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Structure of the Carbon Monoxide Binding Site of Hemocyanins Studied by Fourier Transform Infrared Spectroscopy†

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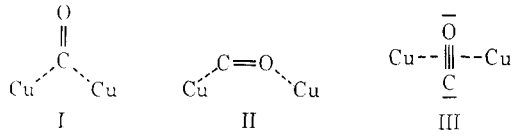
ABSTRACT: Carbon monoxide complexes of hemocyanin from several sources have been studied by infrared spectroscopy. The CO stretching vibrations (νCO) for two molluscs (squid and limpet) occur near 2063 cm^{-1}, at 2054 cm^{-1} for the horseshore crab (*Limulus*) and near 2043 cm^{-1} for two crustaceans (Atlantic rock crab and Ohio crayfish). These frequencies are clear evidence of different local molecular environments for bound CO in hemocyanins from these three groups, and support our proposal that the proteins provide different ligands to coordinate to copper at the active site. The isotopic shift (ν^*/ν) for [^{18}C]carbon monoxide bound to hemocyanin is nearly identical with that for the free gas, while ν^*/ν for [^{18}O]-carbon monoxide bound to hemocyanin is less than that for the free gas for all animal sources except crayfish, which was not tested. These data rule out all model structures in which CO is bridged between two copper atoms or in which the CO is bound perpendicular to a line through the copper nucleus. They are consistent with a structure in which the oxygen atom of CO is coordinated to one atom of copper. A model for the hemocyanin carbonyl complex is proposed which includes a trigonal oxygen of CO coordinated to one copper atom (C–O–Cu angle near 120°), with the second copper atom of the binding site coordinated only to protein.

Complexes of small molecules with heme proteins have been studied in great detail, but relatively little is known about ligand binding to copper proteins. We have therefore undertaken a study of carbon monoxide complexes of hemocyanins from several species. Hemocyanins are the oxygen transport proteins in the hemolymph of many molluscs and decapod crustaceans, and reversibly bind oxygen or carbon monoxide with a stoichiometry of one ligand per two copper atoms (Redfield *et al.*, 1928a; Root, 1934; Kubowitz, 1938; Vanneste and Mason, 1966; Rocca and Ghiretti, 1963). This stoichiometry led to speculation that the ligand might form a bridging structure between two atoms of copper, and Williams (1966) listed possible bridging structures for carbon monoxide bound



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to copper. In a preliminary study, Alben *et al.* (1970) found only a single narrow absorption band due to carbon monoxide bound to hemocyanin from the giant keyhole limpet (*Megathura crenulata*) at a frequency (2063 cm^{-1}) which is consistent with a nonbridging copper carbonyl structure, but which is too high for structures I or II. The frequency of this absorption band was similar to that observed (2069 cm^{-1}, Alben *et al.*, 1970) for copper carbonyl in pyridine solution which is known to be a nonbridging carbonyl coordinated to a single copper (Manchot and Friend, 1908; Wagner, 1931). No bridging carbonyl complexes of copper are known, but they are common for binuclear complexes of iron or cobalt. Absorptions of bridging carbonyls in these complexes are observed in the range of 1800–1900 cm^{-1}, whereas mononuclear complexes are generally observed from about 1950 to 2150 cm^{-1} (Nakamoto, 1963). The range of frequencies is caused by the variety of ligands coordinated to the metal, and to the net charge on the complex. It has been amply demonstrated (Bigorgne, 1961; Bouquet *et al.*, 1968; Alben and Caughey, 1968) that electron-withdrawing effects of ligands are transmitted through a coordinated metal to ligands such as CO, which are coordinated to the same metal. Structure III was not tested by these data, but is ruled out by data presented in this paper.

The nature of ligands coordinated to the metal in copper proteins has been of interest for many years, but has been difficult to determine since any chemical or conformational change in the protein may lead to ligand exchange by the copper, and copper(I) is diamagnetic and not observed by electron spin resonance. Others have speculated that sulfhydryl (Klotz and Klotz, 1955) or histidyl (Lontie, 1958) residues may be coordinated in hemocyanins. We present evidence from the

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infrared spectroscopy of hemocyanin carbonyl complexes that the coordination sphere is different for hemocyanins from various sources. On the other hand, infrared spectroscopy of isotopically substituted carbon monoxide bound to hemocyanins suggests that the nature of the metal–carbonyl complex is the same as in carboxyhemoglobins, in that oxygen appears to coordinate to the copper, and the carbon to be free.

Experimental Section

Hemocyanins. Hemolymph from squids (*Logigo pealii*) and horseshoe crabs (*Limulus polyphemus*) was collected at the Marine Biological Laboratory in Woods Hole, Mass., in June of 1970. Crabs (*Cancer irroratus*) were collected from the Maine coast in August of 1970, and crayfish (a mixture of at least four species from two genera, including *Camberus robustus*, *Camberus loevis*, *Orconectes rusticus*, and *Orconectes sanborni*) from Ohio creeks in July of 1971. Giant keyhole limpet (*Megathura crenulata*) hemocyanin was purchased from Mann Research Laboratories.

The squid hemolymph was collected by slitting the muscular mantel and withdrawing hemolymph from the gill sinus with a syringe. Hemolymph from *Limulus* was drained by cutting a 0.5-in. slit on the ventral side at the hinge between the two flat body sections. Hemocyanin was drained from the crabs after removing the gill cover, and crayfish hemolymph was withdrawn from the ventral sinus with a syringe. Collected hemolymph was stored at −25° until used.

Hemocyanins were prepared by low-speed centrifugation of hemolymph to remove coagulated cells (*Limulus*, crab and crayfish) and insoluble material (squid and keyhole limpet) from the hemolymph solutions. The clear supernatant fluid was centrifuged at 10^5g for 5 hr with a Beckman Model L3-40 centrifuge with a no. 40 or 30 rotor. The colorless or yellowish supernatant was discarded and the dark blue precipitate was redissolved in 0.01 M Tris-HCl buffer (pH 7). Hemocyanin was found to be more stable in Tris buffer than in phosphate, and at neutral pH hemocyanin does not dissociate into its subunits and is readily sedimented in a preparative ultracentrifuge. The small amount of undissolved material was removed by recentrifugation at low speed. The above procedure was repeated at least four times. For *Limulus*, squid, and keyhole limpet, this purification removed most of the small soluble molecules and gave a fairly pure hemocyanin solution. For crab and crayfish hemocyanins the above method was less satisfactory since the hemocyanin solution was blackened by a melanoid polymer. However, it is unlikely that this impurity affects the infrared absorption band of hemocyanin-complexed carbon monoxide. For quantitative studies, the loosely bound or nonactive-site copper was removed by dialysis of purified hemocyanin solutions against EDTA in Tris-HCl buffer (pH 7). The purified, dialyzed hemocyanin generally was used within 2 weeks after it was prepared. For longer storage, addition of Ca^{2+} was used to slow the aging process (Lontie and Witters, 1966).

Formation of Hemocyanin Carbon Monoxide Complexes. Oxygen was removed from concentrated hemocyanin solutions by repeated evacuation and addition of prepurified nitrogen gas to the vacuum-stoppered container. After a final evacuation, the nitrogen was replaced by CO gas and the hemocyanin allowed to equilibrate at one atmosphere.

Measurement of Visible, Ultraviolet, and Infrared Spectra. Visible and ultraviolet spectra were obtained with a Spectracord Model 4000 A Perkin-Elmer split-beam spectrophotometer or manually with a Gilford Model 220 photometer attached to a Beckman Model DU monochrometer. Infrared spectra were measured at approximately 25° with a Digilab Model FTS-14 interferometer at 2-cm^{-1} resolution and with triangular apodization of the time-averaged interferograms before computation of spectra by a fast Fourier transform. Spectra of *Limulus* hemocyanin complexed with $^{12}C^{16}O$, ^{13}CO, and $C^{18}O$ were also measured at 4-cm^{-1} spectral resolution with a Perkin-Elmer Model 102 double-beam dispersive infrared spectrometer with a grating-prism double monochrometer. The results are in excellent agreement with those obtained with the Digilab interferometer, and serve to verify values listed in Tables II and III. ν_{CO} observed for CO, ^{13}CO, and $C^{18}O$, respectively, were 2054.8, 2008.4, and 2008.0 cm^{-1}; α_{mM} (CO) = 1.4 mM^{-1} cm^{-1}; ϵ_{mM} (CO) (area) = 16 mM^{-1} cm^{-2}. Infrared cells were used with CaF_2 windows, and path lengths of approximately 0.05 mm were measured to better than 2% by interference fringes obtained with the empty cell. The vibrational rotational spectra of isotopically substituted carbon monoxide gas samples were obtained by use of a 10-cm path-length gas cell with KBr windows, in order to provide positive identification of the isotopic composition, as was done previously (Alben and Caughey, 1968).

Carbon Monoxide Gas. CP grade $^{12}C^{16}O$ (99.5% pure) was purchased from Linde Division of Union Carbide, [^{13}C]carbon monoxide (60.8 atoms % $^{13}C^{16}O$ in $^{12}C^{16}O$) from Prochem, Ltd., and [^{18}O]carbon monoxide (90.79 atoms % $^{12}C^{18}O$ in $^{12}C^{16}O$) from Miles Laboratories.

Determination of Protein and Copper Concentration. The protein concentrations were measured by the microbiuret method of Westley and Lambeth (1960), with bovine serum albumin (E_{278} = 0.64 1 g^{-1} cm^{-1}) as the standard (Raziro *et al.*, 1961). Copper which was not removed by dialysis against EDTA was determined spectrophotometrically by the method of Peterson and Bollier (1955) after wet digestion with sulfuric acid and hydrogen peroxide. Copper sulfate solutions which contained from 1 to 10 μg of copper were used as standards. The observed extinction coefficients and copper contents of hemocyanin preparations used in this work are listed in Table I. The copper contents of our hemocyanin

TABLE I: Ultraviolet Extinction Coefficients and Copper Contents of Oxyhemocyanin Preparations Used in This Work.

	E_{278} (cm^2 mg^{-1})[a]		A_{340}/A_{278}		Copper Content (%)	
	pH 7	pH 10	Obsd	Lit.	Obsd[i]	Lit.
Squid		1.28	0.17	0.18[j]	0.22	0.25,[b] 0.26[c]
Limpet	1.36			0.15[k]	0.23	
Limulus	1.39	1.19	0.27		0.17	0.17[d]
Crab	1.13[e]		0.115[e]		0.12[e]	0.16–18,[f] 0.18[g]
Crayfish			0.21			0.18[h]

[a] Based on the biuret reaction. [b] Montgomery (1930). [c] Hernler and Philippi (1933). [d] Redfield *et al.* (1928b). [e] These values are assumed to be low due to contaminating protein. [f] Values for *Cancer magister* from Thomson *et al.* (1959). [g] Value for *Cancer borealis* from Allison and Cole (1940). [h] Value for *Procambarus similans* from Larimer and Riggs (1964). [i] Tightly bound copper which is not removed by dialysis against EDTA. [j] Value for *Ommatostrephes sloani pacificus* (Pacific Ocean squid) from data of Omura *et al.* (1961). [k] Alben *et al.* (1970).

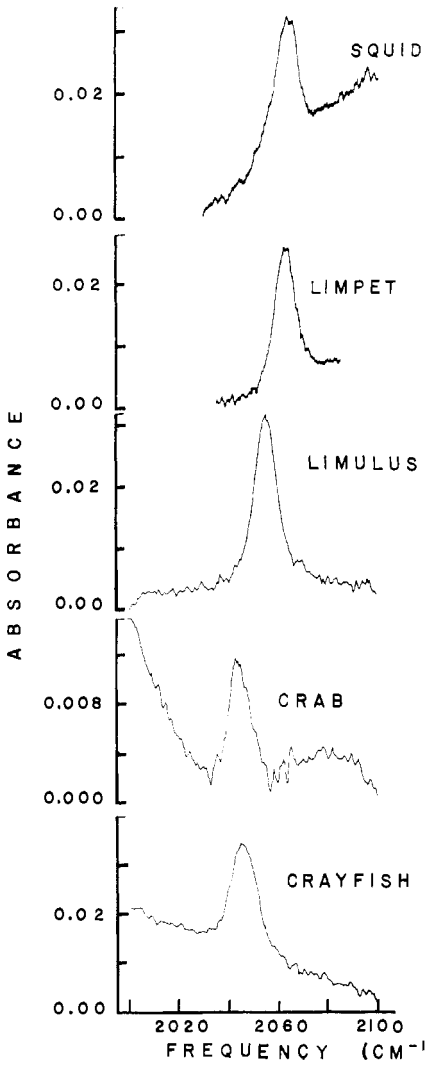


FIGURE 1: Infrared spectra of carbon monoxide ($^{12}C^{16}O$) coordinated to hemocyanins from several invertebrate species. Difference spectra were obtained by use of digitally stored single beam spectra for reference, which were chosen to yield relatively flat base lines. Single-beam spectra were obtained by coherent coaddition of repetitively scanned interferograms, followed by a computed fast Fourier transform, as follows: squid (3330 scans) *vs.* water as reference; limpet (1995 scans) *vs.* water; *Limulus* (4000 scans) *vs.* oxyhemocyanin; crab (2000 scans) *vs.* methemoglobin A; crayfish (3315 scans) *vs.* methemoglobin A. All spectra were obtained at 2-cm^{-1} resolution. Accumulation of 4000 scans requires approximately 3 hr. Neither proteins nor water has any sharp absorption bands in this spectral region. The proteins absorb very little here, and only serve to decrease the effective concentration of water, which has strong, broad, absorption bands. Mismatch in the amount of water in sample and reference cells is the major contributor to sloping base lines. Absorbance (A) is defined as $\log (I_0/I) = \log I_0 - \log I$, where $I_0 > I$, and I_0 is the light intensity in the absence of sample. In the case of solutions, I_0 ideally is the light intensity which would be transmistted if the solute absorbed no light. In the case of single isolated absorption bands such as ν_{CO} in carboxyhemocyanins, I_0 is the base line which would be observed if the absorption band were absent. Therefore, the correct measurement is the difference in absorbance ($\log I_0 - \log I$) between the base line and the absorption band. Millimolar absorptivities listed in Table II were obtained from measurements made in this way.

TABLE II: Quantitation of Infrared CO Absorption Bands of Carboxyhemocyanins from Several Species, Dissolved in 0.10 M Tris-HCl Buffer (pH 7.0).

			App Absorptivities[a]	
	ν_{CO} (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1})	α_{mM} (CO) ((mM CO)$^{-1}$ cm^{-1})	ϵ_{mM} (CO) (Area) ((mM CO)$^{-1}$ cm^{-2})
Mollusca				
Squid	2063.0	10	1.15	13.5
Limpet	2062.0[b]	9.2	1.2	13
Limulus	2054.3	10.7	1.3	18
Crustacea				
Crab	2043.4	10		
Crayfish	2045.5	13		

[a] Apparent absorptivities of bound CO are based on the stoichiometry of one molecule of CO bound per two atoms of copper (from Table I); $\alpha_{mM} = A/cl$ is the millimolar absorptivity of CO at the frequency of maximum absorbance, and $\epsilon_{mM}(CO)(area) = (1/cl)\int A d\nu$ is the millimolar absorptivity integrated over the absorption band. The uncertainty of absorptivities is estimated to be about ±10% of the listed values. [b] A value of 2063 cm^{-1} was reported by Alben *et al.* (1970), for limpet hemocyanin carbonyl complex dissolved in distilled water.

preparations from squid and limpet are only slightly lower than values reported for squid or other gastropods. The lower value may result from loss of some copper from hemocyanin during the preparation. The copper content of our preparation of *Limulus* hemocyanin is the same as that reported by others. Only these three hemocyanin preparations were used to determine apparent millimolar absorptivities of bound carbon monoxide. These absorptivities are based on the established stoichiometry of one molecule of CO bound per two atoms of copper, and the measured content of tightly bound copper, but are only apparent absorptivities since bound CO was not measured directly. The amount of sample in each infrared cell was determined from the hemocyanin concentration and the measured optical path through the cell.

Results

Infrared measurements were made on hemocyanin carbon monoxide complexes from *Limulus*, squid, limpet, crab, and crayfish (Figure 1). For each species, except crayfish, only one absorption band, with a half-bandwidth of 10 ± 0.8 cm^{-1}, was observed between 1800 and 2300 cm^{-1}. This indicates that only a single type of carbon monoxide molecular environment could be present in carboxyhemocyanin from each of these species. The observed narrow half-bandwidths of hemocyanin carbonyls indicate that the protein provides a nonpolar, slightly polarizable solvation shell around the bound carbon monoxide and protects it from water. Similarly narrow half-bandwidths have been observed with hemoglobin ($\Delta\nu_{1/2}$ = 8 cm^{-1}) and myoglobin ($\Delta\nu_{1/2}$ is less than 12 cm^{-1}) carbonyls (Alben and Caughey, 1968; L. Y. Fager and J. O. Alben, unpublished observations) and azide complexes ($\Delta\nu_{1/2}$ = 8–10 cm^{-1}; Alben and Fager, 1972). Aqueous sodium azide ($\Delta\nu_{1/2}$ = 25 cm^{-1}) or denatured hemoprotein carbonyls ($\Delta\nu_{1/2}$ = 15–30 cm^{-1}; J. O. Alben, unpublished observations) exhibit much broader absorption bands. Simple metal carbonyls also

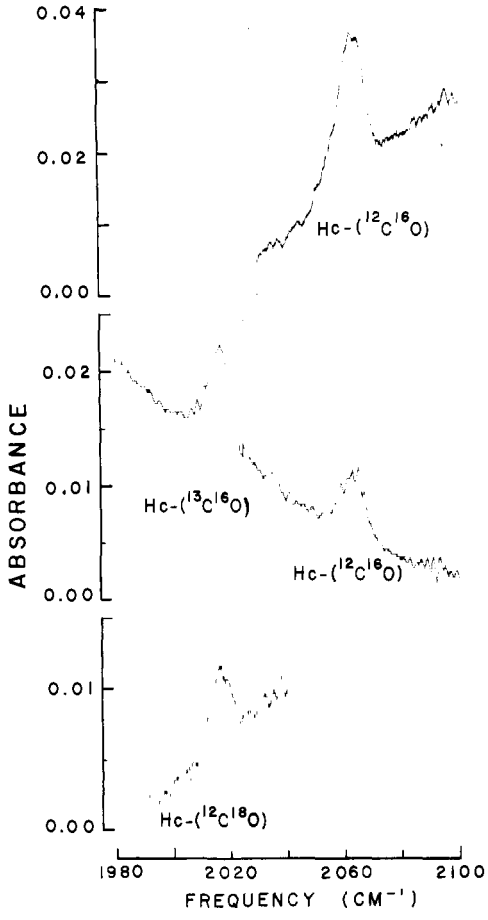


FIGURE 2: Infrared spectra of squid hemocyanin complexed with isotopically substituted carbon monoxide. The difference spectrum for the ^{13}CO complex was obtained by coaddition of 3200 scans *vs.* water as reference; and that for $C^{18}O$ from 3360 scans *vs. Limulus* oxyhemocyanin.

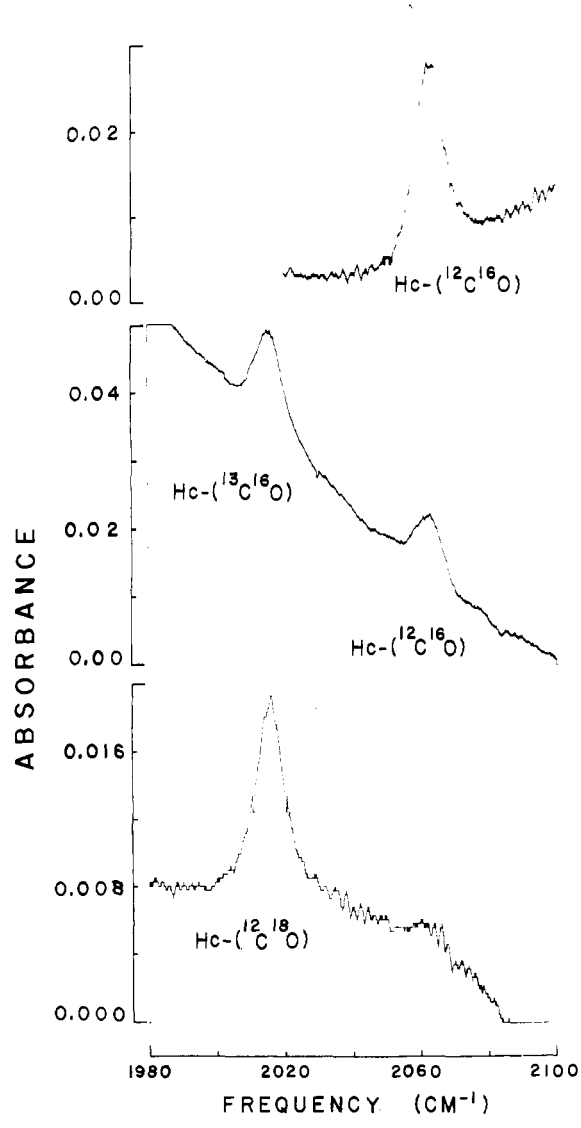


FIGURE 3: Infrared spectra of limpet hemocyanin complexed with isotopically substituted carbon monoxide. The difference spectrum for the ^{13}CO complex was obtained from 2671 scans *vs.* water as reference; and that for $C^{18}O$ from about 3000 scans *vs.* water.

show absorption bands that are sensitive to the solvation shell (Bor, 1962); *i.e.*, polar solvents cause broader absorption bands than nonpolar solvents (the range of $\Delta\nu_{1/2}$ is about 30–3 cm^{-1}). Copper carbonyl in a 10% pyridine solution in benzene absorbs with $\Delta\nu_{1/2}$ = 16 cm^{-1} (N. J. Farrier and J. O. Alben, unpublished observations). The apparently single absorption band of crayfish hemocyanin carbonyl has a half-bandwidth of 13 cm^{-1}, which is 2–3 cm^{-1} wider than half-bandwidths observed for hemocyanin carbonyls of other species. This wider half-bandwidth could indicate that the "solvation shell" of carbon monoxide bound to crayfish hemocyanin is slightly more polar than binding sites of the other hemocyanin carbon monoxide complexes which have been studied. However, since the collected crayfish hemocyanin sample is a mixture of two different genera (including at least four species), it is possible that this broader half-bandwidth is due to the overlap of narrow absorption bands which have center frequencies within 2 cm^{-1} of each other, and have similar intensities. The effects of possible microheterogeneity of crayfish hemocyanins on the frequencies of CO stretching vibrations must await further study.

The frequencies of absorption maxima (ν_{CO}) for various hemocyanin carbon monoxide complexes are indicated in Table II. The carbon monoxide complexes of the hemocyanins from two mollusks, the squid and the keyhole limpet, absorb near 2062 cm^{-1} and those of two crustaceans, crab and crayfish, absorb near 2044 cm^{-1}. The complex of the horseshoe crab, which is of uncertain phylogeny, and of which the close relatives no longer exist, absorbs at 2053 cm^{-1}. These hemocyanin copper carbonyls thus display three greatly different local environments, apparently due to different sets of ligands coordinated to the copper. It is significant that these large differences are phylogenetically determined, whereas the frequencies observed for the mollusks, or for the crustaceans, are similar within each group. The small frequency difference observed within a group is reasonably explained as due to different protein "solvation shells" lining the copper carbonyl pocket.

If we assume that all oxygen bound to the active site has been replaced by carbon monoxide and that one carbon monoxide molecule is bound per two atoms of copper, we can calculate the apparent extinction coefficient of the CO stretching vibration band from the measured protein concentration, copper content and maximum absorbance or area of the infrared absorption band (Table II).

Effects of $^{13}C^{16}O$ and $^{12}C^{18}O$ on ν_{CO}. The identity of ν_{CO} was confirmed by preparing the carboxyhemocyanin from carbon monoxide that was 60% enriched in ^{13}C or 90% enriched in ^{18}O. As shown in Figures 2–6, the complexes of both

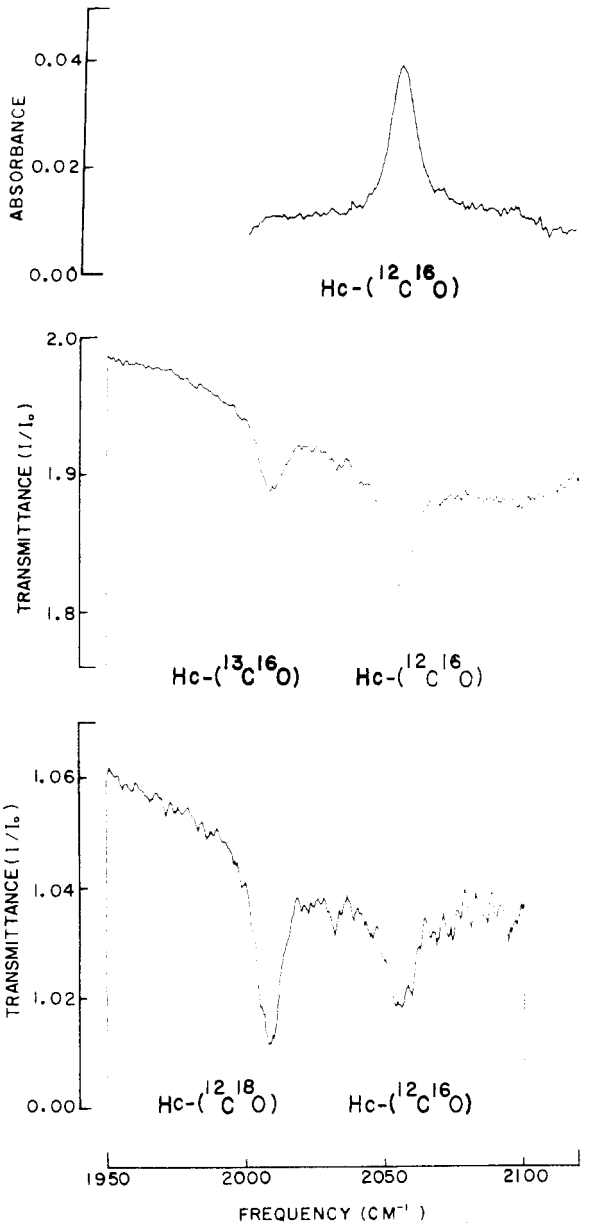


FIGURE 4: Infrared spectra of *Limulus* hemocyanin complexed with isotopically substituted carbon monoxide. The difference spectrum for each complex was obtained from about 4000 scans *vs.* oxyhemocyanin as reference.

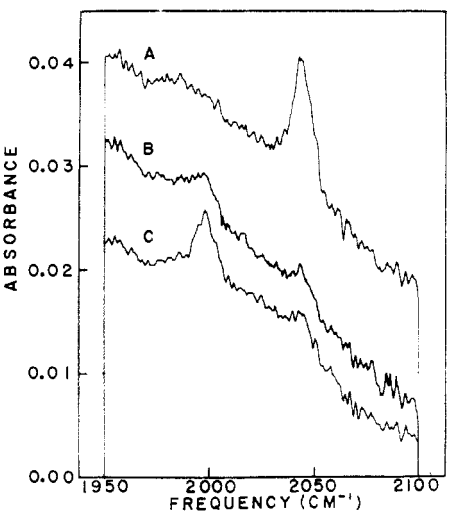


FIGURE 5: Infrared spectra of crab hemocyanin complexed with isotopically substituted carbon monoxide were obtained with carboxymyoglobin as reference, as follows: (A) $^{12}C^{16}O$ complex (2000 scans), (B) $^{13}C^{16}O$ complex (about 4000 scans), and (C) $^{12}C^{18}O$ complex (3857 scans).

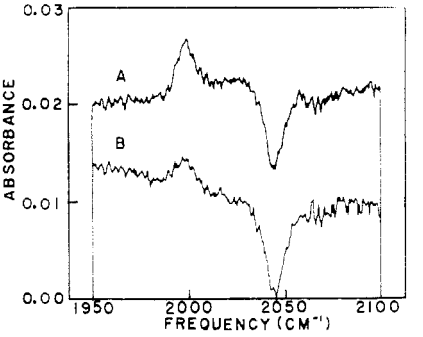


FIGURE 6: Infrared spectra of crab carboxyhemocyanin complexes were digitally replotted from the computer memory as follows: (A) $^{12}C^{18}O$ complex (C, Figure 5) *vs.* $^{12}C^{16}O$ complex (A, Figure 5) and (B) $^{13}C^{16}O$ complex (B, Figure 5) *vs.* $^{12}C^{16}O$ complex (A, Figure 5). The flatter base lines obtained here allowed the very small absorption bands to be measured with greater accuracy than was possible in Figure 5.

isotopic gases show ν_{CO} shifted from that of $^{12}C^{16}O$, and provide positive identification to ν_{CO}. However, the presumed $^{13}C^{16}O$ and $^{12}C^{18}O$ complexes gave values so similar to each other that it was necessary to confirm the identity of the gases by comparison of the vibrational-rotational spectra of the respective gas mixtures with literature values as was done previously (Alben and Caughey, 1968).

The theoretical values for the effects of mass on a simple harmonic oscillator are 0.977749 and 0.975906 for $\nu_{^{13}C^{16}O}/\nu_{^{12}C^{16}O}$ and $\nu_{^{12}C^{18}O}/\nu_{^{12}C^{16}O}$, respectively, where $\nu = (1/2\pi)[f(M_A + M_B)/M_AM_B]^{1/2}$, and $\nu^*/\nu = [(M_A^* + M_B^*)M_AM_B/M_A^*M_B^*(M_A + M_B)]^{1/2}$; M is the mass of each atom in an AB molecule, and f is the force constant. The mass effects found for hemocyanin carbon monoxide complexes are listed in Table III with those of the free gases. The isotopic shifts of the free gases are essentially the same as the theoretical values. In hemocyanin carbon monoxide complexes, the carbon isotopic shift is similar to that in the free gas, while the oxygen isotopic shift is less than in the free gas. This result is the same as that found for hemoglobin carbon monoxide complexes (Alben and Caughey, 1968). Therefore a similar interpretation has been applied, *i.e.*, that carbon monoxide is coordinated to copper through the oxygen. In addition, the isotope shift data rule out structure III as a model of the carbon monoxide binding site, since this model would require that oxygen and carbon be bound similarly, which is clearly not the case.

Discussion

The infrared spectra of various hemocyanin carbon monoxide complexes show great similarities. They each show only one narrow absorption band between 1800 and 2300 cm^{-1} due to bound carbon monoxide. Therefore only one kind of CO environment may exist in the hemocyanin complex from each species. The observed narrow half-bandwidth suggests that, as in myoglobin and hemoglobin, hemocyanin may have a cavity which is surrounded by nonpolar amino acid side chains and the active-site copper is located in this cavity and

protected from the aqueous solution. The half-bandwidths observed in hemocyanin carbonyl spectra (about 10 cm^{-1}) are *slightly* broader than in the case of hemoglobin (8 cm^{-1}), but much less than we found for aqueous sodium azide (25 cm^{-1}) or pyridinocopper carbonyl in benzene (16 cm^{-1}). Thus, while the CO binding sites in hemocyanins and hemoglobins share some similar characteristics and may both be relatively nonpolar, it is much too early to try to specify which amino acid residues actually line the cavity.

The frequencies of absorption maxima (ν_{CO}) for carboxyhemocyanin from various species correlate well with phylogeny, while the CO complexes of hemocyanins from closely related species absorb at nearly the same frequencies. Since the frequency of the absorption maximum is a function of the electronic configuration of the absorbing species and of its surroundings (Alben and Caughey, 1968; Bouquet *et al.*, 1968), the electronic environment of bound CO in the hemocyanins of two mollusks, squid and limpet, must be quite different from that of *Limulus* hemocyanin and differ even more from that in the hemocyanins of two crustaceans, crab and crayfish. Spectra of these hemocyanin carbonyls were all measured as aqueous solutions in Tris buffer (pH 7.0) at about 25°, with similar results obtained from a dispersive spectrometer with *Limulus* hemocyanin and with the previously reported (Alben *et al.*, 1970) spectrum of limpet hemocyanin dissolved in distilled water. In addition, hemocyanin–CO spectra of all species except crayfish (for which insufficient material was available) were replicated by the $^{12}C^{16}O$ which was present in the isotopically enriched gases. Thus, the differences in ν_{CO} appear to be real and associated only with the species of animal which produced the hemocyanin. The major differences in electronic environments of the hemocyanin-bound CO is measured by differences in ν_{CO} between mollusks and *Limulus* (9 cm^{-1}) and between *Limulus* and the crustaceans (an additional 10 cm^{-1}). These differences are similar to those found by Alben and N. J. Farrier (unpublished observations) during the titration of copper carbonyl in benzene solution with pyridine. Absorption bands were observed at 2092 and 2082 cm^{-1} with less than 0.8% pyridine (by volume); the major absorption was at 2082 cm^{-1} with shoulders on either side at 1.6% pyridine; and a band at 2069 cm^{-1} predominated at 10 or 100% pyridine. While these pyridinocopper carbonyls have not been isolated, nor have their structures been established, they illustrate that a change in concentration of one ligand leads to differences in frequency of the carbonyl absorption (ν_{CO}) which are similar to species differences which we observe with hemocyanins. It therefore appears reasonable to suggest that differences between mollusks, *Limulus*, and the crustaceans may be explained by different amino acid groups being coordinated to the Cu–(CO) binding site. We cannot yet speculate as to differences in groups that may be in van der Waals contact with the CO binding site, except to suggest that ν_{CO} frequency differences between the squid and limpet, or between the crab and crayfish, may be explained in this way. The values of ν_{CO} observed for all species are in the region where nonbridging copper carbonyls absorb, and are much higher than would be expected for a bridging carbonyl.

The CO complexes of hemocyanins absorb at much higher frequencies (by about 100 cm^{-1}) than those of myoglobin and hemoglobin. Therefore, the C–O bond strength in hemocyanin carbonyl is much stronger and the metal–(CO) bond much weaker than those in hemoglobin and myoglobin carbonyls. This is consistent with the observed low affinity of hemocyanin for CO, and relatively high affinity for oxygen. In spite of this difference in CO and oxygen binding between the copper(I)–hemocyanins and the Fe(II)–hemoprotein oxygen carriers, the isotope shift data show striking similarities.

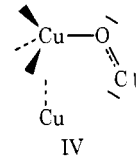
TABLE III: Isotopic Shifts in the CO Stretching Frequency (ν^*/ν) Found for Hemocyanin Carbon Monoxide Complexes.

	$\nu_{^{13}C^{16}O}$	$\nu_{^{12}C^{18}O}$	$\frac{\nu_{^{13}C^{16}O}}{\nu_{^{12}C^{16}O}}$	$10^4\Delta$	$\frac{\nu_{^{12}C^{18}O}}{\nu_{^{12}C^{16}O}}$	$10^4\Delta$
CO (gas)[a]	2096.071	2092.12	0.9777		0.9759	
Squid	2017.5	2016.5	0.9777	0[b]	0.9772	+13[b]
Limpet	2015.5	2015.4	0.9775	−2	0.9775	+16
Limulus	2007.8	2007.7	0.9773	−4	0.9773	+14
Crab	1997.5	1996.5	0.9775	−2	0.9775	+16

[a] Mills and Thompson (1953), and Plyler *et al.* (1955). [b] Difference in ν^*/ν from CO gas.

The isotope shifts ($\nu_{^{13}CO}/\nu_{^{12}CO}$ and $\nu_{C^{18}O}/\nu_{C^{16}O}$) observed for carbon monoxide gas are very nearly those expected where the effects are due only to the mass of the atoms in the diatomic molecule, *i.e.*, they are essentially mass-only shifts. In the case of carbon monoxide complexes of the hemocyanins and hemoglobins, the carbon shows essentially the mass-only shift, while an increase in mass of the oxygen ($^{16}O \rightarrow {}^{18}O$) affects ν_{CO} less than predicted by the change in mass. This is to be expected for atoms in vibrationally coupled systems. The simplest system for strong coupling is obtained for the atom which is bonded directly to the metal. Other possibilities such as H bonds would probably provide much less vibrational coupling than the combined σ and π coordinate covalent bonds found in metal carbonyls, so that regardless of whether CO in hemocyanin might be H bonded in a like manner to that provided by the distal (E-7) histidine of hemoglobin, we suggest that only oxygen bonded to the metal appears to satisfy the isotope shift data. The carbon in bound carbon monoxide has an isotope mass effect which is the same as in the free gas, so we suggest that when bound to hemocyanin or hemoglobin, the carbon is less constrained than is the oxygen. The observed differences in isotope shift for C and O rule out structures such as structure III or ones analogous to the Griffith (1956) model for oxygen bound to hemoglobin, where the C–O axis would be perpendicular to a radius from the metal atom. Such a symmetrically bonded C and O could not produce the observed differences in isotope shift.

We may now complete our model which appears to be in accord with observed data and may be tested by future studies. It should include carbon monoxide bound through oxygen to one of the two copper atoms at the binding site, and be fairly well protected from water by surrounding nonpolar protein groups. Other groups coordinated to the same copper atom as carbon monoxide appear to be different for mollusks, *Limulus*, or crustaceans, but nothing may be said about the second copper atom at the binding site. We suggest that structure IV may correctly represent the structure of the hemocyanin carbonyl complex, with the Cu–O–C angle near 120°, similar to that observed by Huber *et al.* (1970), for erythrocruorin by X-ray difference Fourier synthesis.



IV

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States of Hemoglobin in Solution†

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ABSTRACT: The equilibrium concentrations of partially oxygenated molecules of hemoglobin A, hemoglobin Chesapeake, and hemoglobin Kempsey can be calculated from the parameters of a two-state generalized concerted transition model, where the parameters are determined from a study of the binding of spin label triphosphates to partially liganded hemoglobin solutions. In this model it is essential to use different parameters for the α- and β-heme groups.

Since the discovery by Muirhead and Perutz (1963) that oxyhemoglobin and ligand-free hemoglobin have significantly different molecular structures, there have been many efforts to understand the role played by these two structures in cooperative oxygen binding. In 1965, Monod, Wyman, and Changeux introduced the allosteric transition model to account for cooperativity in hemoglobin and other oligomeric proteins. In this model an oxygen-induced transition between the ligand-free structure (T) and the oxy structure (R) is essential for the cooperative mechanism in hemoglobin, since the hemes in the T and R structures are assumed to have low and high oxygen affinities, respectively. Perutz (1970) has recently given a penetrating discussion of the extent to which the crystallographic studies of hemoglobin are consistent with the allosteric model. This discussion has been amplified in a later article by Perutz and TenEyck (1971). There have also been many attempts to test the validity of the MWC[1] model

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[1] Abbreviations used are: MWC model, Monod–Wyman–Changeux allosteric transition model; GCT, generalized concerted transition model; HbA, hemoglobin A; DPG, 2,3-diphosphoglycerate; ATP, adenosine 5′-triphosphate; IHP, inositol hexaphosphate.