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Diamagnetism of Human Apo-, Oxy-, and (Carbonmonoxy)hemoglobin†

John S. Philo,* Ulrich Dreyer, and Todd M. Schuster

ABSTRACT: In recent years, a controversy has arisen over the magnetic properties of oxyhemoglobin (HbO_2) and (carbonmonoxy)hemoglobin (HbCO). At present, it is unclear which, if any, conditions give a completely diamagnetic state for the heme–ligand complex which can be used as a diamagnetic reference state. In order to establish a diamagnetic reference independent of assignments of electronic configurations, we have measured the magnetic susceptibilities of apohemoglobin solutions and powdered iron-free protoporphyrin IX. We have also reexamined the magnetism of HbO_2 and HbCO solutions at 20 °C and at several ionic strengths. We find no difference in magnetism between HbO_2 and HbCO and no changes in their magnetism with solution conditions. Furthermore, relative to the new (apohemoglobin + porphyrin) diamagnetic reference, our data are consistent with complete diamagnetism for both HbO_2 and HbCO under all conditions we have studied. Our data imply that any low-lying triplet state must lie at least 900 cm^{-1} above the diamagnetic ground states. These results disagree strongly with reports of substantial room temperature paramagnetism for HbO_2 and a smaller paramagnetism for HbCO which disappears at high ionic strength [see Cerdonio, M., Morante, S., Vitale, S., Giacometti, G., & Brunori, M. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Ed.) pp 63–68, Elsevier/North-Holland, Amsterdam, and references cited therein].

In recent years, there has been considerable interest in the magnetic properties of oxyhemoglobin (HbO_2) and (carbonmonoxy)hemoglobin (HbCO). For many years, both had been regarded as "diamagnetic", i.e., as having no unpaired electron spins either on the heme iron or on its ligand. However, in 1977, Cerdonio et al. reported evidence for unpaired spins (paramagnetism) in frozen human HbO_2 solutions at temperatures above 50 K. Since then, they have also reported paramagnetism at room temperature for human HbO_2 in solution, and for human and carp HbCO under certain solution conditions (Cerdonio et al., 1978, 1980, 1982, 1983). These results conflict with the early assignments of HbO_2 and HbCO as completely diamagnetic, spin = 0, compounds by Linus Pauling and co-workers (Pauling & Coryell, 1936) as well with later studies by Havemann et al. (1961).

The recent reports of paramagnetism in HbO_2 and HbCO raise an important question for both past and future studies of the magnetism of heme proteins in solution: What can be used as a "diamagnetic reference" state? In studies of the magnetism of metalloprotein solutions near room temperature, the diamagnetism of the solvent and polypeptide is generally much larger than any paramagnetism of the metallic ions. Thus, when we speak of a metalloprotein being "paramagnetic" what we mean is that the protein solution is found to be less diamagnetic than some diamagnetic reference state. This diamagnetic reference is usually a solution of the protein in a form known to have no unpaired spins. For hemoglobin, this diamagnetic reference has traditionally been a HbO_2 or HbCO solution. Clearly, if either HbO_2 or HbCO has nonzero paramagnetism (or magnetism which varies with solution conditions), then earlier studies which used them as a diamagnetic reference may be in error, and future studies require

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establishing a state with zero paramagnetism (or failing that, at least one whose paramagnetism is accurately known). Cerdonio et al. have suggested that human HbCO is fully diamagnetic in the presence of 450 mM NaCl and have used this as their diamagnetic reference in recent work. This assignment, however, is based only on the fact that this is the most diamagnetic state they have thus far observed.

We recently completed construction of a high-sensitivity magnetic susceptibility instrument for equilibrium and kinetic studies of metalloproteins. This instrument is an order of magnitude more precise than others now operational. For our future work, we felt it essential to establish an accurate diamagnetic reference state for Hb which is independent of any assumptions about which, if any, conditions give zero paramagnetism for the heme–ligand complex. Moreover, because of the great interest in heme electronic configurations and their relation to cooperative ligand binding, we felt that it was important to confirm whether HbO_2 or HbCO does indeed show paramagnetism relative to such a reference state. We find that they both do not.

Materials and Methods

Magnetic susceptibility measurements were made with a superconducting magnetic susceptometer. This instrument is similar to that built previously by Philo & Fairbank (1977), except that nonsuperconducting metals have been removed from the vicinity of the sensing coils to reduce the instrument response time to below 3 μs. This instrument will be described in detail elsewhere. Volume susceptibility measurements were made as described previously (Philo & Fairbank, 1977). Each quartz sample tube is individually calibrated by using air-saturated double-distilled water at 20 °C, to which we assign a susceptibility of -0.71779×10^{-6} emu/cm^3 on the basis of the standard value of -0.7200×10^{-6} emu/g for water at 20 °C, corrected for the paramagnetism of the dissolved oxygen by using its known solubility (Kruis, 1976). With this instrument, the typical precision for repeated insertions of the same sample of water is ±0.001% root mean square, and three different water samples in the same tube gave the same value within ±0.001%. This level of precision is an order of magnitude greater than that of similar laboratory-constructed (Vitale et al., 1982) or commercial (S.H.E. Corp.) susceptometers, although it is not quite as good as the former Philo & Fairbank (1977) instrument due to compromises necessary to improve the kinetic performance. The precision for *protein* solutions is generally worse, due to problems with sample denaturation and purity, but is still typically ±0.01% or better. All susceptibility measurements were made with an applied field of 2345 G and at a sample temperature of 20.0 ± 0.1 °C.

Measurements of the mass susceptibility of protoporphyrin IX were made by placing a small (<40 mg) sample in the bottom of a quartz tube such that the entire sample lies in a region over which the field sensitivity of the superconducting sensing coil varies by less than 1%. For such small samples, the signals are nearly exactly proportional to the sample mass. Calibration of this "mass susceptibility mode" was done by using the known susceptibilities of ferrous ammonium sulfate (Selwood, 1956) and of sucrose (Weast, 1968). We estimate the overall uncertainty in the porphyrin data to be ±2%.

Human hemoglobin was prepared by two different methods in order to assess any differences in the magnetic data due to preparation methods. Hemoglobin A_0 was prepared by the method of Williams & Tsay (1973), except that concentration was done with an Amicon pressure concentrator rather than with a second column. Hemoglobin A, unfractionated with respect to minor components, was prepared by the ammonium sulfate precipitation method (Rossi-Fanelli et al., 1961) followed by removal of the sulfate on a Sephadex G-25 column. Both procedures typically give a residual methemoglobin (MetHb) content of 1–3%. In order to remove this MetHb, in some cases the samples were treated with dithionite as follows. Oxyhemoglobin solutions were first deoxygenated by flushing with N_2. An amount of solid sodium dithionite sufficient to reduce 5 times more MetHb than we estimated to be present was then added, and the sample was immediately placed on a Sephadex G-25 column to remove the excess dithionite and reaction products and allowed to reoxygenate. A similar procedure was used in some cases for removing MetHb from HbCO samples, except that the N_2 flushing was omitted and the column was preequilibrated with CO-saturated buffer. Apohemoglobin (globin) was prepared by the methyl ethyl ketone method (Ascoli et al., 1981) followed by exhaustive dialysis against distilled water and further concentration with an Amicon concentrator. Optical absorption measurements in the Soret band indicated a residual low-spin ferric heme content of 0.5%–0.8%.

Absorption spectra were taken with a computer-controlled Cary 118C spectrophotometer (Philo et al., 1981). Hemoglobin concentrations were determined by using extinction coefficients of 14.3 and 14.3 mM^{-1} cm^{-1} for the 541- and 538-nm peaks of HbO_2 and HbCO, respectively, at 20 °C. These values were determined in our laboratory relative to the standard value of 11.0 mM^{-1} cm^{-1} for MetHbCN at 540 nm (Tentori & Salvati, 1981). Globin concentrations were determined by using an extinction coefficient of 12.7 mM^{-1} cm^{-1} at 280 nm (Ascoli et al., 1981). Concentrations are expressed on a heme or subunit basis. Density measurements of globin, HbO_2, and HbCO solutions at 20 °C were made with a Mettler-Paar vibrating-sample densitometer to determine hydrated volumes.

Metal-free protoporphyrin IX disodium salt was obtained from Sigma (lot 42F-0719) with lot analysis showing less than 0.01% residual iron. Sodium dithionite was obtained from Virginia Smelting Corp., and ultrapure Tris was from Schwarz/Mann; other chemicals were reagent grade.

Results

Establishing a Diamagnetic Reference State. Our approach to establishing a diamagnetic reference independent of assignments of iron electronic states is to make separate measurements of the diamagnetism of all Hb components *except* the heme iron. We have therefore measured the susceptibilities of solutions of apohemoglobin (globin) and of powdered iron-free protoporphyrin IX.

The results of the data for globin solutions in distilled water are shown in Figure 1. A plot of volume susceptibility vs. protein concentration should give a straight line with an intercept at the susceptibility of the solvent and a slope related to the difference in volume susceptibility between the solvent and protein.[1] The exact relationship between the volume susceptibility of the solution, κ(soln), the molar susceptibility

[1] The apparent protein susceptibility from such an analysis may include effects, if any, of the protein on the diamagnetism of the solvent. The diamagnetism of the water molecules bound to or near the surface of the protein may differ by a few percent from the bulk value. This is one reason why it is important to measure globin solutions rather than powder to obtain a reference state appropriate for Hb in solution. The main assumption of such an analysis if that any effects of the protein on the solvent are linear in protein concentration. This assumption may not be valid at very high protein concentrations.

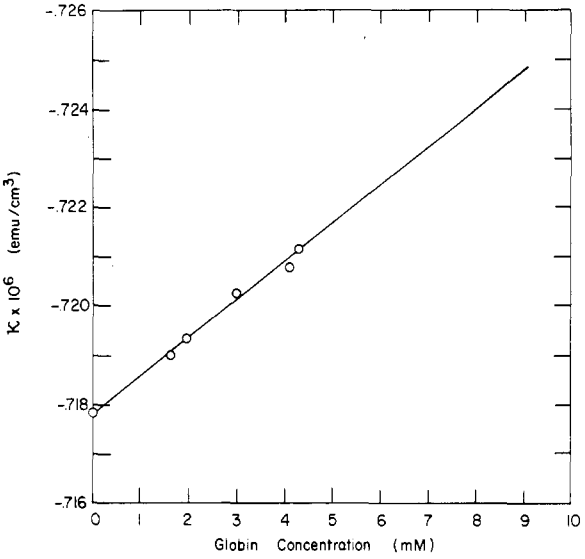

FIGURE 1: Volume susceptibility of globin in distilled water at 20 °C vs. globin concentration. The line is a least-squares fit with a fixed intercept at the value for water.

of the protein, χ_m, and the volume susceptibility of the solvent, κ(buffer), is

$$\kappa(\text{soln}) = \kappa(\text{buffer}) + c[\chi_m - \kappa(\text{buffer})V_m] \times 10^{-3} \quad (1)$$

where c is the molar concentration of the protein and V_m is the molar volume of the hydrated protein (cubic centimeters per mole).

The line shown in Figure 1 is a least-squares fit with a fixed intercept at the value for water. From the slope of this line, the known average subunit molecular weight (15 496.5), and our determination of the hydrated density (1.3317 ± 0.006 g/cm^3), eq 1 gives a molar susceptibility for globin of χ_m = (−9133 ± 17) × 10^{-6} emu/mol. To this value a correction of −11 × 10^{-6} emu/mol must be added to account for the residual heme content of the globin preparation. This gives a molar susceptibility of (−9144 ± 17) × 10^{-6} emu/mol and a gram susceptibility of (−0.5901 ± 0.0011) × 10^{-6} emu/g.

Our data for the mass susceptibility of iron-free protoporphyrin IX disodium salt (three samples) give an average value χ_g = (−0.683 ± 0.017) × 10^{-6} emu/g, and therefore, χ_m = (−414 ± 8) × 10^{-6} emu/mol. Our data are in excellent agreement with those of Rawlinson & Scutt (1951). The molar susceptibilities agree within 0.5% allowing for the sodium in our sample. Our data also agree reasonably well with those of Chow & Cohen (1971) for tetraphenylporphyrin. We find significantly less diamagnetism, however, than that found for several porphyrins by Eaton & Eaton (1980). We note that these latter data show considerable variation for different aliquots of the same sample and involved substantial corrections for ferromagnetic impurities; we observed less than ±1% variation between different samples in our data. To apply these data to the heme in hemoglobin, we must subtract the contribution of the sodium ions and the two hydrogens in the central hole, which we estimate as −24 × 10^{-6} emu/mol by using Pascal's constants (O'Connor, 1982). Further to obtain a reference state relevant for HbO_2 or HbCO, we must add the underlying diamagnetism of the iron–ligand complex, which will be present even for spin-unpaired states. Studies of ionic crystals suggest an underlying diamagnetism for the core electrons of a ferrous iron of −13 × 10^{-6} emu/mol, and we estimated an additional −8 × 10^{-6} emu/mol for the ligand from Pascal's constants (O'Connor, 1982). This gives a value for a diamagnetic oxy or CO heme of χ_m = (−411 ± 11) × 10^{-6} emu/mol. Note that we are implicity assuming that the addition of the iron and its axial ligands to the porphyrin does not change the diamagnetism due to the ring-current effects of the delocalized porphyrin π electrons. We believe this assumption is justified since NMR results show that incorporation of metals into porphyrins produces only small changes in the ring currents (Scheer & Katz, 1975). Furthermore, the total ring-current contribution is only ∼−40 × 10^{-6} emu/mol (Rawlinson & Scutt, 1951), so even a 25% change would not be significant.

Combining the data for globin and heme gives the value to be expected for the molar susceptibility of completely diamagnetic hemoglobin: χ_m = (−9555 ± 20) × 10^{-6} emu/mol, or a gram susceptibility of (−0.592 ± 0.001) × 10^{-6} emu/g. Note that in making this calculation we are assuming that the diamagnetism of the porphyrin is unchanged in going from the porphyrin crystal environment to the globin binding site. This is justified since molar diamagnetism is only weakly dependent on structure and environment— for example, a solid to liquid phase change generally changes the diamagnetism of a molecule by 1% or less. We are also assuming that incorporation of the porphyrin into the globin does not change the globin diamagnetism. While this will produce some change in protein conformation, even the complete thermal denaturation of proteins produced no detectable change in diamagnetism in several studies explicitly designed to look for such changes (Day, 1974).

Susceptibility of HbO_2 and HbCO Solutions. We have also measured the volume susceptibility of solutions of HbO_2 and HbCO under several conditions, including some chosen to be as similar as possible to those used by Cerdonio et al. (1978, 1982). A major problem in such studies is contamination of the samples with traces of MetHb, whose large paramagnetism can significantly alter the data. The sensitivity of our susceptometer is such that the autoxidation of 0.05% of a 5 mM HbO_2 sample is a significant change. Thus, amounts of MetHb which would be negligible in many experiments are a significant concern here. To eliminate this problem, one must either prepare samples which are entirely free of MetHb or accurately account for the paramagnetism of the MetHb which is present.

We have approached the MetHb contamination problem in several ways. We have attempted to prepare samples entirely free of MetHb by reduction with dithionite. For HbO_2, dithionite was added to a deoxy-Hb sample, followed by removal of dithionite and reaction products and reoxygenation on a column. For HbCO, we used both the column technique and direct addition of dithionite to the susceptibility sample. A second approach is to measure the MetHb content and to correct for its paramagnetism. We have done this by two methods. One is to fit the visible absorption spectra of the samples to a sum of MetHb and "pure" HbO_2 or HbCO spectra (i.e., the spectra of the material treated with dithionite). Clearly this procedure gives MetHb concentrations which are relative to the reference material rather than absolute MetHb content. The spectral fitting is itself subject to error, but we believe we can estimate the MetHb content to within ± 1% of the total heme or less.

A second way to determine the MetHb content is to add KCN to the sample to (presumably) change all the ferric heme from the predominantly high-spin aquo to the low-spin cyanomet form. Thus, the susceptibility change induced by KCN addition is a measure of the MetHb content, and with our measured susceptibilities for aquo-MetHb and CN-MetHb, the data can be corrected.

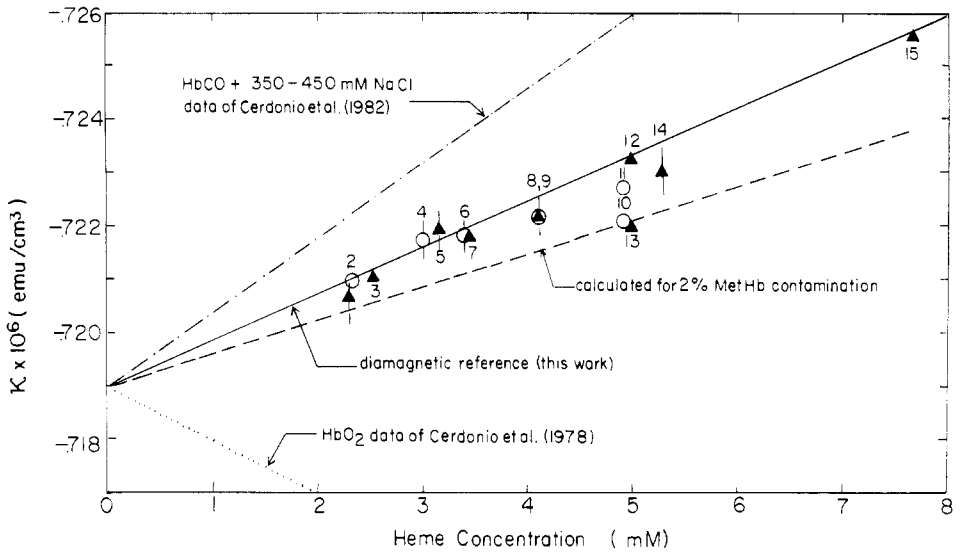

FIGURE 2: Volume susceptibility of human HbO_2 and HbCO solutions at 20 °C vs. heme concentration. Circles represent HbO_2, and solid triangles represent HbCO. Points numbered 2, 3, 7, 10, and 13 are for solutions treated with dithionite to remove MetHb. Points 11, 12, and 15 were corrected for MetHb contamination by addition of KCN (see text). Points with error bars (1, 4, 5, 6, 8, 9, and 14) have been corrected for MetHb content by spectral fitting. Point 14 is for HbCO in the presence of 450 mM NaCl. The solid line (—) represents the diamagnetic reference from the globin and porphyrin data. The intercept is the susceptibility of 50 mM Tris-HCl–0.1 mM EDTA, pH 7.5, buffer. To aid in comparisons, all data taken in different buffer systems have been adjusted for the difference in buffer susceptibility by using eq 1, so all data should share a common intercept. Also, for aid in comparison, the lines representing HbO_2 (···) and HbCO in the high-salt (–·–) data of Cerdonio et al. (1978, 1982) are shown. The dashed line (---) shows the data expected for a sample contaminated with 2% MetHb, relative to the diamagnetic reference line.

We have used all three of these approaches to eliminate errors from MetHb contamination. Figure 2 is a compilation of all the HbO_2 and HbCO data, encompassing three different buffer conditions, two entirely different methods of purifying the hemoglobin, and two separate preparations by each method. The solid line plotted in Figure 2 is not a fit to these data; it represents the diamagnetic reference determined above. That is, if HbO_2 and HbCO are completely diamagnetic, they will fall on this line; if the heme–ligand complex has nonzero paramagnetism, the data will fall below this line. The buffer conditions were either unbuffered in distilled water (pH ~7.3), unbuffered in 450 mM NaCl (HbCO only), or 50 mM tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)–0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5. For easier comparison of data taken in different buffers (which have different intercepts and slopes in such a plot), we have used eq 1 to adjust the raw data to common buffer conditions. Since our own determinations of the hydrated volume of HbCO and HbO_2 agree closely with those of Cerdonio et al. (1977), we will use their value for the molar volume, 48 315 cm^3/mol (tetramer), to facilitate comparing data. In no case was the MetHb content above 7%.

Several features of these data are noteworthy. First, we find no significant differences between HbO_2 and HbCO. This fact is also confirmed by experiments in which HbO_2 samples were converted to HbCO directly in the susceptometer sample tubes. Second, we find no significant changes with solution conditions or between Hb purification methods. Third, the agreement of the data using different methods of correcting for MetHb is quite good.

Despite our efforts to minimize the influence of MetHb contamination on these measurements, we still believe this problem is the major source of uncertainty in the data. Error bars are shown only for the points which have been corrected for MetHb content by spectral fitting and indicate our estimate of the errors in this procedure. For the data corrected by cyanide addition, the error bars should be about equal to the size of the data points in Figure 2, assuming that all MetHb present is readily is readily converted to the cyanide form and assuming that sufficient cyanide was added. For the dithionite-treated samples, it is always uncertain whether the treatment was completely successful, and indeed our data show that it is not. The data points numbered 10 and 13 in Figure 2 are for samples treated with dithionite, yet it was clear from cyanide addition and from the absorption spectra that these samples were not entirely free of MetHb. We have included these points as an indication of the range of possible errors from the MetHb removal procedures. We believe the incomplete removal of the MetHb was probably the result of trying to minimize the amount of dithionite added.

Our conclusion from these data is that, within experimental uncertainty, both HbO_2 and HbCO are essentially completely diamagnetic under all conditions we have studied. That is, we believe the deviations of the data from the diamagnetic reference line are not outside the range of errors from MetHb contamination. A fit of all the data, both HbO_2 and HbCO, excluding only the two points known to contain MetHb, gives a molar susceptibility of $(-9512 \pm 14) \times 10^{-6}$ emu/mol, i.e., a paramagnetism of $(43 \pm 24) \times 10^{-6}$ emu/mol relative to our diamagnetic reference. This is equivalent to the paramagnetism due to 0.36% MetHb. If this paramagnetism is due to a low-lying triplet state, this state would be 1100 cm^{-1} above a diamagnetic ground state. If we take a 3σ upper limit of 115×10^{-6} emu/mol for the paramagnetism, then any triplet state must lie at least 910 cm^{-1} above a diamagnetic ground state.

Discussion

Clearly our results and conclusions are at variance with those of Cerdonio et al. (1978, 1982). These differences are both qualitative and quantitative. To aid in comparison, we have shown a dotted line in Figure 2 corresponding to their HbO_2 data (Cerdonio et al., 1978), and the uppermost broke line corresponds to their HbCO data at high salt concentrations (which is their diamagnetic reference state) (Cerdonio et al., 1982).

The largest qualitative difference is for HbO_2. We invariably find HbO_2 solutions to be more diamagnetic than the buffers, whereas Cerdonio et al. (1978) report HbO_2 solutions to always be less diamagnetic than the buffers, with a molar paramagnetism of $(2460 \pm 600) \times 10^{-6}$ emu/mol. Since they have reported that the magnetic properties of human and carp HbCO vary with solution conditions, one question which arises is whether the differences in the HbO_2 data reflect differences in solution conditions or sample preparation. Cerdonio et al. (1978) saw no differences in the magnetism of HbO_2 with removal of organic phosphates or with different solution conditions. We also find no systematic differences in the magnetism of HbO_2 either in distilled water or in 50 mM Tris + 0.1 mM EDTA, pH 7.5, and no difference between highly purified Hb A_0 and hemoglobin unfractionated with respect to minor components (whose preparation is essentially the same as that used by Cerdonio et al.). We therefore doubt that the different results are due to minor differences in sample preparation or solution conditions.

With regard to the HbCO data, the important qualitative difference is that we fail to see changes in its magnetism with solution conditions. Cerdonio et al. (1982) report that addition of 300–450 mM NaCl to HbCO solutions increases their diamagnetism by $(667 \pm 54) \times 10^{-6}$ emu/mol, and they believe that it is only under these high salt conditions that HbCO achieves full diamagnetism. As shown in Figure 2, we do not see a significant difference between high salt and other solution conditions. We have also made a more direct test of the influence of NaCl on the magnetism by comparing the susceptibility of a sample of HbCO in distilled water before and after addition of a concentrated NaCl solution to give 450 mM NaCl. Such a comparison is less sensitive to absolute determination of MetHb content. This experiment showed that addition of NaCl gave a very slight increase in diamagnetism of $(-71 \pm 40) \times 10^{-6}$ emu/mol, i.e., only slightly greater than our estimate of the instrumental uncertainty. We also believe part of this small difference is due to the effect of NaCl on the paramagnetism of the ~3% MetHb in this sample. The increase in ionic strength will lower the pK' of the acid–alkaline transition in MetHb, which will lower its paramagnetism (George & Hanania, 1953). We estimate that this effect produces a change of -33×10^{-6} emu/mol, leaving a net change of $(-38 \pm 40) \times 10^{-6}$ emu/mol in the HbCO. Furthermore, this analysis assumes the diamagnetism and the hydrated volume of the globin are independent of salt addition, which may not be exactly true. We therefore conclude that our data are consistent with no change in the magnetism of the heme–CO complex upon addition of NaCl. With regard to the salt effects on HbCO, we also note that there seems to be an inconsistency between the two sets of data reported by Cerdonio et al. The 1978 data in zero or low salt conditions[2] appear to agree with the 1982 data under high salt conditions.

Quantitatively, the agreement between our HbCO data and those of Cerdonio et al. in the *absence* of NaCl is quite good. From their 1982 data, we derive a molar susceptibility for HbCO in low salt conditions of $(-9436 \pm 54) \times 10^{-6}$ emu/mol, a value quite close to our diamagnetic reference, especially considering that no corrections for MetHb were made in their data (the difference from our diamagnetic reference is equivalent to that from about 1% MetHb). That is, the same data which they interpret as showing a magnetic moment of 1.25 μ_B for HbCO under these conditions, we would interpret as consistent with complete diamagnetism for HbCO with a small MetHb contamination. This significant difference in interpretation is due, of course, to the differences in diamagnetic references.

Obviously, the major differences between our data and conclusions and those of Cerdonio et al. raise two key questions: (1) Do the differences reflect true multiple magnetic states for HbO_2 and HbCO, with subtle differences between samples producing different magnetic states in the two laboratories? (2) Are some of the measurements in error, and if so, why? Unfortunately, neither question can be answered with certainty. The different results *might* reflect real variability in magnetic states, but we believe this is unlikely. In some of our measurements, we attempted to reproduce closely the conditions used by Cerdonio et al. but obtained different results. Other than possible differences in the protein samples, the only real differences in measurement conditions are temperature (12 °C in their studies, 20 °C in ours), applied magnetic field (~30 vs. ~2300 G), and concentration (5–25 vs. 3–8 mM). We known of no reason to believe that either of the first two conditions should be significant. With regard to concentration, none of the data suggest any concentration dependence, nor do we expect any. However, we feel the most compelling argument against different magnetic states is our inability to produce any. We have seen no evidence for variability despite deliberate attempts to produce it by varying buffer conditions and hemoglobin preparation methods. Certainly, *if* HbO_2 or HbCO shows paramagnetism due to low-lying paramagnetic excited states, it is reasonable to expect variation in this paramagnetism between species and with conditions known to influence ligand binding. However, since we find no conclusive evidence for any paramagnetism, it is hardly surprising that we cannot vary the amount.

If the differences in data are due to errors, what could be the source? Here we are able to shed little light. The MetHb contamination problem could produce small differences but cannot account for the major discrepancies between the data. It is clear that the problem is not one of random errors due to insufficient instrument sensitivity; the differences are systematic and large compared to the precision of the instruments involved. Our discussions with Dr. Cerdonio have not revealed any potential sources of systematic errors in our respective instruments or measuring procedures which could explain the differences.

This leaves us with the most difficult question: Which (if any) of the data are correct? Is there any independent evidence which is relevant? First, let us examine the question of the diamagnetic reference. Clearly, if our diamagnetic reference is correct, the HbCO in the high salt data of Cerdonio et al. (1982) *must* be in error, for one cannot observe a larger diamagnetism than the reference. From their data, we compute their reference as -10079×10^{-6} emu/mol, i.e., 524×10^{-3} emu/mol more diamagnetic than ours. It seems most unlikely that an error of this size could come from our determination of the porphyrin component, since it would require increasing the porphyrin diamagnetism by more than a factor of 2. Therefore, the major question is what is the correct diamagnetism for the globin? One independent method of determining the diamagnetism of globin is to calculate its value based on the susceptibilities of its constituent amino acids. It has been known for many years that the diamagnetism of compounds is nearly additive and can generally be calculated to within ±1–2% with a set of empirical parameters

[2] There is some confusion about the solution conditions in these data. Some of the data points are clearly in ion-free conditions. The legend to Figure 1 in Cerdonio et al. (1978) states that the other samples are in "unbuffered water", but elsewhere in the paper the solvents are referred to as "saline solutions", and in later papers (Cerdonio et al., 1981), these same data are described as being for samples in 150 mM NaCl.

known as Pascal's constants. We have calculated the diamagnetism of globin by using measured values for the amino acids (Weast, 1968), where available, and Pascal's constants (O'Connor, 1982) to correct for the water molecule lost in polypeptide formation. For those amino acids without experimental data, we have calculated susceptibilities by using Pascal's constants and the data from the chemically most similar amino acid for which experimental data are available. Our calculation gives a molar susceptibility for globin of -9005×10^{-6} emu/mol, with a probable uncertainty of $\pm 180 \times 10^{-6}$ emu/mol. This agrees well with our determination of -9144×10^{-6} emu/mol. To be consistent with the HbCO in high salt diamagnetic reference used by Cerdonio et al. (1982, 1983), this calculated value would have to be in error by over 7%, far outside the range expected for such a calculation.

The diamagnetism of globin was also measured by Havemann et al. (1961). They measured $(-8600 \pm 100) \times 10^{-6}$ emu/mol for lyophilized globin, a value significantly smaller than ours. This same paper also contains measurements of some amino acids and HbCO solutions. These HbCO data have been strongly criticized by Cerdonio et al. (1980). We agree that the HbCO data are questionable, and we note that all the values in this paper seem abnormally small. (We have specifically excluded these amino acid data from the analysis described above.) However, it is interesting that the conclusions from their study are identical with ours: they find that the diamagnetism of globin is very close to that calculated from their amino acid data and that it is, within error, the same as that of HbCO.

In principle, another way of settling the issue of the diamagnetic reference is to measure hemoglobin in a state whose paramagnetism is accurately known or predictable. Unfortunately, we know of no such state. For a completely high-spin ferric heme, the magnetic moment can be calculated exactly if the zero-field splitting is measured by some independent method. Fluoro-MetHb may be in such a state at low temperatures, but it is doubtful that it is in a completely high-spin state at room temperature. Most room temperature susceptibility studies have found magnetic moments of 5.73–5.85 μ_B for fluoromethemoglobins or fluorometmyoglobins, i.e., values significantly less than the 5.92 μ_B for a fully high-spin form (Theorell & Ehrenberg, 1951; Scheler et al., 1957; Havemann & Haberditzl, 1958; Beetlestone & George, 1964). Our own data give 5.77 μ_B for human fluoro-MetHb in the absence of phosphates, relative to the diamagnetic reference reported here (J. S. Philo and U. Dreyer, unpublished results). The large temperature dependence of the visible absorption spectrum of fluoro-MetHb is also evidence for a mixed-spin state (Cho & Hopfield, 1979). On the other hand, Cerdonio et al. (1983) report a moment of 6.2 μ_B for human fluoro-MetHb at room temperature, relative to their HbCO in high salt diamagnetic reference. This value is much in excess of the theoretical limit of 5.92 μ_B, which may be further evidence that their diamagnetic reference is too diamagnetic. Lacking clear evidence for any completely high-spin form of MetHb at room temperature, we must look elsewhere for a possible magnetic standard.

Cyanomethemoglobin has been suggested as a magnetic standard by Cerdonio et al. (1980, 1981, 1982). However, the magnetic moment of low-spin ferric hemes contains a considerable contribution from the orbital angular momentum and cannot be predicted on a theoretical basis. The measured values at room temperature (which show significant variation) also depend on the choice of diamagnetic reference, so they are of no help. A measurement of the Curie law temperature dependence of the susceptibility can give the magnetic moment without knowledge of the diamagnetic reference. The reported moments for cyano-MetHb from such temperature-dependent studies, 2.23 μ_B (Iizuka & Kotani, 1969) and 2.59 μ_B (Messana et al., 1978), differ by more than can be accounted for by the difference between our diamagnetic reference and that of Cerdonio et al., so these data are also of no help. We also note that our data for MetHbCN give a magnetic moment of 2.41 μ_B relative to our diamagnetic reference, which is very close to the 2.43 μ_B we derive from the solution data of Cerdonio et al. (1982) relative to their diamagnetic reference (showing again that the differences between the laboratories are, in part, systematic). Finally, the third principal paramagnetic form of hemoglobin, high-spin ferrous deoxy-Hb, again can have variable contributions of orbital angular momentum, and its magnetic moment cannot be predicted. In summary, there does not appear to be any "standard" paramagnetic form of Hb which can help to establish which diamagnetic reference is correct.

Turning now to the HbO_2 and HbCO data, is there any independent evidence for which of these data are correct? Here we may consider other susceptibility studies, theoretical calculations, and other experimental techniques. The previous susceptibility studies have been reviewed by Cerdonio et al. (1981), and we agree that it is difficult to draw any firm conclusions from them. Indeed, detailed quantitative comparisons with the earlier room temperature studies are impossible since the susceptibilities of the solvents are not reported (this is particularly a problem for the whole blood measurements). However, a qualitative comparison shows that the only data showing HbO_2 solutions to be less diamagnetic than water, and the only data showing substantial differences in magnetism between HbO_2 and HbCO, are those of Cerdonio et al. (1978). We also note that the solution studies of (carbonmonoxy)myoglobin (MbCO) by Theorell & Ehrenberg (1951) gave a gram susceptibility for MbCO of -0.591×10^{-6} emu/g, in excellent agreement with our diamagnetic reference of -0.592×10^{-3} emu/g and the fit to all our HbCO and HbO_2 data, which corresponds to -0.589×10^{-6} emu/g, considering that the polypeptide diamagnetism should differ slightly between Mb and Hb.

The issue of whether there are paramagnetic ground states or low-lying excited states in HbO_2 or HbCO has also been addressed by a number of theoretical studies. All the theoretical studies of HbO_2 seem to agree that the ground state has no paramagnetism, but there is no agreement on whether there is a paramagnetic triplet state that is significantly populated at room temperature. Herman & Loew (1980) calculated that a paramagnetic triplet state is only 129 cm^{-1} above the ground state, in agreement with the data of Cerdonio et al. (1978), whereas a variety of other calculations (Karplus, 1982) place this state at least 5000 cm^{-1} above the ground state, which is consistent with our data. With regard to HbCO, we know of no theoretical studies consistent with the low-lying paramagnetic state implied by the data of Cerdonio et al. (1982).

Finally, we must ask whether there is any other experimental evidence for paramagnetism in HbO_2 or HbCO. The question of the magnetic states of HbO_2 and HbCO has often been strongly linked in the literature to the controversy over the formal charge states for the iron and ligand. While there is considerable experimental evidence for a formal ferric state for the heme iron and a negatively charged ligand, this is *not* direct evidence for paramagnetism. Since paramagnetic states, if present, would probably not be EPR detectable, the most

likely experimental technique other than magnetic susceptibility which could verify the existence of paramagnetism is NMR. Paramagnetism of the heme can produce hyperfine shifts and increased relaxation rates for nuclei of the porphyrin, ligand, and nearby amino acids. However, there is no clear evidence that such effects have ever been observed in HbO_2 or HbCO. In their most recent studies of carp HbCO, Cerdonio et al. (1983) report small shifts of two proton resonance peaks. These shifts have a dependence on the presence of chloride and inositol hexaphosphate which is similar to the reported changes in paramagnetism. They have suggested that these shifts may be due to heme paramagnetism. We do not believe this interpretation can be supported. For human HbCO, shifts in these same resonances were studied extensively (Lindstrom et al., 1972; Lindstrom & Ho, 1973). These studies showed that the positions of these resonances are sensitive to pH and the addition of a variety of anions (chloride, sulfate, inorganic and organic phosphates). However, according to the magnetic data of Cerdonio et al. (1978, 1983), only chloride affects the magnetism of human HbCO, while inorganic phosphate and 2,3-diphosphoglycerate do not. Moreover, if paramagnetism in human and carp HbCO is shifting these resonances upfield, then the larger paramagnetism they report for HbO_2 should produce even larger upfield shifts. However, a comparison of the NMR spectra of human HbO_2 and HbCO (Lindstrom & Ho, 1973) appears to show a downfield shift of the 5.68 ppm peak and an upfield shift of the 6.58 ppm peak [the assignments for the HbO_2 spectra are not entirely clear, but certainly the shifts relative to HbCO do not scale with the paramagnetism reported by Cerdonio et al. (1982)]. The positions of these ring-current shifted peaks are highly sensitive to motion relative to the heme, and the original interpretation of the anion-induced shifts was that they reflected subtle changes in geometry around the heme. The existing data cannot exclude the possibility that some of the changes in these resonances are due to heme paramagnetism; on the other hand, we do not feel the data support a new interpretation of these changes as positive evidence for paramagnetism.

For HbCO, one might expect that paramagnetism at the heme would produce large shifts and broadening of the ^{13}C NMR of bound ^{13}CO and indeed that the resonance might be unobservable if paramagnetism were present. However, the NMR data for a wide variety of hemoglobins fail to provide evidence for such effects. The chemical shifts and line widths for human HbCO in 0.1 M NaCl are pH independent over a 6.4–8.0 range and are not affected by the addition of 2,3-diphosphoglycerate or by carbamylation of the lysines and terminal valines (Moon & Richards, 1974). Furthermore, the ^{13}CO NMR spectra of fetal, sickle cell, and the isolated α- and β-subunit (carbonmonoxy)hemoglobins are very similar to that of human adult HbCO, and there are differences in the chemical shift of only a few tenths of a ppm between human HbCO and a variety of animal (carbonmonoxy)-hemoglobins. If there is no paramagnetism in these (carbonmonoxy)hemoglobins, it is not surprising that the NMR spectra are so similar. The data of Cerdonio et al. imply that human HbCO does show paramagnetism under these conditions, so to be consistent with these NMR data, the paramagnetism of all these (carbonmonoxy)hemoglobins would have to be very similar. That is, for consistency between the paramagnetism reported by Cerdonio et al. and these ^{13}CO NMR data (Moon & Richards, 1974), the paramagnetism would have to be nearly invariant over several human hemoglobins with significant functional differences, and between human and a range of animal hemoglobins, and yet be significantly different with the addition of salt and between human and carp hemoglobins. This, we believe, is rather unlikely. Certainly ^{13}C NMR seems the most promising technique for an independent verification of the magnetism of carboxy hemes, and further studies of human and carp HbCO and changes with salts and organic phosphates would be helpful.

In conclusion, our data support the original assignments of human $HbCO_2$ and HbCO as diamagnetic compounds. While we have established an independent diamagnetic reference state, our data also support the use of human HbO_2 or HbCO as a diamagnetic reference if sufficient caution against MetHb contamination is exercised.

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Registry No. HbO_2 A, 9062-91-3; HbO_2 A_0, 61912-95-6; HbCO A, 9072-24-6; HbCO A_0, 87901-13-1; protoporphyrin IX disodium salt, 50865-01-5.

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Allosteric Modulation of *Callinectes sapidus* Hemocyanin by Binding of L-Lactate†

Bruce A. Johnson,* Celia Bonaventura, and Joseph Bonaventura

ABSTRACT: Hemocyanin of the blue crab *Callinectes sapidus* has the typical structure of crustacean hemocyanins in that its smallest in vivo structure is a hexamer of subunits each having a molecular mass of approximately 75 000. As found in the blood, *Callinectes* hemocyanin consists of a mixture of hexamers and dodecamers (typically 1:4). As in other crustacean hemocyanins, the affinity with which oxygen binds to the binuclear copper site has been reported to be very sensitive to pH and to a variety of inorganic allosteric effectors. We report here the interaction of L-lactate, a natural metabolite, with the native hemocyanin and with chromatographically purified hexamers and dodecamers. Under ionic conditions that approximate those found physiologically, the addition of 10 mM L-lactate to native *Callinectes* hemocyanin substantially increases its oxygen affinity ($\Delta \log P_{50} = -0.28$). The data from lactate titrations were fit to a theoretical equation, and the best fit was obtained with a lactate dissociation constant of 1.8 mM for the oxy state and 2.2 lactate binding sites for every 6 oxygen binding sites. Independent measurements by ultrafiltration techniques indicated a dissociation constant of 3.2 mM with 2.8 lactate binding sites per 6 oxygen binding sites. The two sets of data clearly indicate that there is less than one lactate binding site per oxygen binding site. The fit to the titration was not improved with the assumption of more than one class of lactate binding site. The hexamers and dodecamers of native *Callinectes* hemocyanin are not in equilibrium and are stable after separation by gel-filtration chromatography. Polyacrylamide gel electrophoresis of the subunits of the dissociated dodecamers shows five major bands. Two of these bands, which constitute one-sixth of the total dodecameric hemocyanin, do not appear upon gel electrophoresis of dissociated hexamers. The oxygen affinities of the hexameric and dodecameric hemocyanin forms are similar to one another but show differences in their sensitivity to L-lactate. The oxygen affinity of native *Callinectes* hemocyanin was increased appreciably more by L-lactate than by glycolate, D-lactate, and pyruvate (listed in decreasing order of effectiveness). Propionate, acetate, succinate, D-alanine, and L-alanine were without effect, thus illustrating the selectivity of the L-lactate effect on the hemocyanin. We found the magnitude of the Bohr effect to be unchanged by the addition of L-lactate over the pH range 7.5-8.0. Moreover, there is no significant effect of L-lactate on the aggregation state of the hemocyanin or on its 340-nm copper–oxygen absorption band. The foregoing results are consistent with the role of L-lactate as a specific allosteric effector of *Callinectes* hemocyanin that acts by preferential binding to a stereospecific site of the oxyhemocyanin.

Hemocyanins are the copper-containing, oxygen-transport proteins that occur in some arthropods and molluscs. In these proteins, oxygen is bound between two copper atoms as a peroxide bridge (Solomon, 1981). The oxygen affinity of the binuclear copper site can be altered by a variety of allosteric ligands in a manner analogous to the effect of protons and organic phosphates on the oxygen affinity of vertebrate hemoglobins (Van Holde & Miller, 1982). In recent years, the allosteric interactions of protons, chloride, calcium, and magnesium with various hemocyanins have been extensively studied and modeled by using various extensions of the two-state model of Monod, Wyman, and Changeux (Brouwer et al., 1977, 1982b). We still know little, however, about the exact mechanisms of action of allosteric effectors of hemocyanin. Direct measurements of the number of binding sites and their affinities for allosteric effectors have been made in only a few cases (Arisaka & Van Holde, 1979; Kuiper et al., 1979; Chiancone et al., 1981; Andersson et al., 1982). Additionally, almost no information concerning the location or identity of amino acid residues involved in these interactions has been obtained.

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