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Effect of Magnetic Susceptibility on Nuclear Magnetic Resonance Signals Arising from Red Cells: A Warning†

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ABSTRACT: We have demonstrated that magnetic susceptibility effects can strongly influence nuclear magnetic resonance spectra, particularly in cells, and lead to spurious line broadening and chemical shifts which will result in false conclusions about molecular motion and intracellular pH and equilibrium constants. Three specific instances of a general phenomenon resulting from the difference in magnetic susceptibility inside and outside deoxygenated red cells have been examined. We find that at 95 MHz the resonance of ^{19}F covalently bound to both hemoglobin and glutathione as the trifluoroacetonyl compound undergoes substantial line broadening in deoxygenated red cells. Line broadening is not observed in oxygenated solutions or oxygenated cells nor is it observed in deoxygenated hemoglobin solutions. The broad lines in the deoxy red cell can be narrowed by matching the magnetic susceptibility of the suspending medium to that of the cell interior by adding suitable amounts of a paramagnetic metal ion such as manganese or dysprosium to the suspending medium. Similarly, we have observed line broadening of the ^{31}P resonance of 2,3-diphosphoglyceric acid which occurs only in deoxygenated red cells and has a field dependence greater than the first power. This line broadening does not occur in oxygenated solutions or oxygenated cells nor does it occur in deoxygenated hemoglobin solutions. Again, the broadened lines in the deoxygenated cells can be narrowed by matching the magnetic susceptibility. Because the line-broadening effects reported here do not depend on the nucleus, the chemical nature of the molecule, or the molecular size, they cannot be attributed to specific chemical interactions or interactions with the cell membrane. Because they can be eliminated by matching the internal and external magnetic susceptibility, we attribute the observed broadening to magnetic susceptibility differences. Differences in magnetic susceptibility will also result in systematic displacement of the chemical shift of resonances arising inside the cell which may either add to or cancel effects due to biochemical interactions. We show that the appropriate corrections can be estimated but that due to the presence of magnetic field gradients use of internal references may be necessary.

Nuclear magnetic resonance (NMR)[1] is uniquely suited to the study of events occurring inside intact cells. It is frequently desirable to conduct such studies at high magnetic fields to overcome the problems of low signal to noise and overlapping resonances. However, if line-broadening effects occur which are more pronounced at high field, the expected enhancement in sensitivity and resolution will not be attained. Hemoglobin is present at high concentrations (5 mM) in the red cell and, particularly in its met and deoxy forms, has a magnetic susceptibility which differs significantly from that of water. Chemical shifts and line broadening due to magnetic susceptibility effects have been recognized in nonbiological systems since the earliest days of NMR. However, it has generally been assumed that such effects were negligible in biological

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[1] Abbreviations: 2,3-DPG, 2,3-diphosphoglyceric acid; Hb-TFA, hemoglobin with a trifluoroacetonyl group on both β-93 sulfhydryls; PBS, equal volumes of 0.15 M pH 7.2 phosphate buffer and isotonic saline; NMR, nuclear magnetic resonance; BTFP, 3-bromo-1,1,1-trifluoropropanone; PMB, *p*-(hydroxymercuri)benzoate; GSH, glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MCHC, mean corpuscular hemoglobin concentration; DTPA, *N*,*N*-bis[2-[bis(carboxymethyl)-amino]ethyl]glycine (diethylenetriaminepentaacetic acid); T_2, transverse relaxation time; T_1, longitudinal relaxation time.

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systems. We will show that these effects are not small and must be considered if intracellular data are to be correctly interpreted.

Discontinuities in magnetic susceptibility will result in magnetic field gradients. The effect of field gradients on line width (which is inversely proportional to T_2, the transverse relaxation time) was considered by Hahn (1950) and Carr & Purcell (1954). Line broadening in lithium metal suspensions in oil which was ascribed to local field inhomogeneities was observed by Zamir et al. (1964); and Murday & Cotts (1968) also considered the effect of restricted diffusion inside a small volume. More recently (Stoll & Majors, 1982), the use of susceptibility matching to eliminate some line-broadening effects in powdered solid samples was reported. The effect of discontinuities of magnetic susceptibility on line width in biological systems was considered by Packer (1973), who concluded it would have minimal effects in systems such as muscle. Glasel & Lee (1974) discussed the effect of spherical glass beads in the micrometer size range and of various magnetic susceptibilities on the line width of the water protons in the solvent and pointed out the relevance of such effects to biological systems. More recently, magnetic susceptibility differences induced by adding various paramagnetic metal ions to the suspending medium of red cells were used to measure transport of small molecules into the red cell (Brindel et al., 1979). However, field-dependent line broadening of signals from the inside of intact cells which are the result of spontaneously occurring differences in magnetic susceptibility between compartments has not to date been considered a limiting factor in studies of intracellular properties.

Materials and Methods

Fluorination and Assay for Fluorination in Cells and Solutions. In solutions, fluorination was carried out on DE52-purified hemoglobin by using 3-bromo-1,1,1-trifluoropropanone (BTFP) (PCR Research Chemicals, Gainsville, FL) (Huestis & Raftery, 1972a). Excess reagent was removed by filtration through Sephadex G-25 (Pharmacia, Piscataway, NJ) equilibrated with the buffer of choice. In cells, 0.1 mL of BTFP was added to 1 mL of 1 volume of packed cells suspended in 4 volumes of pH 7.2 PBS, and the pH was maintained at 7.1 with 0.5 N NaOH for 30 min. The cells were then washed 5 times with cold isotonic saline. This procedure eliminates all free BTFP and results in 99+% fluorination of the Hb. The percent fluorination of the Hb was determined by titration with PMB (Boyer, 1954). This procedure determines the content of free sulfhydryls, and full fluorination masks all sulfhydryls. Under these conditions, glutathione (GSH) is also fluorinated in the red cell. We can demonstrate that the second resonance arises from GSH by comparing the chemical shifts of authentic glutathione which has been fluorinated with the second chemical shift observed in the red cell and by the fact that GSH is the second most prevalent sulfhydryl-containing species in the red cell, having a concentration of about one-fourth that of the hemoglobin concentration (Beutler, 1975). Glutathione can be assayed for by reaction with DTNB (Beutler et al., 1963). Since this reaction depends on the presence of a free GSH sulfhydryl, the percent fluorinated glutathione can be determined by assay before and after fluorination. It is possible to fluorinate GSH only by carrying out the above procedure including the washes under deoxy conditions. Assays for fluorination of Hb and GSH indicated that <10% of the Hb and >90% of the GSH were fluorinated under these conditions.

Mean corpuscular hemoglobin concentrations were determined by using hand-spun hematocrits, and hemoglobins were determined by using Drabkin's reagent. Osmolarity was determined by using a Precision System microosmometer (Precision Systems Inc., Sudbury, MA).

Deoxygenation was achieved by alternate exposure to mild vacuum (25 psi) and water-saturated nitrogen with gentle agitation to expose the maximum surface area; under these conditions, foaming is minimized. The percent oxygenation was then determined by using an Instrumentation Laboratory CO oximeter.

NMR spectra were measured on a Jeol 100 pulsed Fourier-transform NMR spectrometer and on a Varian XL-200 spectrometer in the laboratory of Dr. R. Gupta at Albert Einstein College of Medicine. Line widths were measured either by the width at half-height for simple spectra or by determining the best fit of synthesized spectra. Chemical shifts were measured in ppm from a reference signal from 10 mM P_i (inorganic phosphate) in D_2SO_4 contained in the inner concentric tube. In the case of the Varian XL-200 spectrometer, it was possible to accurately demonstrate that the reference signal is independent (to ±0.02 ppm) of the magnetic susceptibility of the solution in the outer tube. The same observation was made of the Jeol 100 spectrometer but to a lesser degree of precision (±0.1 ppm).

Magnetic susceptibilities were measured by using the Jeol 100 spectrometer, which is an electromagnet, by the static (nonspinning) method of Engel et al. (1973) as corrected by Orrell & Sik (1980). We therefore used eq 10 of Orrell & Sik (1980), that is

$$\chi_M = \left(\frac{\Delta\Delta\nu}{\Delta c}\right)\left(\frac{1 + 4\pi\chi_5}{1 + 4\pi\chi_3}\right)\left(\frac{a_3^2}{4\pi\nu_0 a_1^2}\right) \qquad (1)$$

where $\Delta\nu$ is the splitting of the reference resonance in the outer concentric tube in hertz, Δc is the concentration in moles per liter, χ_5 and χ_3 are the magnetic susceptibilities of the air and the solvent, respectively, a_3 is the inner radius of the outer tube, a_1 is the inner radius of the inner tube, and ν_0 is the operating frequency of the spectrometer in hertz. This results in a magnetic susceptibility expressed in terms of cgs-emu units. The magnetic susceptibilities of deoxygenated Hb solutions, manganese solutions, and the dysprosium tripolyphosphate complex (Gupta & Gupta, 1982) were measured. The values obtained for the manganese solutions of 15.3×10^{-2} compared favorably with the literature values (*Handbook of Chemistry and Physics*, 1971–1972), and the dysprosium tripolyphosphate was found to have a magnetic susceptibility of $(59.3 \pm 2) \times 10^{-2}$ (Figure 1). The 5 mM deoxygenated hemoglobin solution was found to have a magnetic susceptibility of -0.50×10^{-6}. This value can also be calculated by using either Weideman's rule or the equation (Cerdonio et al., 1981)

$$\chi_{mL} = \chi_{mL}^{H_2O} + n_{heme}(\chi_M^{dia} + \chi_{heme}^{para} - \upsilon_M\chi_{mL}^{H_2O}) \qquad (2)$$

where χ_{mL} is the volume magnetic susceptibility, $\chi_{mL}^{H_2O}$ is the volume magnetic susceptibility of the solvent, n_{heme} is the molar concentration of heme, χ_M^{dia} is the molar diamagnetic susceptibility of a single globin chain, and υ_M is the average molar volume of a single globin chain. The value for the protein diamagnetic contribution to the magnetic susceptibility can be derived either from the data given by Cerdonio et al. (1981) or from the value given by Cerdonio et al. (1978) for CO-Hb.

Results

Fluorinated Hemoglobin and Red Cells. The line width of the ^{19}F resonance at 95 MHz of hemoglobin fluorinated at the β-93 sulfhydryl with BTFP was measured as a function

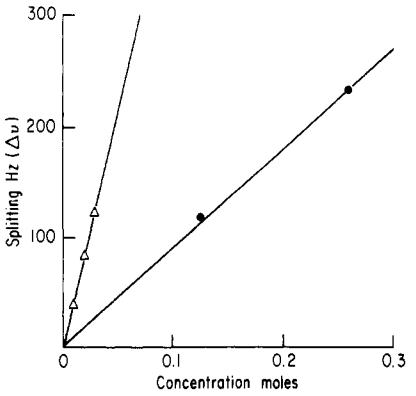

FIGURE 1: Separation between the two observed peaks as a function of paramagnetic ion concentration for solutions of $MnCl_2$ (●) and $Dy(PPP)_2$ (△) by using the static method (Engel et al., 1973) to determine magnetic susceptibility. The tubes used for this set of data were Wilmad 513-7PP and GS-10BL tubes which have an inner radius for the outer tube of 4.48 mm and an inner radius of the inner concentric tube of 1.62 mm which results in a geometric factor of 0.0153×10^{-6} Hz^{-1}.

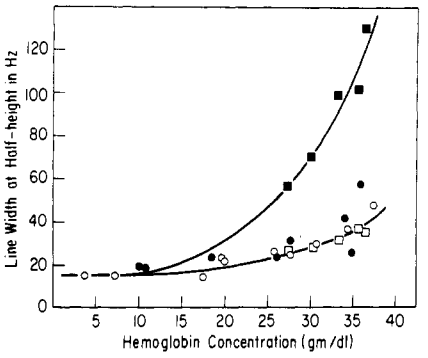

FIGURE 2: ^{19}F NMR line widths of HbA-TFA in carbon monoxy (○) and deoxy (□) solutions and carbon monoxy (●) and deoxy (■) red cells as a function of hemoglobin concentration. All deoxy samples were 95% or more deoxygenated.

of hemoglobin concentration under oxy and deoxy conditions and was found to increase somewhat with increasing hemoglobin concentration (Figure 2). The increased line width is probably due to hindered rotation of the entire hemoglobin molecule, reflecting the increased viscosity at hemoglobin concentrations over 25 g/dL. If the same hemoglobin concentration range is compared in oxy and deoxy red cells where the MCHC was altered by changing the extracellular osmolarity, we find that the line widths observed for oxygenated cells are nearly identical with those observed for oxyhemoglobin solutions; however, the line widths for deoxygenated cells are much broader than those for either oxy or deoxy solutions or oxy cells are comparable hemoglobin concentrations (Figure 2). Similarly, the line width of the glutathione, which can be separately studied by fluorinating deoxygenated red cells, is found to increase as the percent deoxygenation is increased (Figure 3).

If we calculate the magnetic susceptibility by using eq 2 for a 95% deoxygenated hemoglobin solution with a MCHC of 33 g/dL, we find that the Mn–DTPA concentration required to match the internal magnetic susceptibility is 18 mM. When deoxy red cells are suspended in 8, 18, and 22 mM Mn–DPTA, maximum narrowing is observed at 18 mM as predicted, and the narrow glutathione signal can once more be observed (Figure 4). At these concentrations, Mn^{2+} is excluded from the red cell only for short periods of time (Fabry & Eisenstadt, 1978). That it was excluded during the period of the measurements was demonstrated by atomic absorption spectroscopy of the external medium and the red cell hemolysate after the NMR spectra had been taken.

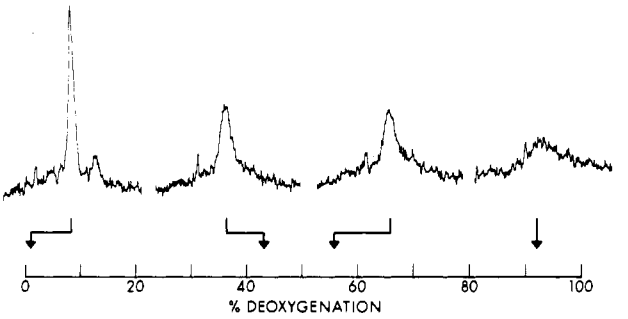

FIGURE 3: ^{19}F NMR spectra as a function of deoxygenation of fluorinated glutathione made by reacting red cells under deoxy conditions.

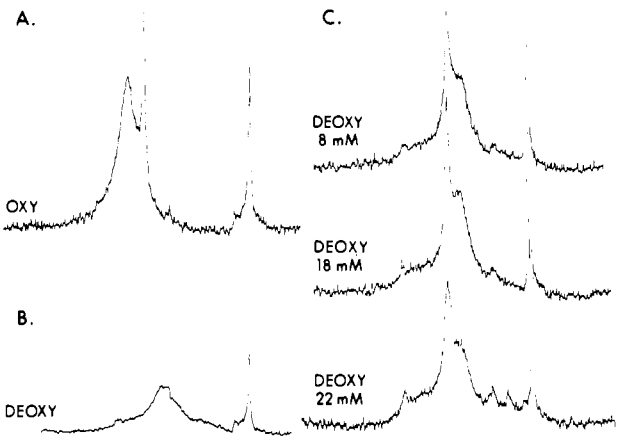

FIGURE 4: ^{19}F NMR spectra of fluorinated red cells showing a signal from Hb-TFA and fluorinated glutathione. In the top left spectra, the cells are oxygenated, and in the bottom left spectra, the cells are deoxygenated. Note the loss of intensity due to broadening and the loss of the sharp glutathione peak. On the right are a series of spectra of deoxygenated cells to which Mn–DTPA has been added. Note the reappearance of the resolved glutathione resonance when the magnetic susceptibility is matched with 18 mM Mn. The resonance due to Hb-TFA is closer to the reference signal in the deoxy samples because the ^{19}F resonance of Hb-TFA shifts on deoxygenation.

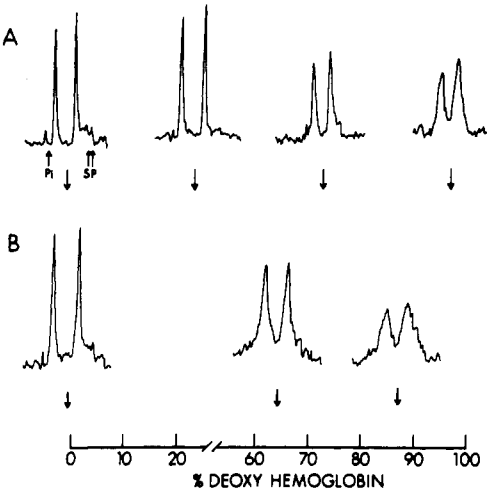

FIGURE 5: ^{31}P spectra of 2,3-DPG in intact HbA red cells at 40.5 MHz as a function of deoxygenation at two different MCHC's. Note that the resonances broaden as the percent deoxyhemoglobin increases. (A) MCHC = 33 g/dL. (B) MCHC = 38 g/dL.

^{31}P NMR of 2,3-DPG in Red Cells. The ^{31}P resonances from 2,3-DPG were observed as a function of deoxygenation at two different MCHC's (Figure 5). Again, the line width

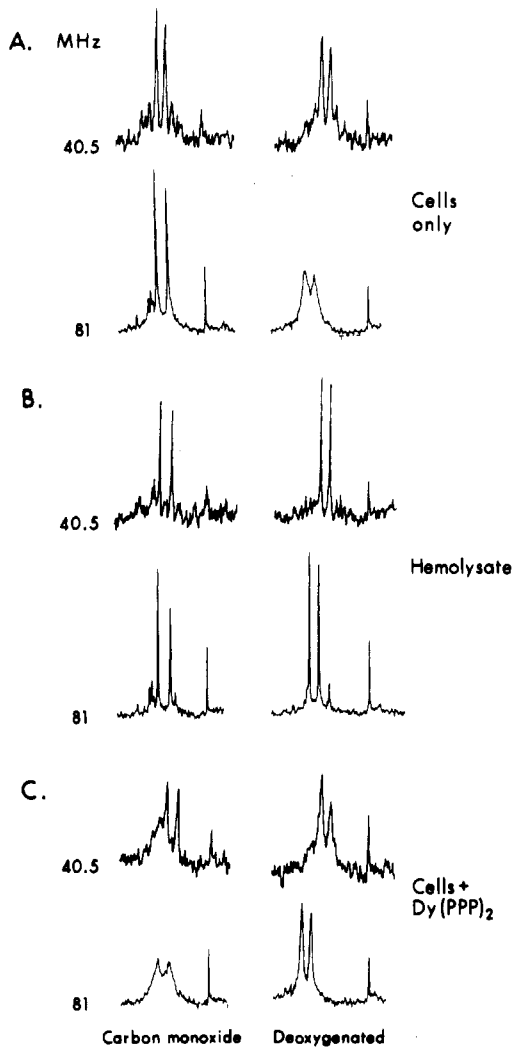

FIGURE 6: ^{31}P NMR spectra at 40.5 and 81 MHz. The same sample was run in two different spectrometers within 1 h without opening the NMR tubes. Spectra on the left represent >99% CO; spectra on the right represent >90% deoxyhemoglobin. (A) Carbon monoxy and deoxy red cells in plasma only. (B) Carbon monoxy and deoxy hemolysates of red cells; hemoglobin concentration equals 2 mM. (C) Carbon monoxy and deoxy red cells with 3 mM $Dy(PPP)_2^{7-}$ added.

gradually increases as the solution is deoxygenated. The effect of increasing the MCHC at 40.5 MHz (Figure 5) is that the line width at a given percent deoxygenation is broader. If the ^{31}P signals are compared at two different field strengths, we find that there is no increase in the line width at higher field in either carbon monoxy or deoxy solutions (Figure 6B). However, in intact cells, the carbon monoxy line widths are similar at 40.5 and 81 MHz, but in deoxygenated cells, there is a substantial increase in line width at the higher field (Figure 6A). Again, this line broadening can be decreased by adding a paramagnetic metal ion to the suspending medium at a concentration sufficient to match the internal magnetic susceptibility. In this case, we added dysprosium tripolyphosphate to both the carbon monoxided solution and the deoxygenated solution. We observed (Figure 6C) that now the lines remain narrow in the deoxy solution at high field and are broadened in the carbon monoxy sample, because it is now this sample for which a susceptibility mismatch occurs. An advantage of $Dy(PPP)_2^{7-}$ is that it can be used at lower concentrations (about 3 mM for deoxy-Hb in red cells) due to its higher magnetic susceptibility; furthermore, it is excluded from the cell for longer periods of time. This can be demonstrated by holding the cells in the $Dy(PPP)_2^{7-}$ medium overnight after the NMR spectra have been run, washing the cells, hemolyzing them, and looking for the chemical shift which would result from traces of $Dy(PPP)_2^{7-}$ in the hemolysate.

Calculation of Anticipated Line-Broadening Effects. In the earliest days of NMR, Hahn (1950) and Carr & Purcell (1954) calculated the effect of macroscopic magnetic field inhomogeneity on T_2, the transverse relaxation time. Line width ($\Delta\nu_{1/2}$, the width of the line at half-height expressed in units of hertz) is related to T_2 by

$$\Delta\nu_{1/2} = 1/(\pi T_2) \quad (3)$$

where T_2 is expressed in seconds. One way of measuring T_2 is by spin-echoes; the simplest sequence consists of a $90°-\tau-180°-(2\tau-180°)_x$ sequence (Carr–Purcell sequence) which produces a spin-echo at 2τ after the 90° pulse where τ is a short time interval. The echo amplitude in the presence of a field gradient was given by Packer (1973) as

$$E(t) = E_0 \exp\{-[t[1/T_2 + (1/3)\gamma^2 g^2 D\tau^2]]\} \quad (4)$$

where $E(t)$ is the spin-echo amplitude at time t ($=2N\tau$), E_0 is the original amplitude of the signal following the 90° pulse, T_2 is the transverse relaxation time due to all other effects, γ is the gyromagnetic ratio characteristic of each nucleus, g is the magnetic field gradient, and D is the diffusion coefficient of the observed molecule in solution. This equation is for diffusion of a molecule in an unbounded fluid; diffusion within a small cavity has been considered by Murday & Cotts (1968), who concluded that restricted diffusion would make diffusion appear to be retarded and that this effect would be more severe for smaller cavities. By examining eq 3 and 4, we can see that the net result of a field gradient will be to create an effective T_2 which is shortened and which will in turn be reflected in broader resonances. The field gradient dependent term adds to the line width due to other causes to produce a broadening which is dependent on the gyromagnetic ratio squared, the field gradient squared, the diffusion constant, and the spacing between the echoes. the dependence on the gyromagnetic ratio implies that at a constant field nuclei with a higher resonant frequency (recalling that $\nu = \gamma H_0$) will show stronger line-broadening effects. The dependence on τ implies that for very closely spaced pulses, where τ is short with respect to the time it takes to diffuse across a significant fraction of the field gradient, the effect of a field gradient would be minimized. This suggests that it should be possible to eliminate broadening due to field gradients by shortening 2τ, the time between the echoes. However, the closest pulse spacing we were able to achieve in a Carr–Purcell sequence was 1 ms, and no narrowing was observed for 2,3-DPG (the Jeol spectrometer is not capable of multiple pulse sequences). We can estimate how close one would need to space the pulses by considering the time required for a water molecule to diffuse across half of the narrowest part of the cell. We find that about 0.2 ms is required to diffuse across the 0.7-μm distance by using the diffusion rate of water in protein solutions (Wang et al., 1954). 2,3-DPG diffusion will be somewhat slower but not enough to lead us to believe that broadening can be eliminated with the echo spacing attainable with our instrumentation.

Glasel & Lee (1974) studied the effect of small glass beads of different magnetic susceptibilities on the water (deuterium in D_2O) T_2 and demonstrated that the observed line broadening could be estimated by using

$$\Delta H = \pi H_0 \Delta\chi \quad (5)$$

where ΔH is the field inhomogeneity, H_0 is the magnetic field

strength, and $\Delta\chi$ is the difference in magnetic susceptibility between the bead and solvent. Using this approach, we estimate a line broadening due to 5 mM deoxyhemoglobin in the red cell of about 65 Hz for ^{19}F at 95 MHz. The observed value is about 60 Hz. The exactness of the agreement is probably fortuitous. A value of 27 Hz at 40.5 MHz was estimated for ^{31}P. Glasel's results apply to the region outside a sphere, but we can extend them to the concave region around the central dimple inside of the red cell. These results also imply that there will be effects due to magnetic susceptibility differences both inside and outside the cell.

Packer (1973) suggests that, in equations having the form of eq 4, $\Delta\omega$ can be used to approximate the term γgX. In this case, X is a dimension characteristic of the region to which the spins are confined, and $\Delta\omega$ represents the range of frequencies allowed and is proportional to ΔH in eq 5. This implies that effects due to susceptibility differences will be proportional to the magnetic field squared.

Calculation of Chemical Shift Effects. The chemical shift experienced by a nucleus is proportional to the magnetic field experienced by that nucleus. One effect of magnetic susceptibility is to change the effective magnetic field strength. This is a particularly deceptive effect in hemoglobin solutions because there will be a continuous change in the magnetic susceptibility of the solution as it is deoxygenated; furthermore, the magnetic susceptibility of deoxyhemoglobin solutions and hence the observed chemical shift will also depend on the hemoglobin concentration itself. We will therefore use a variation of Cerdonio's equation (Cerdonio et al., 1981) with a term added for the percent deoxyhemoglobin present and another term added to account for the paramagnetic contribution of oxyhemoglobin (Cerdonio et al., 1978) to determine the magnetic susceptibility as a function of oxygenation:

$$\chi_{mL} = \chi_{mL}^{H_2O} + n_{Hb}[\chi_{Hb}^{dia} + (1 - f_{deoxy})\chi_{Hb}^{oxy} + f_{deoxy}\chi_{Hb}^{deoxy} - v_M\chi_{mL}^{H_2O}] \quad (6)$$

where $\chi_{mL}^{H_2O}$ is the magnetic susceptibility of the water ($-0.719 + 10^{-6}$), n_{Hb} is the concentration of hemoglobin tetramers in moles per milliliter, χ_{Hb}^{dia} is the molar diamagnetic susceptibility of the globin only (-4.03×10^{-2}), f_{deoxy} is the fraction of the hemoglobin which is in the deoxy form, χ_{Hb}^{oxy} is the molar paramagnetic contribution of oxyhemoglobin (9.84×10^{-3}), χ_{Hb}^{deoxy} is the molar paramagnetic contribution of fully deoxygenated hemoglobin ($+4.96 \times 10^{-2}$), and v_M is the molar volume of hemoglobin. Furthermore, the corrections for the effect of magnetic susceptibility on the chemical shift depend on the orientation of the cylindrical sample tube in the magnetic field. As originally reported by Live & Chan (1970) and discussed by Lagodzinskaya & Klimenko (1982), the appropriate correction is

$$\delta_{cor} = \delta_{obsd} + k(\chi_{sample} - \chi_{ref}) \quad (7)$$

where $k = 2\pi/3$ for an electromagnet and $k = -4\pi/3$ for a solenoidal magnet. We will call the corrections $\delta_\perp$ for the electromagnet and $\delta_\parallel$ for the solenoidal magnet. Both magnetic susceptibility and the chemical shift corrections are plotted as a function of deoxygenation in Figure 7. Figure 7 illustrates two important points. (1) In both the carbon monoxy and oxy states, there are significant chemical shift corrections because the magnetic susceptibility of the protein (globin only) is more diamagnetic than water and oxyhemoglobin has a small paramagnetic contribution. Cerdonio et al. (1978) have demonstrated that all proteins have approximately the same magnetic susceptibility so that the diamagnetic correction is a general effect. (2) Note that the correction is added to the

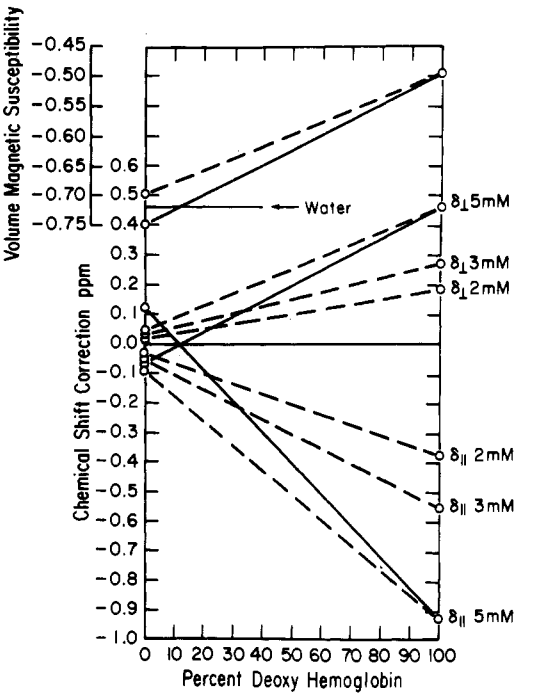

FIGURE 7: Calculated chemical shift corrections for electromagnets and solenoidal magnets as a function of deoxygenation for 5, 3, and 2 mM hemoglobin solutions. The symbols $\delta_\perp$ and $\delta_\parallel$ stand for corrections appropriate for electromagnets and solenoidal magnets, respectively. The solid lines represent values calculated for carbon monoxy–deoxy mixtures. The dashed lines represent calculated magnetic susceptibility corrections for 5, 3, and 2 mM oxyhemoglobin–deoxyhemoglobin mixtures. The lines at the top represent the magnetic susceptibility of oxy–deoxy and carbon monoxy–deoxy 5 mM hemoglobin solutions (dashed and solid lines, respectively) as a function of deoxygenation.

spectra from the electromagnet and subtracted from the spectra of the solenoidal magnet to bring the deoxy spectra in Figure 6 into alignment. The calculation of chemical shift corrections for intermediate oxygenations assumes a highly cooperative oxy–deoxy transition; that is, at a given degree of deoxygenation, most Hb molecules are in either a fully paramagnetic or a fully diamagnetic conformation.

The preceding discussion applies to a homogeneous sample in a cylindrical tube. The complex shape of the red cell and the field gradients induced in the interior by differences in magnetic susceptibility make such simple analysis questionable. However, after the magnetic susceptibility of the medium is matched to that of the cell interior, we can examine the chemical shift under much simpler conditions. We find that there is a small additional chemical shift following matching which implies that because of the presence of magnetic field gradients the full magnetic susceptibility appropriate to the red cell (e.g., that calculated for 5 mM hemoglobin) cannot be used to make corrections to chemical shifts of resonances from species inside the red cell.

Discussion

NMR is an invaluable tool for the nondisruptive study of events occurring within intact cells. Frequently, simple interpretations can be made of changes in NMR spectra. Chemical shifts can be interpreted in terms of modifications of the immediate environment of the nucleus observed by ionization (such as pH changes) or chemical modification (binding, formation of bonds), and increases in line width (shorter T_2's) can be interpreted in terms of slower molecular motions. However, in these experiments, we have observed changes in line width and chemical shifts due to differences

in magnetic susceptibility between the interior and exterior of the red cells. The chemical shift correction is larger for the sample configuration typical of superconducting magnets than for electromagnets, but is otherwise not field dependent. On the other hand, line broadening observed is field dependent and may be more severe as biological samples are investigated at higher fields.

Both hemoglobin and the red cell have been the target of numerous investigations using NMR. Binding of the allosteric effector 2,3-DPG to deoxyhemoglobin was studied by ^{31}P NMR (Huestis & Raftery, 1972b). They observed a chemical shift but no line broadening of the 2,3-DPG ^{31}P NMR signal in deoxyhemoglobin solutions. They also studied oxygenation and cooperativity by using the trifluoroacetonyl derivative of hemoglobin (Hb-TFA) (Huestis & Raftery, 1972a, 1975) which was formed by reaction of 3-bromo-1,1,1-trifluoropropanone with the β-93 sulfhydryl of hemoglobin. This derivative shows substantial chemical shifts but no broadening in deoxyhemoglobin solutions; a further shift is observed when 2,3-DPG is bound. Costello et al. (1976) and Marshall et al. (1977) studied 2,3-DPG binding both in solution and in intact red cells. They used the solution ^{31}P chemical shifts observed when 2,3-DPG binds to deoxyhemoglobin to calibrate the extent of binding and then studied binding in deoxy red cells. They reported that 2,3-DPG was much more weakly bound in the red cell than in solution.

In agreement with the results described above, we also observe narrow lines in both oxy- and deoxyhemoglobin solutions and in oxy red cells irrespective of the nucleus observed at field strengths of 2.3 and 4.6 T. However, in deoxy red cells, we observe broadening of the ^{19}F signal from both the Hb-TFA derivative and the TFA derivative of glutathione at 95 MHz (2.3 T). We observe a modest broadening of the ^{31}P resonance of 2,3-DPG in deoxy red cells at 41 MHz (2.3 T) and a much more pronounced effect at 81 MHz (4.6 T). Because these line-broadening effects are independent of the nucleus (^{31}P or ^{19}F), molecular size, and chemical nature of the molecule, we conclude that they are not due to specific chemical interactions or interactions with the cell membrane. At constant field (2.3 T), the line broadening was greater for the nucleus with the higher resonant frequency (Figure 2 vs. Figure 4) as predicted by eq 4. We have demonstrated that the lines in deoxy cells can be narrowed by matching the susceptibility of the medium with that of the interior of the cell by adding suitably chelated paramagnetic metal ions to the suspending medium. The magnitude of the anticipated line broadening can be estimated (Glasel & Lee, 1974), and good agreement is found between the observed line-broadening and the calculated effects. However, the magnitude of this effect cannot be calculated exactly due to the complex shape of the red cell.

Similarly, the displacement of the chemical shift due to the difference in magnetic susceptibility inside the deoxy red cell can be calculated by using eq 7. That the shifts observed for the samples containing cells in both magnet/sample configurations were in the same direction and magnitude as those observed for the hemolysates indicates that the effective susceptibilities both inside and outside the cells are partially averaged values, as would be anticipated from the slow decline of the magnetic field gradient from the surface of the cell and the close proximity of the cells. However, because of the presence of field gradients, molecules in the interior of the deoxy red cell will not experience a single chemical shift. In the absence of binding, we can estimate the number of times a small molecule traverses the red cell before relaxation occurs if we know the rate of diffusion and the dimensions of the red cell. From this, we can estimate that the molecule should experience most chemical shift environments in the cell before relaxation occurs. Hence, the chemical shift in the absence of binding represents an average value of the chemical shift in the cell, and, indeed, a small additional chemical shift is observed when the magnetic susceptibility of deoxy cells is matched. This suggests that the chemical shift correction for hemoglobin concentrations appropriate to the red cell cannot be used directly and that an intracellular reference will be needed. These corrections are important because the chemical shift correction of 2,3-DPG in deoxy red cells is of the same order as the total shift observed when 2,3-DPG binds to deoxyhemoglobin.

The chemical shift correction for carbon monoxy red cells is much smaller, but it is still significant when the controversy of the pH between the interior and the exterior of the red cell is considered. It is well accepted from biochemical measurements that this value is 0.2 pH unit under physiological conditions (i.e., an external pH of 7.2–7.4 and an osmolarity of 280–300 mosM). The chemical shift of inorganic phosphorus (P_i) has been used to measure intracellular pHs, and in its most sensitive region (near the pK), it has a chemical shift of about 0.1 ppm/0.1 pH unit, a readily measurable quantity. The correction for chemical shifts of resonances inside the red cell in an electromagnet is −0.06 ppm, and the same correction for a solenoidal magnet is +0.12 ppm for carbon monoxy red cells. For oxygenated red cells, the corrections are −0.9 ppm and +0.5 ppm for solenoidal magnets and electromagnets, respectively. Clearly, in the presence of met- or deoxyhemoglobin, the corrections for intracellular pH will be even larger. Due to the difficulty of calculating or measuring the intracellular magnetic susceptibility precisely, the best approach is to use an intracellular reference. The separation between the 2- and 3-phosphorus atoms in 2,3-DPG after appropriate correction for pH and complexation with Mg^{2+} is a possible reference for DPG binding to hemoglobin. Because of the interaction of ATP with Mg^{2+}, the α-phosphorus has been demonstrated (Gupta et al., 1978a,b) to be pH independent in the physiological range (pH 6–9) and may serve as an internal reference for pH measurements using inorganic phosphate.

Conclusions

We have demonstrated that magnetic susceptibility differences between the interior and exterior of the deoxy red cell can lead to line broadening and chemical shifts which are of the same magnitude as and add to chemical shifts and T_2 effects due to biochemical interactions. We have demonstrated that because of the complex shape of the red cell, the calculated magnetic susceptibility cannot be used to correct the much larger chemical shifts of signals arising from the inside to deoxy red cells and that the best approach is the use of intracellular references.

Added in Proof

In the 74th Annual Federation Proceedings, May 1983, Philo, Dreyer, and Schuster (abstract 1609) question Cerdonio's values for the magnetic susceptibility of both the oxy and carbon monoxy forms of hemoglobin. Corrected values for the chemical shifts can be obtained by appropriate substitutions in eq 6.

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Registry No. 2,3-DPG, 138-81-8; GSH, 70-18-8; Mn, 7439-96-5; dysprosium, 7429-91-6.

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Characterization of the Histidine Proton Nuclear Magnetic Resonances of a Semisynthetic Ribonuclease†

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ABSTRACT: The proton magnetic resonance spectrum at 300 MHz of the histidine residues in a semisynthetic derivative of bovine pancreatic ribonuclease (RNase A) has been determined. The derivative RNase 1–118·111–124 was prepared by enzymically removing six residues from the COOH terminus of the protein (positions 119–124) and then complementing the inactive RNase 1–118 with a chemically synthesized peptide containing the COOH-terminal 14 residues of ribonuclease (RNase 111–124) [Lin, M. C., Gutte, B., Moore, S., & Merrifield, R. B. (1970) *J. Biol. Chem. 245*, 5169–5170]. Comparison of the line positions of the C(2)-^{1}H resonances of these residues and of their pH dependence with those reported by other workers has allowed assignment of the resonances to individual residues, as well as the determination of individual pK values for histidine-12, histidine-105, and histidine-119. The assignment of histidine-119 was confirmed by the use of a selectively deuterated derivative. The titration behavior of all four histidine residues is indistinguishable from that observed by others for bovine pancreatic ribonuclease A. Partial dissociation of the noncovalent semisynthetic complex was evident at 30 °C, pH 4.0, 0.3 M NaCl; pertinent spectra were analyzed to provide an estimate of the association constant between the component chains under these conditions of 1.9×10^3 M^{-1}.

Successive digestion by pepsin and by carboxypeptidase A of bovine pancreatic RNase A[1] removes six residues from the COOH terminus of the molecule to provide a shortened chain, RNase 1–118, that is devoid of enzymatic activity (Lin, 1970). Complementation of this inactive segment with a chemically synthesized tetradecapeptide comprising the COOH terminus of RNase, viz., residues 111–124, results in the formation of

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[1] Abbreviations: DSS, sodium 3-(trimethylsilyl)-1-propanesulfonic acid; DCC, dicyclohexylcarbodiimide; RNase A, bovine pancreatic ribonuclease A; RNase 1–118, polypeptide composed of residues 1 through 118 of RNase; RNase 111–124, tetradecapeptide composed of residues 111 through 124 of RNase; RNase 1–118·111–124, noncovalent complex of RNase 1–118 and RNase 111–124; Boc, *tert*-butyloxycarbonyl; RNase S, noncovalent complex of residues 1 through 20 and 21 through 124 formed by limited subtilisin digestion of RNase.

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