1880 $cm^{-1}$ to interfere with the CO determination. Lower left: Spectra of tissue exposed to $^{12}C^{16}O$. Lower right: Spectra of tissue exposed to $^{13}C^{16}O$. The effect of isotope on the carbon-oxygen stretching frequency confirms that CO is the origin of these bands.

globin which may be formed during the mincing process. The intact heart is then exposed to either $^{12}C^{16}O$ or $^{13}C^{16}O$ for 10-30 minutes. The heart is finely minced with surgical scissors. Approximately 30 mg of minced tissue is pressed between two $CaF_2$ windows and placed in the sample compartment. A variable pathlength reference cell containing water is adjusted to compensate for the water in the sample.

Other methods for the preparation of mouse heart muscle have been as successful, or nearly so, as the method described above. Also, exposure of live animals to a CO atmosphere allowed the detection of the CO infrared bands without further exposure of the heart tissue to CO. Other tissues in addition to heart (e.g. lung, liver, kidney, skeletal muscle) were explored but due to a lower concentration of hemeproteins the CO infrared bands were less intense.

All spectra were recorded on a Perkin-Elmer Model 180 infrared spectrophotometer using absorbance mode and expanded ordinate and abscissa at a resolution of 3 $cm^{-1}$.

Results and Discussion. Infrared difference spectroscopy provides a direct measure of any CO present. With whole blood the CO bound to Hb is determined without need of removal of bound CO or lysis of erythrocytes.

The portion of the Hb present as HbCO can be directly calculated from the relative peak heights for untreated and CO saturated blood as the intensity of the infrared CO band for HbCO has been shown to follow Beer's Law (10). Figure 1 illustrates the infrared difference spectra of blood from a nonsmoker (Fig 1 A) and a moderate cigarette smoker (about half a pack a day) (Figure 1 B). The blood from these persons contained 1.5% and 4.5% HbCO respectively. The signal to noise ratio of these spectra is adequate to determine the HbCO levels to within ± 0.2%.

The direct determination of blood CO by this method while being technically simple compared to many other CO determination methods would be expected to be one of the most accurate. No standard curves need to be pre-

pared and the independent determination of total Hb is not necessary except when a significant amount (> 10%) of metHb is present.

The technique has an additonal advantage of detecting isotope effects upon the carbon-oxygen stretching frequency (11). Use of $^{13}C$ or $^{18}O$ enriched substrates makes possible the determination of the carbon or oxygen source of bound CO. The CO noted upon exposure of humans to methylene chloride (12) appears to arise directly from the halide since in rats the carbon of $^{13}C$ dihalomethanes gives $^{13}CO$ bound to Hb (13).

Because different proteins give different CO stretching frequencies, infrared spectroscopy has the unique capability of quantitating the amount of CO at each site or quantitating the various CO binding proteins in a given tissue. Bovine and mouse heart tissue when exposed to $^{12}C^{16}O$ revealed absorption bands at 1964 $cm^{-1}$ and 1951 $cm^{-1}$ and a shoulder at 1944 $cm^{-1}$ which correspond to isolated carbonyl cytochrome c oxidase (14,15), HbCO (11) and MbCO (16) respectively (Figure 2 lower left). These assignments for mouse heart tissue were confirmed by use of $^{13}C^{16}O$. The expected isotopic shifts were clearly observed for the cytochrome oxidase (1920 $cm^{-1}$) and Hb (1908 $cm^{-1}$) (Fig 2 lower right). No bands were seen in the 1965-1940 $cm^{-1}$ region.

Many Hb variants containing altered oxygen binding sites can also be detected and quantitated in whole blood by the shift in the stretching frequencies of the CO bound to the variant even though these variants may not be detectable by other methods (17). Rabbit blood serves as an excellent example. Infrared spectra of CO saturated rabbit whole blood demonstrate two distinct CO stretching bands at 1951 $cm^{-1}$ and 1928 $cm^{-1}$ while clinical electrophoresis capable of separating hemoglobins A, $A_2$, F, C, and S and could not resolve the second component in rabbit Hb. Thus infrared spectroscopy should prove uniquely useful to the hematologist for detecting and quantitating abnormal Hbs containing pathological, but electrophoretically silent, substitutions affecting the oxygen binding site (18,19).

The infrared technique has the advantage of allowing measurement under

physiological conditions of pH, temperature, medium, and concentration. Furthermore, the effects of scattering phenomena responsible for the relative opacity of blood and other tissues to visible and ultraviolet radiation are minimized in the infrared technique due to the longer wavelength radiation employed. The study of CO distribution throughout the whole organism at various CO and $O_2$ tensions awaits improved sampling procedures and increased instrument sensitivity however these problems appear readily surmountable and should provide much important data. Tissues with lower concentration of CO binding sites of course require greater sensitivity. Also, the infrared methods are by no means restricted to probing for CO. Any infrared active molecule or group with a frequency sufficiently near a window in the infrared spectrum of aqueous or $D_2O$ solutions of proteins to permit a small amount of energy to pass is potentially observable by infrared spectroscopy.

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