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An Infrared Study of Bound Carbon Monoxide in the Human Red Blood Cell, Isolated Hemoglobin, and Heme Carbonyls*

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ABSTRACT: The binding of carbon monoxide to hemes has been studied by high-resolution infrared spectroscopy. Inductive and resonance effects of substituents both on the porphyrin ring (*cis* effects) and on pyridine that is coordinated to the iron(II) (*trans* effects), as well as solvent effects in simple synthetic heme derivatives are reported and compared with the infrared difference spectrum of carbonyl-hemoglobin (HbCO − HbO_2) to yield a picture of intramolecular interactions that is consistent with hemoglobin structure. Assignment of the infrared absorption bands (ν_{CO}) of HbCO and heme–CO has been confirmed by isotopic substitution with [^{13}C]- or [^{18}O]carbon monoxide. The isotopic data for HbCO appear to be more consistent with Hb–Fe–O≡C (oxygen coordinated to the iron) than with the more conventional structure, Hb–Fe–C≡O (carbon coordinated to the iron), while a structure in which CO is bound parallel to the heme appears unlikely.

The reactions of carbon monoxide with hemoglobins, cytochrome *c* oxidase, and other hemoproteins, as well as with simple iron(II) porphyrins (hemes), have long been of both biochemical and chemical interest. Because structural and chemical interpretations of bonding in transition metal carbonyls have been based on studies of carbon–oxygen stretching frequencies (*cf.* Cotton and Wilkinson, 1966), the availability of infrared instrumentation of high sensitivity and resolution suggested the possibility that CO stretching frequencies (ν_{CO}) could also be observed and interpreted for carbon monoxide bound to the intact red blood cell or to isolated hemoglobins. Here we report and discuss such ν_{CO} values obtained with the normal human red blood cell and isolated hemoglobin and with variously substituted deuterohemes. A portion of this work has appeared in preliminary communications (Alben and Caughey, 1962, 1966; Caughey *et al.*, 1965, 1967).

Experimental Section

Materials. The hemes (Alben *et al.*, 1968) and metal-free porphyrins (Caughey *et al.*, 1966a) were prepared and characterized as reported earlier. Normal human hemoglobin A that was used with $^{12}C^{16}O$, kindly donated by Professor Keith Richardson, Department of Physiological Chemistry, Ohio State University, was prepared from washed red blood cells by lysis through freezing and thawing, removal of the stroma, and passage through a column of diethylaminoethylcellulose in 0.005 Tris-phosphate buffer (pH 8). Normal human hemoglobin A that was used with $^{13}C^{16}O$ or $^{12}C^{18}O$ was obtained in concentrated solution as a biproduct from the purification of genetically abnormal hemoglobin by Professor Samuel Charache, Department of Medicine, The Johns Hopkins School of Medicine. The carbon monoxide complex

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of hemoglobin[1] was prepared by equilibration of oxyhemoglobin with a CO atmosphere for 30 min. Solid ammonium sulfate was added to 70% saturation and the precipitate was collected by centrifugation. This precipitate was drawn into a syringe for injection into the infrared cell. Oxyhemoglobin was similarly precipitated for use in the reference beam of the infrared spectrophotometer. The isotopically labeled CO complexes were obtained by admission of the gas to a previously evacuated solution of hemoglobin in a stoppered vial. Infrared reference cells were filled with the corresponding oxyhemoglobin solutions.

Red cells from freshly drawn normal human blood were exposed to oxygen or $^{12}C^{16}O$ and packed by centrifugation. The packed cells were drawn into syringes for injection into their respective infrared cells.

Infrared Studies of Monopyridinemonocarbonylheme Esters and Metal-Free Porphyrins. The solid heme was dissolved in liquid pyridine derivative or in a concentrated solution of solid pyridine derivative in bromoform followed by dilution, under a CO atmosphere, with bromoform to a final pyridine derivative concentration of 0.13 M. Although all solutions were saturated with CO, we later found it unnecessary to exclude oxygen or to have a large excess of CO present to prevent autoxidation under these conditions. Infrared spectra were obtained for these solutions with a Perkin-Elmer Model 421 spectrophotometer adjusted to resolve water vapor peaks at 1792.65 and 1790.95 cm^{-1}. All carbonyl-stretching frequencies were calibrated to the water vapor peak at 1942.6 cm^{-1}. The precision of measurement for the maximum of carbonyl absorption is estimated to be ± 0.5 cm^{-1}. (The absorption maximum of protoheme carbonyl was reproduced to ± 0.1 cm^{-1} with a Perkin-Elmer Model 521 spectrometer by use of the same calibration procedure.)

Melting points were obtained on a hot stage apparatus and are corrected. Pyridine (J. T. Baker Chemical Co., Phillipsburg, N. J.) was distilled: bp 115.0° (761 mm), n_D^{25} 1.5074; lit. (Leis and Curran, 1945) bp 114.5° (740 mm), n_D^{25} 1.5074. The 4-substituted pyridines obtained from Aldrich Chemical Co. were purified further. 4-Aminopyridine was recrystallized from benzene, sublimed, and recrystallized: mp 158°, lit. (Leis and Curran, 1945) mp 159–160°. α-Picoline was distilled: bp 144.5° (762 mm), n_D^{25} 1.5030, lit. (Leis and Curran, 1945) bp 143–145°, n_D^{25} 1.5031. 4-Acetylpyridine was distilled: bp 81–82° (5 mm), n_D^{25} 1.5273; lit. (Burns and Powell, 1945), bp 77° (5 mm). 4-Cyanopyridine was recrystallized from benzene: mp 83°, lit. (Murmann and Basolo, 1955) mp 78–80°. The infrared spectra of the substituted pyridines were found to be in essential agreement with literature values in each case (pyridine, Corssin *et al.*, 1953; 4-aminopyridine, Katritzky and Gardner, 1958; Costa *et al.*, 1956; Angyal and Werner, 1952; α-picoline, Katritzky and Gardner, 1958; Cannon and Sutherland, 1951; 4-acetylpyridine and 4-cyanopyridine, Katritzky and Gardner, 1958). Benzene (J. T. Baker Chemical Co., Phillipsburg, N. J.) was distilled: bp 79.8–79.95°, n_D^{25} 1.5016, or bp 80.2–80.4°, n_D^{25} 1.5010; lit. (Handbook of Chemistry and Physics, 1966) bp 80.1°, n_D^{20} 1.5011. Bromoform was distilled: bp 25° (3 mm), n_D^{25} 1.5948 (a sample with bp 25° (3 mm), n_D^{25} 1.5978, mp 8.1° was used in experiments illustrated in Figure 3); lit. (Handbook of Chemistry and Physics, 1966) n_D^{20} 1.5976, mp 8.3°. Carbon monoxide was used as obtained from manufacturers. $^{12}C^{16}O$ was obtained from Matheson Co., reported as CP grade and 99.5% pure. The ^{13}C derivative was obtained from Merck Sharp & Dohme of Canada, Ltd., and reported as 53% $^{13}C^{16}O$ in $^{12}C^{16}O$. The ^{18}O derivative was obtained from Isomet Corp. and was reported to contain 86.1% $^{12}C^{18}O$ in $^{12}C^{16}O$. The NH-stretching frequencies of the metal-free porphyrins in bromoform (estimated precision ± 0.5 cm^{-1}) were calibrated from the 3447.20-cm^{-1} water peak.

Infrared Studies of Hemoglobin–Carbon Monoxide Complexes. The absorption band of carbon monoxide bound to hemoglobin was observed by means of difference spectroscopy with HbCO in the sample beam and HbO_2 in the reference beam of the spectrometer. This allowed absorption bands due to CO or oxygen to be isolated from all other absorptions by compensation for the broad absorption bands due to protein and water. The latter absorptions passed between 10 and 25% of the incident radiation through the reference and sample cells, in the spectral region of interest. Spectroscopy was conducted on a Perkin-Elmer Model 521 double-beam grating spectrometer with demountable cells (Connecticut Instruments Division of Barnes Engineering Co.) equipped with CaF_2 windows and 0.05-mm Teflon spacers. Spectra were obtained by use of a standard slit-width program (approximately 135-μ mechanical slit width at 1950 cm^{-1}), which was just insufficient to resolve water vapor bands at 1910.1 and 1908.2 cm^{-1}, a fivefold expanded ordinate (per cent transmission), a 20-fold expanded abscissa (wave number), and a scanning time that was sufficiently long to permit a satisfactory signal-to-noise ratio.

The concentrations of hemoglobins used for the infrared spectra in the present study were not determined accurately. However, approximate concentrations were estimated from the visible spectra recorded for the loaded infrared cells and were in the range of 10^{-2}–10^{-3} M.

Results and Discussion

Effects of 2,4-Substituents on ν_{NH} of Pyridinecarbonyldeuterohemes and on ν_{NH} of Deuteroporphyrins. Carbonyl-stretching frequencies (ν_{CO}) for the heme carbonyls in bromoform–pyridine increased with changes in the 2,4-substituent in the order ethyl < hydrogen < vinyl < acetyl (Table I). This order has also been found to represent the order of increasing electron-withdrawing effect of the 2,4-substituents as reflected

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[1] HbCO denotes the hemoglobin–carbonyl complex and HbO_2 oxyhemoglobins.

TABLE I: ν_{CO} Values for Heme Carbonyls and ν_{NH} and pK_3 Values for Metal-Free Derivatives of Substituted Deuteroporphyrin IX Dimethyl Esters.

		Metal-Free Porphyrin	
2,4-Substituent	Pyridine-carbonylheme ν_{CO} (cm^{-1})[a]	ν_{NH} (cm^{-1})[b]	pK_3[c]
Ethyl	1973.0	3316.8	5.8
Hydrogen	1975.1	3316.4	5.5
Vinyl	1976.6	3315.9	4.8
Acetyl	1983.7	3310.7	3.3

[a] In bromoform with 0.12 M pyridine. [b] In bromoform. [c] Caughey *et al.* (1966b).

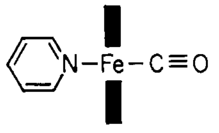
TABLE II: Effects of 4-Substituents on Pyridine Basicity and on Carbonyl-Stretching Frequencies of 4-Substituted Pyridinecarbonyliron(II)-2,4-diacetyldeuteroporphyrin IX Dimethyl Esters.

4-Substituent	ν_{CO} (cm^{-1})[a]	pK_a[b]
Amino	1971.1	9.12
Methyl	1982.3	6.03
Hydrogen	1983.7	5.21
Acetyl	1986.5	3.51
Cyano	1988.5	1.86

[a] In bromoform with 0.13 M 4-substituted pyridine. [b] Fischer *et al.* (1964).

in nitrogen basicities (pK_3 values) of metal-free porphyrins (Caughey *et al.*, 1966b). The differences found among ν_{NH} values for metal-free porphyrins in bromoform (Table I) also reflected a similar order. The diacetyl compound gave the lowest frequency, as expected for the least basic compound.

The order found for the ν_{CO} values can be rationalized in terms of differences in electron density or availability at the iron atom. The greater the electron density the better the iron atom can serve as a π donor to CO. The simplest representation is one based on the valence bond formalism as depicted by contributions by forms I and II. Here, the stronger the Fe–(CO) bond, the greater will be the bond order of the Fe–(CO) bond, the smaller will be the CO bond order, and the weaker will be the CO bond; *i.e.*, the ν_{CO} frequency can be expected to decrease as the bond order of the CO bond decreases. The data of Table I, therefore, indicate that the strength of the iron to CO bond decreases as the electron-withdrawing character of the 2,4 substituent increases and support the idea that CO bonding is strengthened by increased ability of the iron atom to serve as a π donor.



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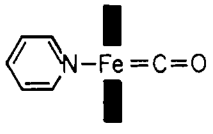

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Effects of Pyridine Substituents on the ν_{CO} of 4-Substituted Pyridine-2,4-diacetyldeuteroheme Dimethyl Ester Carbonyls. Substituents on the 4 position of the pyridine ligand also influenced ν_{CO}. Infrared measurements were made for bromoform solutions with 0.13 M substituted pyridine and diacetyldeuteroheme. As shown in Table II, ν_{CO} increased as the basicity of the pyridine decreased. These findings can likewise be explained in terms of valence bond structures I and II. (The substituted pyridines exert a *trans* effect whereas the substituted porphyrins exert a *cis* effect.) A factor which could complicate interpretation of the substituted pyridine data is the difference in solvent which results upon changing the pyridine derivative. However, although solvent differences have been shown to alter both the frequency and the band width of carbonyl absorption bands (Bor, 1962; Beck and Lottes, 1964), it appears unlikely that solvent differences could account for more than a small portion of the differences noted among the pyridine derivatives, particularly in view of the low (0.13 M) concentrations of substituted pyridine used in the polar solvent (bromoform).

Effects of 2,4-Substituents on the Competition of Pyridine and Carbonyl Ligands for Deuterohemes. The order of strength of CO binding indicated by ν_{CO} values were also compared with the degree to which carbonyl complexes of these deuterohemes are formed in benzene–pyridine solutions. In 1926, Hill reported that in aqueous pyridine one pyridine ligand of dipyridinehemes was reversibly replaced by one CO ligand; the relative amounts of dipyridine and monopyridinemonocarbonyl species were dependent on the relative concentrations of pyridine and CO. Subsequently Wang *et al.* (1958) demonstrated reversible formation of carbonylprotoheme ester in benzene solutions. Figure 1 illustrates our determination of the reversibility of carbonylheme formation for the protoheme; similar results were found for each of the other three hemes. [Differences between the spectra reported by Wang and coworkers (1958) and those reported here have been discussed previously (Alben *et al.*, 1968).] The amounts of both dipyridine and pyridine carbonyl species were computed from visible spectra. Thus in benzene solutions at 20° with 3.5×10^{-5} M total heme, 1.3 M pyridine, and 4.5×10^{-4} M CO, the dipyridine heme spectra were converted to carbonylheme spectra to the extents of 77, 60, 56, and 17% for diethyl-, dihydro-, divinyl-, and diacetyldeuterohemes, respectively. In solutions identical except that the CO concentration was increased to 6.7×10^{-3} M, the extents of conversion to carbonylheme spectra was 96, 94, 88, and 65%, respectively. When

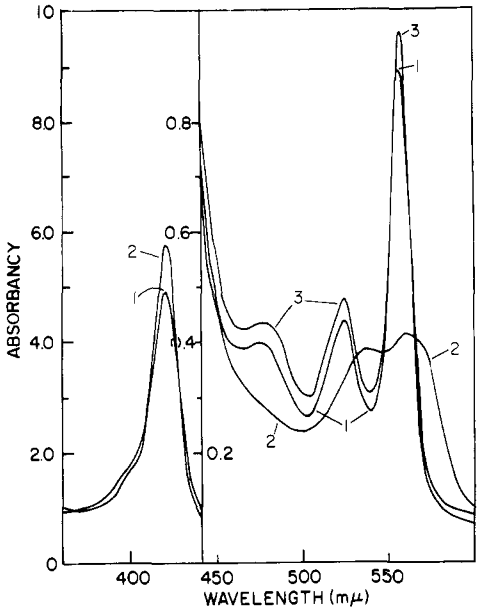

FIGURE 1: Spectra of protoheme dimethyl ester (3.2×10^{-5} M) in 1.3 M pyridine in benzene solution illustrate the reversibility of heme carbonyl formation: (1) before treatment; (2) after passage of CO through the solution for 2 min; (3) after purge of solution 2 with nitrogen (Matheson, prepurified, 99.996%) for 2 min. Increased absorbance in curve 3 compared with curve 1 is due to solvent evaporation during the purge.

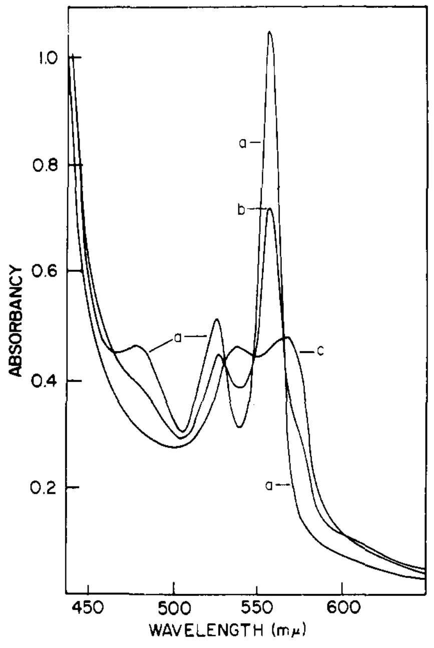

FIGURE 2: Relative affinity of heme for carbon monoxide or pyridine, determined by visible spectra. Protoheme dimethyl ester (3.4×10^{-5} M at 20°) in 1.00-cm cuvets: (a) no CO in 1.3 M pyridine in benzene; (b) 4.5×10^{-4} M CO in 1.3 M pyridine in benzene; (c) 6.7×10^{-3} M CO in 0.26 M pyridine in benzene.

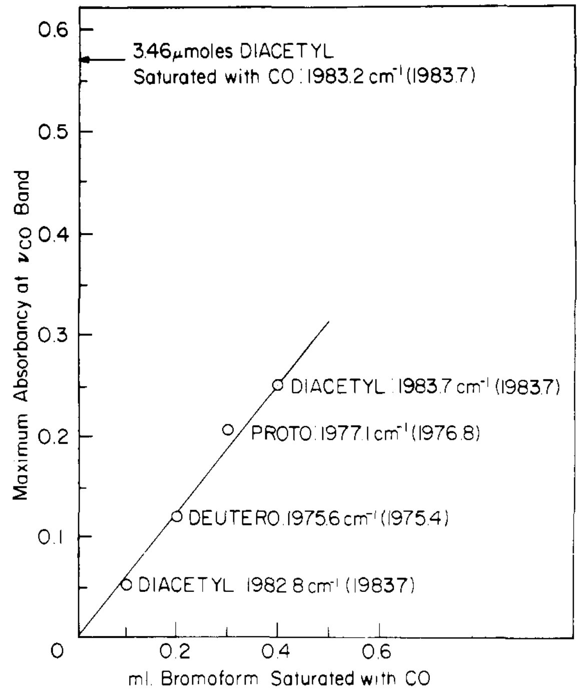

FIGURE 3: Infrared spectrophotometric titration of hemes with carbon monoxide in bromoform. The heme derivative and frequency of maximum absorbance of the heme carbonyl are indicated for each experiment, with peak frequencies previously obtained for the respective hemes saturated with carbon monoxide placed in parentheses. The solution of diacetyldeuteroheme which contained 0.40 ml of bromoform saturated with CO was subsequently saturated with CO, giving a maximum absorbance of 0.57, as shown. Half-band widths for the lower three CO concentrations were 25–26 cm^{-1}, and for the two higher concentrations, approximately 27 cm^{-1}. Diacetyl, proto, and deutero signify diacetyldeutero-, proto-, and deuteroheme complexes, respectively. Final volume was 1.30 ml.

the original conditions were modified by lowering the pyridine concentration to 0.13 M, the diethylheme was essentially 100% carbonyl and the diacetylheme was 97% carbonyl. These results demonstrate that carbon monoxide becomes a less effective competitor with pyridine as a ligand as the 2,4-substituents on the deuterohemes become more electron withdrawing in character. These findings can be rationalized in terms of the greater importance of the π donor (and lesser importance of σ acceptor) role for the iron atom in the case of CO bonding than in the case of pyridine binding.

Consideration of Possible Heme–Heme Association and Formation of Dicarbonyls. Both heme–heme association and the formation of dicarbonyl species, if present, could complicate the thus far relatively straightforward interpretations of the infrared spectra and equilibrium experiments. These complications do occur with heme solutions in benzene. The increased

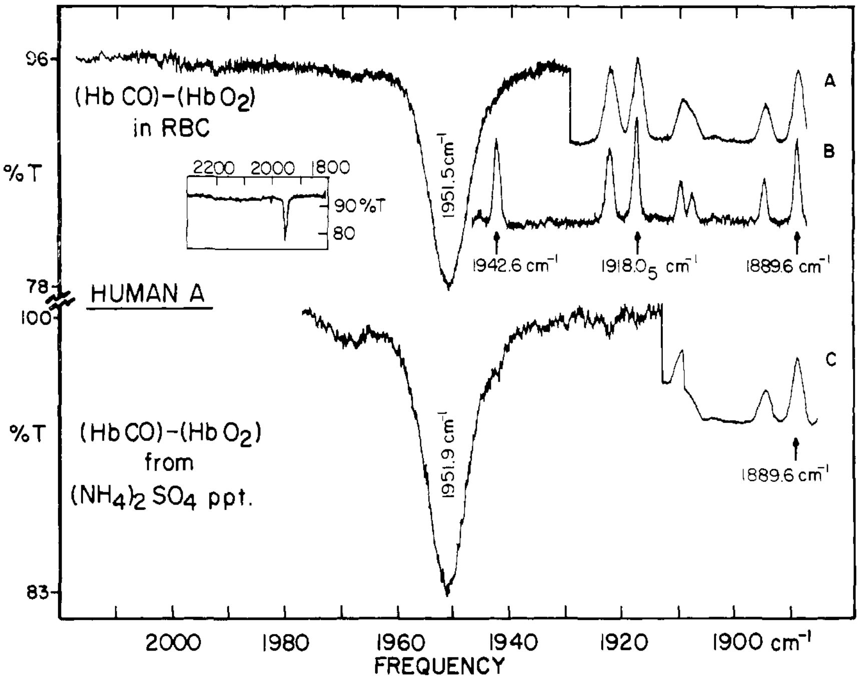

FIGURE 4. Infrared difference spectra of carboxyhemoglobin minus oxyhemoglobin in the region of the carbon–oxygen stretching vibration (ν_{CO}). Upper spectra: packed red blood cells; lower spectrum: aqueous hemoglobin precipitated with ammonium sulfate. Inset: hemoglobin in red blood cells scanned from 2300 to 1800 cm^{-1} with no scale expansion. Other HbCO spectra were obtained with a fivefold expansion of the ordinate and a 20-fold expansion of the abscissa. A and C are atmospheric water vapor spectra, obtained by conversion to single-beam operation with no alteration of the mechanical scanning system, and were obtained at the same resolution as the corresponding helmoglobin spectra. B was obtained at higher resolution. Water vapor peaks used for calibration are indicated by arrows.

absorbance observed between 445 and 460 mμ in the presence of 4.5×10^{-4} M CO relative to no CO, and the decreased absorbance at high CO concentrations indicate at least two heme carbonyl species (Figure 2). Similar results were also obtained with deutero- and mesoheme carbonyl formation, and at least two sets of isosbestic points were observed when diacetyldeuteroheme or mesoheme was titrated with CO in 0.13 M pyridine in benzene.

The possibility that species other than monopyridinemonocarbonylhemes exist in bromoform solution under the conditions used for the infrared studies was examined in the following way. Solutions of hemes in pyridine–bromoform were prepared as for Table I except that CO was added as an aliquot of CO-saturated bromoform. The amounts of heme carbonyl formed depended directly on the amount of CO added (the heme was less than half saturated) and was independent of the nature of the heme (Figure 3); thus no evidence for heme–heme interactions in our system was obtained. In addition, the peak frequencies obtained at one-tenth to one-half saturation were identical with those obtained at saturation. The molecular species that were observed at low and high concentrations of CO must, therefore, have been the same, since a marked shift in CO-stretching frequency would be expected if the heme carbonyl species varied from, for example, a monocarbonylheme to either a dicarbonylheme or a binuclear complex which contained two hemes per carbon monoxide molecule. The absence of shift in frequency from that obtained at saturation with CO appears to rule out the possibility that more than a single carbonylheme species was present. The absorption intensities of CO peaks listed in Tables I and II (0.79–0.9 and 0.9–1.2 μmole^{-1} cm^2, respectively), although measured less quantitatively than those in Figure 3, are consistent with the interpretation that all species observed by infrared spectroscopy were monocarbonyl–monopyridine–heme complexes. Interpretation of the data for substituted-pyridine complexes (Table II) as *trans* effects is also consistent with formulation as a monocarbonylmonopyridine species, as is the presence of a single infrared absorption due to CO.

If only 1 mole of CO is bound/heme, then from the per cent saturation of the diacetyldeuteroheme (Figure 3) bromoform is estimated to be 3.8 mM in CO when saturated at 20°. The corresponding values for benzene and chloroform are 6.84 and 7.88, respectively (Just, 1901).

Infrared Studies of Carbon Monoxide Bound to Hemoglobin. The sensitivity to environmental influence

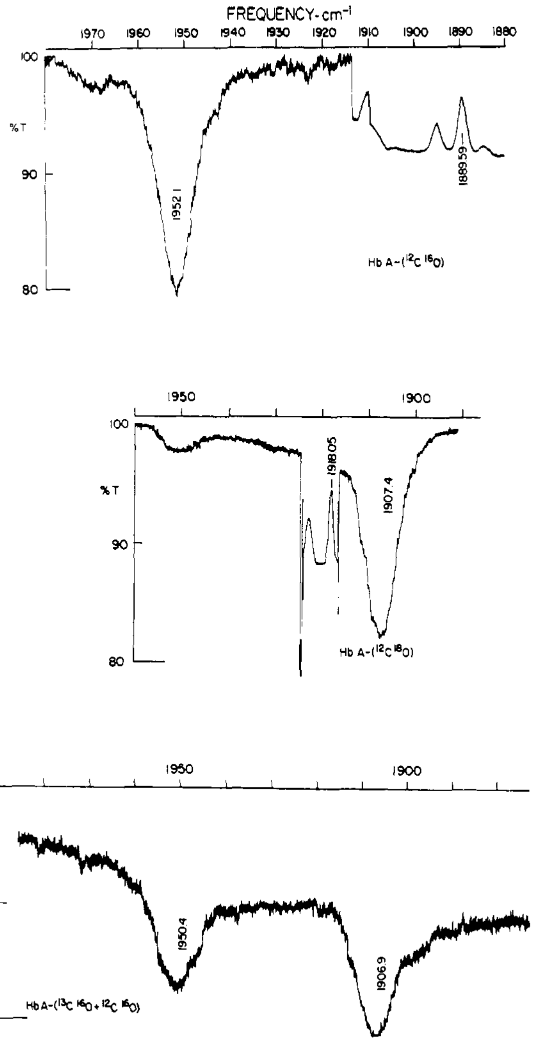

FIGURE 5: Isotopic mass effects on ν_{CO} for carboxyhemoglobin. Hb^{12}C^{16}O spectrum from Figure 4 (upper tracing) for comparison; Hb^{12}C^{18}O and Hb^{13}C^{16}O spectra were obtained under similar conditions as in Figure 4 except that hemoglobin was in aqueous solution.

TABLE III: Isotope Shifts in Infrared Spectra of Human Hemoglobin–Carbonyl Complexes and Pyridine–(Protoheme dimethyl ester)–Carbonyl Complexes.

	ν_{CO} (cm^{-1})		
	^{12}C^{16}O	^{13}C^{16}O	^{12}C^{18}O
Hemoglobin–CO complex			
In red blood cell	1951.5		
Ammonium sulfate precipitate	1951.9		
Aqueous solution	1950.3	1906.8	
Aqueous solution	1950.8		1907.3
Pyridine–(protoheme-dimethyl ester)–CO complex	1976.5	1933.65	
In bromoform solution	~1976		1930.8

of the carbon–oxygen stretching frequency in carbonylhemes suggested that measurement of this frequency would be of great value in the understanding of carbonyl-heme–protein interactions in compounds such as hemoglobin and myoglobin. To establish that measurements could be made on native hemoproteincarbonyl complexes without spectral interference from absorption bands other than the C–O stretching vibration of interest,[2] infrared measurements have been made of human carbonyl hemoglobin in the intact red blood cell, as an ammonium sulfate precipitate, and in concentrated aqueous solution. The absorption bands were similar for all three cases, with maxima near 1951 cm^{-1} and half-band widths of about 8 cm^{-1} (Figures 4 and 5, and Table III). The inset in Figure 4 illustrates the absence of any other absorption band in the difference spectrum between 2300 and 1800 cm^{-1}. It also illustrates the need for adequate scale expansion for accurate work.

The single, sharp absorption band at 1951 cm^{-1} indicates that only a single type of carbon monoxide environment can be present in carbonyl hemoglobin, and indeed that differences between the α and β chains do not affect the bound CO. Shifts in ν_{CO} have been observed with genetically abnormal hemoglobins which contain an amino acid substitution in the vicinity of the heme (J. O. Alben, S. McCoy, S. Boyer, P. Hathaway, S. Charache, and W. S. Caughey, to be published). The lower frequency of the absorption maximum observed with HbCO relative to that with pyridine–heme–CO may be due in part to the presence of imidazole rather than pyridine coordinated to the sixth position of the iron. The other major difference between the spectra of HbCO and pyridine–heme–CO in bromoform solution is the much narrower half-band width of the former (8 cm^{-1} compared with about 27 cm^{-1} for the heme carbonyls). The half-band width of ν_{CO} is highly sensitive to the nature of the solvent in the case of simple metal carbonyls, with values of about 4 cm^{-1} in alkanes, 8 cm^{-1} in tetrachloroethylene, 19 cm^{-1} in toluene, and 22 cm^{-1} in chloroform, reported for nickelcarbonyl and cobaltnitrosylcarbonyl (Bor, 1962), and corresponding values for a variety of metal carbonyls in hexane and tetrachloroethylene (Noack, 1962). A carbonyl half-band width of 8 cm^{-1} is thus consistent with a nonpolar, slightly polarizable solva-

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[2] A low-resolution spectrum of "carboxyhemoglobin" was presented with no background compensation (Wang *et al.*, 1958) from protein that had been "crystallized" in a vacuum rotary evaporator at 2°, then pressed into a KBr disk. It is not likely that the native structure of hemoglobin could survive such treatment, and indeed the value reported does not agree with the solution values obtained here.

TABLE IV: Identification of Carbon Monoxide Isotopic Mixtures by Rotational Bands at ν_{CO} Fundamental Band (cm^{-1}).

Obsd $^{13}C^{16}O$ + $^{12}C^{16}O$	(J)[a]	$^{13}C^{16}O$	(J)[b]	$^{12}C^{16}O$	(J)[a]	$^{12}C^{18}O$	Obsd $^{12}C^{18}O$ + $^{12}C^{16}O$
2159.0	R18	2159.53	3	2158.30	19	(2158.41)[c]	2158.2
2157.3							
2155.2	17	2156.41			18	(2155.42)	2155.2
2154.1	16	2153.42	2	2154.59	17	2152.35	2152.2
2151.0	15	2150.33	1	2150.86			2150.9
2147.6	14		R0	2147.08	16	2149.43	2149.3
2144.4	13	2144.02			15	2146.19	2146.1
2141.2	12	2140.81	P1	2139.43	14	2143.08	2142.9
2137.9	11	2137.59			13	(2139.95)	2139.6
2135.8			2	2135.55	12	2136.74	2136.6
2134.6	10	2134.34			11	2133.52	2133.6
2131.2	9	2131.01	3	2131.63	10	2130.24	2130.4
2127.6	8		4	2127.68	9	(2126.95)	2126.9
2124.1	7	2124.24	5	2123.70	8	(2123.62)	2123.5
2120.8	6	2120.86					
2119.5			6	2119.68	7	(2120.25)	2120.1
2117.1	5	2117.43					
2115.4			7	2115.63	6	2116.84	2116.7
2113.6	4	2113.94			5	2113.40	2113.2
2111.6			8	2111.55			2111.3
2110.2	3	2110.45			4	2109.96	2109.7
2107.0	2	2106.92	9	2107.42	3	2106.45	2106.2
2103.2	1		10	2103.27	2	(2102.92)	2102.4
2099.1	R0	2099.70	11	2099.09	1	(2099.35)	2099.9
2094.8			12	2094.86	R0	(2095.75)	2095.6
2092.4	P1	2092.40					
2090.4			13	2090.61			2090.6
2088.7	2	2088.69			P1	(2088.46)	2088.2
2086.2			14	2086.32			
2085.0	3	2084.93			2	(2084.76)	2084.8

[a] Mills and Thompson (1953). [b] IUPAC (1961). [c] Numbers in parentheses are calculated in footnote *a*.

tion shell about the bound CO ligands as suggested by the Perutz (1965) model for hemoglobin. The narrow half-band width in HbCO confirms the constancy of amino acid environment around the heme carbonyl. This indicates a similarity of environment in the α and β chains, since differences arising from any cause would result in a broader absorption band.

Effects of Ligand Isotopic Mass on ν_{CO}. In order to confirm the identity of ν_{CO}, HbCO was prepared from carbon monoxide that was 53% enriched in ^{13}C or 86% enriched in ^{18}O. The results are in accord with expectation (Figure 5 and Table III) in that complexes of both isotopic gases show ν_{CO} shifted from that of $^{12}C^{16}O$ and lend positive identification to ν_{CO}. The $^{12}C^{16}O$ complexes in the isotopic mixtures gave the same ν_{CO} as in the red blood cells or precipitated hemoglobin. However, the presumed $^{13}C^{16}O$ and $^{12}C^{18}O$ complexes gave ν_{CO} values sufficiently similar to each other that it was necessary to prove the composition of the gases by other means. The vibrational–rotational spectra of the respective gas mixtures were therefore determined at 1 atm with a 3-mm path, and compared with literature values (Table IV). It is evident that the gases were correctly identified qualitatively and quantitatively.

It was of further interest to compare the isotope shifts of the complexes with those of the free gases, as well as with theoretical mass effects. Such mass effects (ν^*/ν) found for free gases (Mills and Thompson, 1953; Plyler *et al.*, 1955) are

$$\frac{\nu(^{13}C^{16}O)}{\nu(^{12}C^{16}O)} = \frac{2096.071}{2143.774} = 0.977749$$

$$\frac{\nu(^{12}C^{18}O)}{\nu(^{12}C^{16}O)} = \frac{2092.12}{2143.77} = 0.975906$$

and for hemoglobin complexes (Table III) are

$$\frac{\nu(Hb^{13}C^{16}O)}{\nu(Hb^{12}C^{16}O)} = \frac{1906.88}{1950.3} = 0.97769$$

$$\frac{\nu(\mathrm{Hb}^{12}\mathrm{C}^{18}\mathrm{O})}{\nu(\mathrm{Hb}^{12}\mathrm{C}^{16}\mathrm{O})} = \frac{1907.3}{1950.8} = 0.97770$$

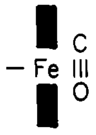
The mass effects were calculated from the relation

$$\frac{\nu^*}{\nu} = \frac{(M_A{}^* + M_B{}^*)/M_A{}^*M_B{}^*}{(M_A + M_B)/M_AM_B}$$

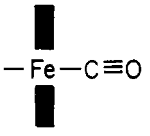
where M is the mass of atom A or B, and * denotes the isotopic derivative. The calculated ratios are 0.977775 and 0.975900 for $\nu(^{13}\mathrm{C}^{16}\mathrm{O})/\nu(^{12}\mathrm{C}^{16}\mathrm{O})$ and $\nu(^{12}\mathrm{C}^{18}\mathrm{O})/\nu(^{12}\mathrm{C}^{16}\mathrm{O})$, respectively.

The predicted position from the theoretical isotopic shift due to mass only for ^{13}C(HbCO) is (1950.3)·(0.977775) = 1906.5 cm^{-1}, which is the same as the observed value. The position predicted from the mass-only shift for ^{18}O(HbCO) is (1950.8)(0.975900) = 1903.8, which differs significantly (3.5 cm^{-1}) from the observed value.

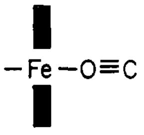
It is of interest to compare these isotopic shifts with structural models for HbCO. The observed frequencies clearly require that CO be bound directly to iron as, for example, in III, IV, and V as above. π-Complex structures such as that between hemoglobin and xenon (Schoenborn *et al.*, 1965) and clathrate bonding (Steinhardt *et al.*, 1966) would not be compatible with the HbCO data, since a value of ν_{CO} much closer to that of the free gas would then be expected. Structure III would be fully consistent with the ^{13}C isotopic shift. However, the ^{18}O isotopic shift would seem to require some mass coupling with other vibrations which is not present in the ^{13}C isotopic shifts.[3] *That is, the carbon isotopic shift behaves as that in the free gas, while the oxygen isotopic shift appears to be coupled with other vibrations.* This would suggest structure V rather than the conventionally written structure IV. It is doubtful if structure III could be made compatible with the isotopic shift data even if oxygen were hydrogen bonded to an imidazole group, as may be the case in HbO$_2$. That hydrogen bonding is too weak to affect the isotopic shift is supported by the observation of similar isotopic shifts upon replacement of the E-7 histidine of hemoglobin with arginine or tyrosine by use of Hb-Zurich or Hb-M$_{\mathrm{Emory}}$, respectively (J. O. Alben, S. McCoy, S. Boyer, P. Hathaway, S. Charache, and W. S. Caughey, to be published). Thus structure V appears to give the best fit to the infrared data from hemoglobin.



III



IV



V

The isotopic shifts found with pyridine–(protoheme dimethyl ester)–carbonyl in bromoform could be interpreted less clearly in terms of models for CO bonding than was the case for the hemoglobins. The observed shift with each isotope was less than the mass-only shift computed from a $\nu(^{12}\mathrm{C}^{16}\mathrm{O})$ value of 1976.5 × 1 cm^{-1} for ^{13}CO (calcd 1932.6 cm^{-1}, found 1933.6 cm^{-1}) and × 2 cm^{-1} for C^{18}O (calcd 1928.9 cm^{-1}, found 1930.8 cm^{-1}). The unresolved absorption bands of a mixture of species may be responsible for the wide half-band widths observed in the case of these solutions of simple hemes in bromoform.

Use of Difference Infrared Spectroscopy in Studies of Substrate and Inhibitor Binding to Proteins. Difference infrared spectroscopy allows selective observation of certain molecular vibrations in the presence of massive background absorption. The problems of this technique are common to all types of difference spectroscopy and include determining whether the apparent absorption band is due to a unique molecular vibration, to a slight mismatch of reference and sample cells, or to an unrecognized instrumental artifact. Assignment of absorption bands is made possible by their reproducibility and occurrence in the triple-bond region of the spectrum which contains some broad absorptions but no other sharp bands. The carbon monoxide, concentrated manyfold from aqueous solution by adsorption to the iron, has a single sharp absorption band which is clearly different from any incompletely compensated broad absorption which might be present.

This use of infrared difference spectroscopy provides a new way to study metal–protein complexes with ligands such as carbon monoxide, cyanide, azide, and others which absorb in the triple-bond region. This method of study can be applied profitably to all metal proteins which bind ligands of this sort in their native states. This includes diamagnetic iron and copper proteins which cannot be observed by electron paramagnetic resonance or pulsed nuclear magnetic resonance methods. Since our first report (Alben and Caughey, 1966) the use of infrared difference spectroscopy has been extended to include a variety of normal and abnormal human and animal hemoglobins and myoglobins (Caughey *et al.*, 1967; McCoy and Caughey, 1967; J. O. Alben, S. McCoy, S. Boyer, P. Hathaway, S. Charache, and W. S. Caughey, to be published) as well as the CO$_2$ complex of carbonic anhydrase (Riepe and Wang, 1967). This list will undoubtedly grow rapidly.

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[3] Deviations in the metal–carbonyl isotope shifts from mass-only effects have been reported and ascribed to both vibrational coupling and anharmonicities (Braterman *et al.*, 1967, and references therein).

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