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Magnetic susceptibility of oxy- and carbonmonoxyhemoglobins

(diamagnetism/hemoglobin)

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ABSTRACT **The room temperature magnetic susceptibilities of human and carp oxy- and carbonmonoxyhemoglobin solutions were measured in a 30-gauss (1 G = 10^{-4} T) field with a superconducting magnetometer. To within experimental uncertainty, the susceptibility was the same for both the oxy and carbonmonoxy forms, and salt concentration did not affect it. A variety of sample preparations were used; the iron chemical state was verified by Mössbauer spectroscopy. A value of $-0.580 \pm 0.010 \times 10^{-6}$ centimeter-gram-second (cgs) system was obtained for the mass susceptibility of the protein. We attribute the paramagnetism sometimes observed in oxyhemoglobin solutions to the presence of a small amount of the deoxy form.**

A controversy has recently grown over the magnetic susceptibility of oxy- and carbonmonoxyhemoglobin under various solution conditions. The data of Pauling and Coryell (1), Taylor and Coryell (2), and Havemann *et al.* (3) indicate that both forms of the protein are totally diamagnetic at room temperature. Recent work (4, 5) shows spin unpairing in oxyhemoglobin with a singlet–triplet separation of 144 cm^{-1}. Further evidence for an excited state was the observation of temperature-dependent quadrupole splittings and linewidths in the Mössbauer spectra of oxyhemoglobins (6). The diamagnetism of carbonmonoxyhemoglobin came under question with reports of salt-dependent effects (7). Most recently, one researcher has again found oxyhemoglobin to be diamagnetic (8) but in solution conditions vastly different from those in which paramagnetism was seen.

The resolution of this conflict is imperative for the development of valid electronic models of reversible oxygen binding to hemes. Therefore, we have constructed a superconducting magnetometer to measure the magnetic susceptibilities of oxy- and carbonmonoxyhemoglobin at room temperature and have attempted to reproduce the conditions of the work which showed paramagnetism (4, 5, 7) as closely as possible.

MATERIALS AND METHODS

A superconducting magnetometer built in our laboratory was used to make the measurements. A SQUID (Superconducting QUantum Interference Device), connected by superconducting wires, was used to detect magnetic flux changes in one of two five-turn, 1.27-cm diameter pickup coils, which were wound in opposition and had an axial separation of 5 cm. Typically, a 2.5-cm^3 liquid sample filled a 4.2-mm-i.d. high-purity quartz tube (Wilmad, Buena, NJ, no. 703 PQ) to a height of 14 cm. This was lowered into the upper of the two coils until a maximum response of the SQUID was observed and recorded. The sample was then withdrawn, and the SQUID output was read again. The difference, $\Delta\phi$, between these two signals was determined for sample, salt/buffer solution, distilled water, and empty tube. All solutions were equilibrated with air. The samples were always long with respect to the pickup coil radii. With a 1-Hz filter on the SQUID output, the system noise, as measured by the fluctuations in the measured susceptibility of a water-filled tube, was less than 0.1% of the susceptibility of water. This is consistent with a calculation of the Johnson noise produced by the copper sample chamber and radiation shield tubes and by the aluminum pickup coil form. We used an applied field of 30 gauss (1 G = 10^{-4} T) for all measurements, and the temperature was maintained at 20.0 ± 0.2°C.

A variety of sample preparations were used. Most of the hemolysate samples were prepared by drawing blood over EDTA and washing four times in a 0.9% NaCl solution. The cells were lysed by sonication and spun to remove the membranes. In two cases, the susceptibility was measured directly after addition of KCN to 15% of the heme concentration (see samples 1 and 4 in Fig. 1). These samples proved to be more paramagnetic than water. A Mössbauer spectrum of one of these, frozen to 77 K immediately after the susceptibility measurement, indicated about 9% deoxyhemoglobin, a spin 2 compound. After this discovery, Mössbauer spectra were taken immediately after all susceptibility measurements. Mössbauer measurements, although less sensitive than optical spectroscopy, avoid dilution and the consequent risk of changing the oxy/deoxy equilibrium.

We found that it was difficult to prepare samples free of deoxyhemoglobin by exposing concentrated solutions (15 mM in heme) to air. If a concentrated, mostly oxygenated sample were exposed to CO, all deoxyhemoglobin would be replaced by carbonmonoxyhemoglobin, but a significant fraction of the protein remained in the oxygenated form. By diluting a sample to about 5 mM in heme, we could reliably prepare pure oxy or carbonmonoxy proteins. More concentrated solutions were then obtained through the use of a Millipore Immersible CX-30 ultrafilter. Mössbauer spectroscopy proved these samples to have a deoxy fraction of less than 3%, our sensitivity limit.

After much data had been taken, we were informed that spinning very concentrated solutions of hemolysate would not effectively remove all cell membranes. To see if this had any effect on our measurements, we prepared several samples by lysing with distilled water and spinning the diluted hemolysate. The toluene preparation of Antonini and Brunori (9) also was employed. Further tests were done by drawing blood over sodium citrate to see if the anticoagulant had any effect. Hemoglobin preparations stripped of 2,3-diphosphoglycerate were measured to verify that nothing was left unaccounted for in the solutions.

Two of the human stripped samples (samples 9 and 11 in Fig. 1) were prepared by washing the cells four times with a 0.9% NaCl solution. They were hemolyzed by adding 1.5 volumes of distilled water and 0.5 volume of toluene and then shaking for 5 min. A clear hemoglobin solution was ob-

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Abbreviation: cgs, centimeter-gram-second system.

tained by centrifuging this mixture (10,000 rpm for 20 min) after adjusting the sodium chloride concentration to 0.9%. Hemoglobin was stripped of organic phosphate by passage through a Dintzis column (10). Hemoglobin solution was concentrated first by use of a Millipore CX-30 and then by an Amicon Centriflo CF25 ultrafilter.

In all cases, the samples were adjusted to have KCN at 15% of the heme concentration. All concentrations were measured spectrophotometrically by using the extinction coefficients of Antonini and Brunori (9).

In order to facilitate a comparison of measurements made under a variety of solution conditions, the salt/buffer contribution was in each case subtracted before plotting the solution volume susceptibility as a function of heme concentration. Different preparation techniques dictated that data be analyzed in different ways. In the case of stripped samples, a volume of hemoglobin in distilled water and an equal volume of water were measured out. Equal volumes of buffer were added to each, producing one sample and one standard. When all solutions are measured in the same quartz tube, the volume susceptibility of a solution of hemoglobin in distilled water is given by

$$\chi_{cc} = \chi_{water}\left[1 + \frac{\Delta\phi(\text{sample}) - \Delta\phi(\text{buffer})}{\Delta\phi(\text{water}) - \Delta\phi(\text{empty tube})}\right]. \quad [1]$$

In the case of the hemolysate samples, hemoglobin-free buffer specimens were sometimes obtained by ultrafiltration of the hemoglobin sample. The volume susceptibility was in each case indistinguishable from that of the solution used to wash the cells and dilute the hemolysate. Its use as the buffer value in Eq. 1 for the sample susceptibility introduces an error due to the volume fraction actually occupied by hemoglobin. For example, this would be about 25% at 20 mM heme concentration. The required correction is made by adding

$$\Delta\chi_{cc} = \nu_{hb}\,(\chi_{sol} - \chi_{water}) \quad [2]$$

where ν_{hb} is the volume fraction of hemoglobin in the sample and χ_{sol} is the susceptibility of the solution used as a buffer. Although χ_{cc} is smaller than the experimental uncertainty,

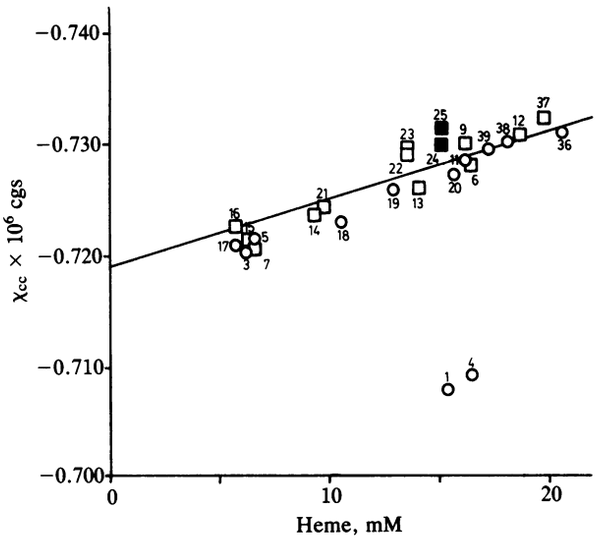

FIG. 1. Buffer-corrected volume susceptibility of hemoglobin solutions, χ_{cc}, as a function of heme concentration. ○, Human oxyhemoglobin; □, human carbonmonoxyhemoglobin; ■, carp carbonmonoxyhemoglobin.

Table 1. Sample preparation and composition

Sample	Mössbauer results, %			Lysing method	Toluene used	Buffer/salt	Heme, mM	Remarks
	HbO_2	HbCO	Hb					
1	ND	ND	ND	Sonication	No	0.9% NaCl	15.5	
3	95	4	1	Water	No	0.32% NaCl	6.25	
4	86	4	10	Sonication	No	0.9% NaCl	16.5	
5	94	6	0	Sonication	No	0.31% NaCl	6.6	
6	55	45	0	Sonication	No	0.9% NaCl	16.5	
7	25	75	0	Sonication	No	0.31% NaCl	6.6	
9	96	4	0	Water	Yes	0.9% NaCl/Tris Cl	16.4	pH 7 stripped
11	35	63	2	Water	Yes	0.9% NaCl/Tris Cl	16.4	pH 7 stripped
12	25	75	0	Sonication	No	0.9% NaCl	18.8	
13	17	83	0	Sonication	No	0.9% NaCl	14.1	
14	12	88	0	Sonication	No	0.9% NaCl	9.4	
15	7	90	3	Sonication	No	0.9% NaCl	6.3	
16	7	90	3	Sonication	No	2.8% NaCl	5.8	
17	92	7	1	Sonication	No	0.9% NaCl	5.8	
18	94	6	0	Sonication	No	0.9% NaCl	10.6	
19	96	3	1	Sonication	No	0.9% NaCl	13.0	
20	98	2	0	Sonication	No	0.9% NaCl	15.8	
21	24	76	0	Sonication	No	2.9% NaCl	9.7	
22	3	96	1	Water	No	Phosphate	13.6	pH 7.2 stripped
23	3	96	1	Water	No	Phosphate/2.3% NaCl	13.6	pH 7.2 stripped
24	1	98	1	Water	No	0.05 M Cl/Bis Tris	15.2	Carp pH 5.6 stripped
25	1	98	1	Water	No	0.05 M Cl/Bis Tris/2.3% NaCl	15.2	Carp pH 5.6 stripped
36	98	2	0	Water	Yes	0.9% NaCl	20.6	
37	4	96	0	Water	Yes	0.9% NaCl	19.9	
38	96	4	0	Water	Yes	0.9% NaCl	18.2	Na citrate
39	97	3	0	Water	Yes	0.9% NaCl	17.4	Na citrate

Some of the indicated carbonmonoxyhemoglobin may be due to hemochromogen, which has a similar Mössbauer spectrum. ND, not done.

we have in fact made this correction in all relevant cases. We used a value of -0.719×10^{-6} centimeter-gram-second (cgs) system as the volume susceptibility of water and a hemoglobin density of 1.335 g/cm^3. In all cases, χ_{cc} should extrapolate to χ_{water} in the limit of zero hemoglobin concentration.

RESULTS AND DISCUSSION

In Fig. 1 we have plotted the buffer-corrected volume susceptibilities of our solutions as a function of heme concentration. Solution conditions are given in Table 1. Samples 1 and 4 were measured early in the series. We attribute their paramagnetism to the presence of deoxyhemoglobin. Its presence in sample 4 was verified by Mössbauer spectroscopy, but sample 1 was subjected to this technique only after a considerable delay. Apart from the anomalies of samples 1 and 4, all data points lie within about 2 times the system noise of the straight line drawn from the susceptibility of air-equilibrated water at zero heme concentration. From this we find the mass susceptibility of hemoglobin to be -0.577×10^{-6} cgs. This is based on the values of 1.335 g/cc for the density of hemoglobin and 64,500 for the molecular weight of a hemoglobin tetrameter. An important source of uncertainty is the possibility of up to 3% of the hemes being in the deoxy form (the sensitivity limit of our Mössbauer measurements). Our value could be at most 0.005×10^{-6} cgs too paramagnetic from this source. The scatter between our points also indicates an error of $\pm 0.008 \times 10^{-6}$ cgs. The possible effect of methemoglobin in the presence of cyanide is dwarfed by comparison. Recognizing the possibility of the above systematic errors, we estimate our final hemoglobin mass susceptibility value to be $-0.580 \pm 0.010 \times 10^{-6}$ cgs.

The most important result that we report is our confirmation of a diamagnetism in oxyhemoglobin under conditions in which paramagnetism has been reported (5) and our identification of the deoxy component as the likely source of error. M. Cerdonio (personal communication) believes that this could account for his apparent room-temperature paramagnetic result and is separately publishing more recent results that confirm our findings. We think it likely that deoxyhemoglobin also is involved in the apparent paramagnetism detected at lower temperature (4).

A second result is that we do not confirm the reported salt effect on the susceptibility of carp carbonmonoxyhemoglobin (7). This has led to a reexamination of the problem and the conclusion that the reported result was in error (M. Cerdonio and R. Noble, personal communication).

Finally, a number of variations in the preparation and solution conditions were found to have no observable effect on the susceptibility measurement. Specimens drawn over EDTA and sodium citrate gave similar results. Stripped hemoglobin, hemolysate, and hemolysate prepared with the toluene method showed no evidence for any membrane effects.

In summary, it appears that there is no good evidence for a low-lying paramagnetic excited state in either oxyhemoglobin or carbonmonoxyhemoglobin. The excitation which the Mössbauer spectra of the former imply must be such as to preserve spin pairing in the electronic system—a vibrational or conformational excitation, for example.

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