Carbon-13–Proton Nuclear Magnetic Double-Resonance Study of Deoxyhemoglobin S Gelation†

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ABSTRACT: Recently developed ^{13}C–^{1}H nuclear magnetic double-resonance techniques have been used to study the gelation of deoxyhemoglobin S molecules, both in hemoglobin solutions and within sickle erythrocytes. Only isotropically mobile hemoglobin molecules ($\tau \lesssim 10^{-6}$ s) are detected in a scalar-decoupled ^{13}C NMR spectrum, whereas only the motionally restricted molecules of polymerized hemoglobin are detected in a proton-enhanced ^{13}C spectrum. The latter spectrum is obtained with a matched Hartmann–Hahn contact to transfer polarization from protons to carbons (cross-polarization). Both isotropically mobile and polymerized hemoglobin molecules are detected in a dipolar-decoupled ^{13}C spectrum. A comparison of integrated intensities obtained from the various types of spectra show that, at 37 °C, approximately 0.4 of the molecules in a 28 g/dL preparation of deoxyhemoglobin S enters the polymer phase, while at 37 g/dL a significantly higher fraction (ca. 0.6) of the hemoglobin is in the polymer phase. As much as 0.8 of the hemoglobin is polymerized in deoxygenated sickled erythrocytes. The cell-free deoxyhemoglobin S preparation at 28 g/dL has the spectral characteristics of a two-phase system. The motionally narrowed signals observed in the scalar-decoupled spectrum indicate that one phase behaves as a low-viscosity solution of isotropically mobile molecules. In contrast, the width of the signal ($\gtrsim$150 ppm) observed in the carbonyl–aromatic region of the proton-enhanced ^{13}C spectrum indicates that the second phase, containing the polymerized molecules, behaves as a crystalline solid.

Although the pathophysiology of sickle cell disease is linked to the intracellular gelation of deoxyhemoglobin S molecules (Dean & Schechter, 1978), studies of hemoglobin polymerization in cells have lagged behind studies of hemoglobin solutions. Structural analyses by X-ray diffraction and electron microscopy (Magdoff-Fairchild et al., 1972; Finch et al., 1973; Dykes et al., 1978) have suggested that the deoxyhemoglobin S gel in cell-free hemoglobin preparations and in erythrocytes is the same or similar. Studies of the mechanism of intracellular hemoglobin polymerization, however, have been largely restricted to proton nuclear magnetic resonance studies of water relaxation parameters (Cottam et al., 1974; Lindstrom et al., 1976; Zipp et al., 1976). Such magnetic resonance spectroscopy, including parallel studies with hemoglobin S solutions (Ho et al., 1976), only permit indirect measurements of the polymerization process.

Considerably more progress has been made in understanding the physical chemistry of deoxyhemoglobin S gelation in cell-free preparations (Hofrichter et al., 1976a,b). A variety of physical data supports the view that the highly viscous gel is composed of paracrystalline polymerized hemoglobin molecules in equilibrium with largely monomeric (64 000 M_r) hemoglobin in solution. The gel can be separated into two components by high-speed ultracentrifugation (Bertles et al., 1970; Hofrichter et al., 1976a,b; Ross et al., 1977). All of the polymer phase is contained in a pellet which can be readily separated from the supernatant. The fraction of hemoglobin in the polymer phase, f_p, has been calculated by assuming that the pellet is composed entirely of polymer. Values of f_p have been so obtained in cell-free preparations, as a function of temperature and hemoglobin concentration (Hofrichter et al., 1976b; Ross et al., 1977).

We describe herein a ^{13}C NMR procedure for measuring f_p in cell-free preparations of deoxyhemoglobin S as well as in deoxygenated sickle erythrocytes. This procedure utilizes the different ^{13}C spectral characteristics of isotropically mobile and motionally restricted molecules to discriminate between hemoglobin S monomers and polymers. The line widths of isotropically mobile ($\tau \lesssim 10^{-6}$ s) molecules are less than 1 kHz since the strong, angularly dependent interactions which broaden lines in solids (static dipolar interactions and chemical shift anisotropy) are averaged out by molecular rotation. The small remaining indirect or scalar couplings are removed by low level ($\gamma H_2/2\pi \sim 3$ kHz) irradiation of the protons at their Larmor frequency (scalar decoupling). In randomly oriented rigid solids (powders), the strong, angularly dependent ^{13}C–^{1}H static dipolar interaction produces a ^{13}C powder line shape that is too broad to detect. However, the ^{13}C–^{1}H static dipolar contribution to the powder line width can be removed (Bloch, 1958; Pines et al., 1973; Mehring, 1976) by strong ($\gamma H_2/2\pi \sim 60$ kHz) resonant irradiation of the protons (dipolar decoupling). The powder line shape of rigid proteins in the presence of dipolar decoupling arises from chemical shift anisotropy (Pines et al., 1973; Mehring, 1976) and ^{13}C–^{14}N static dipolar coupling (Torchia & VanderHart, 1979). The powder line width arising from these interactions is narrow enough ($\lesssim$3 kHz) to detect, and the powder width and shape provide information about molecular motion on the millisecond time scale (Pines et al., 1973; Mehring, 1976).

While the line narrowing produced by dipolar decoupling permits detection of signals arising from immobilized molecules, it is often difficult to measure the unsaturated ^{13}C signal intensity in solids since rigid molecules have very large ^{13}C spin–lattice relaxation times. A matched Hartmann–Hahn (Hartmann & Hahn, 1962; Pines et al., 1973) contact circumvents this problem by transferring polarization from the protons (which often have small T_1 values in solids) to the carbon nuclei. After cross-polarization, the proton-enhanced ^{13}C signal is detected with dipolar decoupling.[1] The signal intensity can be up to four times that obtained in the usual dipolar-decoupled ^{13}C spectrum acquired without proton

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[1] In proton-enhanced spectra the ^{13}C polarization is obtained from the protons, whereas in dipolar-decoupled spectra the ^{13}C polarization arises from ^{13}C spin–lattice relaxation processes. In both procedures, dipolar decoupling is applied during acquisition of the free-induction decay signal.

enhancement. Since polarization transfer occurs only between ^{13}C and ^{1}H spins having a nonvanishing static dipolar interaction (Hartmann & Hahn, 1962; Pines et al., 1973; Demco et al., 1975), carbons which are isotropically mobile (on the time scale of $\lesssim 10^{-5}$ s) will not contribute signal to the proton-enhanced spectrum. Hence, a scalar-decoupled spectrum contains signal intensity from only isotropically mobile molecules, whereas a proton-enhanced spectrum contains signal from only motionally restricted molecules. Dipolar decoupling reveals the sum of the two, provided that ^{13}C T_1 values are sufficiently small.

This paper presents the scalar-decoupled, dipolar-decoupled, and proton-enhanced spectra of hemoglobin A and hemoglobin S, in solutions and in intact cells. Comparison of integrated signal intensities in these three types of spectra permits estimates of the degree of polymerization of the hemoglobin under a variety of conditions. Furthermore, a lower limit for the rotational correlation time of polymerized hemoglobin can be estimated from the line shapes observed in the proton-enhanced spectrum.

Materials and Methods

Sample Preparations. Hemoglobin S was prepared according to the ion-exchange procedure of Huisman & Dozy (1965) as follows. Fresh hemolysates were prepared from erythrocytes obtained from individuals homozygous for the sickle globin gene. These hemolysates were made 0.4 M in NaCl, and membranes were removed by centrifugation at 30000*g* for 30 min. The supernatants were dialyzed into 0.05 M Tris-HCl at pH 8.3 and then applied to DEAE-Sephadex A-50 from which the hemoglobin was eluted with a gradient of pH from 8.2 to 7.3. The chromatographed hemoglobin was concentrated by ultrafiltration and vacuum dialysis and then dialyzed from the chromatographic buffer into 0.15 M potassium phosphate buffer at pH 7.35. Hemoglobin concentrations were determined by the cyan-met procedure (van Assendelft, 1970), with an extinction coefficient at 540 nm of 11.0 mM^{-1} cm^{-1} per heme unit of molecular weight 16000. In those cases where NMR samples were not prepared directly from freshly chromatographed hemoglobin, the hemoglobin was frozen dropwise in liquid nitrogen and stored at −70 °C. The concentration of methemoglobin in each oxygenated sample was determined spectrophotometrically. In no case did the methemoglobin constitute more than 10% of the sample.

Hemoglobin, at appropriate concentrations, was transferred at 5 °C and in a nitrogen atmosphere into either 5- or 8-mm NMR tubes, which were then subjected to repeated flushing and evacuation of nitrogen. Samples were deoxygenated by means of small aliquots of freshly prepared buffered sodium dithionite (Vine Chemicals, Ltd., Manchester, England), which were injected into the solution from a Hamilton syringe fitted with a 15-mm needle, such that the final concentrations of dithionite were 0.05 M. The tubes were immediately sealed with Lexan polycarbonate plugs fitted with "O" rings. The sealed NMR tubes were rapidly agitated by hand in an ice–water mixture and then gently agitated at 5 °C for a 24-h period. Final hemoglobin concentrations in the 5-mm NMR tubes were determined with a Cary 17 spectrometer and the equation (Hofrichter et al., 1976a)

$$c\ (g/dL) = 20.06[(A_{910} - A_{1090}) + 0.04]$$

based upon the hemoglobin extinction coefficients at 910 and 1090 nm. (The correction factor 0.04 accounts for differences in the absorption of buffer at these wavelengths, and the multiplicative constant, 20.06, differs from the value 24.56 used by Hofrichter et al. (1976a) since the 5-mm NMR tube has a larger internal diameter than the 5-mm quartz EPR tube.) The ability of the resulting deoxygenated samples to polymerize was then verified by monitoring the turbidity increase of these samples at 800 nm in a temperature-jump experiment, performed as described elsewhere (Hofrichter et al., 1976a), except that it was carried out on a Cary 15 spectrometer with a specially constructed light-proof housing designed to accommodate long NMR tubes.

Whole blood from individuals homozygous for sickle hemoglobin was collected in EDTA solutions to prevent coagulation and then gently centrifuged, and the plasma was removed. The erythrocytes were resuspended and washed three times with 0.15 M NaCl at 4 °C. The cells were then pelleted directly in 8-mm NMR tubes, the latter being reinforced against breakage by means of Teflon centrifuge tube mountings. As with the hemoglobin, the cells were deoxygenated with sodium dithionite at a final concentration of 0.05 M and sealed with Lexan plugs. Data collection was normally begun within approximately 3 h of blood drawing.

^{13}C Fourier Transform Spectra. ^{13}C spectra were obtained on a Nicolet TT-14 spectrometer modified for high-power double-resonance experiments in solids (Torchia et al., 1977; Jelinski and Torchia, unpublished experiments). All experiments were performed in a Dewared probe containing a ^{13}C coil having a length of 18 mm and an inside diameter of 8 mm. Most experiments were carried out with 0.65-cm^3 preparations of hemoglobin in 8-mm Wilmad glass tubes. Some early experiments were carried out on 0.2-cm^3 samples in 5-mm tubes. Although the signal to noise ratio was three times smaller in the 5-mm tubes, it was possible to measure the optical properties and NMR spectra in the same tube. The large hemoglobin absorbance precluded optical measurements in 8-mm tubes. The ^{13}C transmitter delivered 50 W (at 15.09 MHz) to the probe, which rotated the ^{13}C magnetization through 90° in ca. 5 μs. The probe was tuned to 50 Ω before each spectrum was obtained. Scalar decoupling was carried out with a resonant field of 0.7 G ($\gamma_2 H_2/2\pi$ = 3 kHz) applied to the protons, whereas a 14-G field ($\gamma_2 H_2/2\pi$ = 60 kHz) was used for dipolar decoupling. Nuclear Overhauser enhancements were eliminated in all decoupled spectra by the use of gated decoupling.

Proton-Enhanced Spectra. The Hartmann–Hahn condition ($\gamma_1 H_1 = \gamma_2 H_2$) was established in the following manner. A sample of adamantane was placed in the probe, and the impedance of the ^{13}C channel was tuned to 50 Ω. The standing wave ratio in the proton channel was adjusted to a minimum, and proton-enhanced spectra of adamantane were obtained as a function of the power level in the ^{13}C channel which could be attenuated in 0.1-dB steps. The best match attenuation level was that which gave the largest amplitude adamantane signal. After replacing the adamantane sample with the hemoglobin sample, the impedances in the proton and ^{13}C channels were tuned as described for adamantane. All proton-enhanced spectra were obtained by alternating the proton spin temperature (Stejskal & Schaefer, 1975) to suppress artifacts. For instance, isotropically mobile hemoglobin does not contribute intensity to a proton-enhanced spectrum when the sign of the proton spin temperature is alternated. This procedure was also incorporated into the pulse sequence used to measure the partially relaxed spectra (Torchia, 1978), ensuring that the time dependence of proton-enhanced ^{13}C magnetization alone was detected.

Temperature Measurements. The sample temperature in the probe was regulated with a Varian temperature controller.

Table I: Comparison of Fractions of Polymerized Deoxyhemoglobin S Determined from ^{13}C Spectra of Various Preparations at 37 °C

sample	method[a]	fraction polymerized[b]
28 g/dL	A	0.42
	B	0.35
37 g/dL	A	0.58
	B	0.45
S erythrocytes	B	0.75

[a] Methods A and B are defined under Discussion. As discussed in the text, method B provides an underestimate of the fraction polymerized. [b] Uncertainty, due to errors in integrated intensities: ±10% in solution and ±15% in erythrocytes.

The NMR tube, the ^{13}C coil, and the ^{1}H coil were located within a Dewar flask, and the sample temperature was measured in the following manner. After setting up the desired pulse sequence, we placed a hemoglobin preparation having the same composition as the analytical sample in the probe, and a copper-constantan thermocouple was suspended a few centimeters above the hemoglobin. Several hundred transients were accumulated. Immediately following data acquisition, the thermocouple was immersed in the hemoglobin and the temperature was read. Temperature variation over the hemoglobin volume was found to be <2 °C.

Signal Intensity Measurements. Differences in signal intensities between spectra were measured two different ways. First, appropriately normalized spectra were subtracted in the NIC-80 computer with the Nicolet software. One spectrum was scaled relative to the other until a nulled difference signal was measured. Second, the areas of the signals of interest were determined by numerical integration, with the NIC-80 software. The first method was employed when the line shapes of the two spectra were the same, e.g., proton-enhanced spectra at two different concentrations. Due to limitations in the signal to noise ratio, uncertainties in the intensity measurements produced 10–15% uncertainties in the determinations of f_p (see Table I).

Results

Comparison of ^{13}C Spectra. ^{1}H scalar-decoupled ^{13}C NMR spectra of 28 g/dL solutions of oxy- and deoxyhemoglobin A are presented in Figures 1a and 1b. Four signal areas are readily distinguished: carbonyl carbons, 15–25 ppm; aromatic carbons, 60–75 ppm; aliphatic carbons (primarily backbone α carbons), 120–150 ppm; and aliphatic carbons in the side chains, 150–200 ppm. The dipolar-decoupled deoxyhemoglobin A spectrum (Figure 1c) is indistinguishable from the scalar-decoupled spectrum, as evidenced by the absence of a signal in the difference spectrum (Figure 1d). Consistent with this was the finding that a proton-enhanced signal was not observed (Figure 1e) for deoxyhemoglobin A. Likewise, dipolar- and scalar-decoupled spectra of oxyhemoglobin A solutions were found to be indistinguishable, and a proton-enhanced ^{13}C oxyhemoglobin A spectrum was not obtained. These remarks also apply to spectra of 28 g/dL oxyhemoglobin S solutions.

The behavior of deoxyhemoglobin S solutions contrasts with that of the oxyhemoglobin A, deoxyhemoglobin A, and oxyhemoglobin S solutions in the following ways. First, the signal intensity observed in the scalar-decoupled spectrum of the 28 g/dL gel of deoxyhemoglobin S (Figure 2b) is significantly less than the intensities observed in the scalar-decoupled spectra of oxyhemoglobin A (Figure 1a), deoxyhemoglobin A (Figure 1b), and oxyhemoglobin S (Figure 2a). Quantitative comparison of the integrated signals in oxyhemoglobin

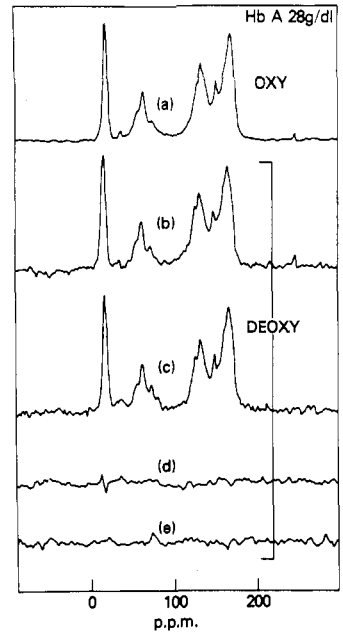

FIGURE 1: Comparison of spectra of a 28 g/dL preparation of hemoglobin A at 37 °C. (a) Oxygenated sample, 90° – t pulse sequence, t = 1 s, scalar decoupled, 32 768 transients accumulated; (b) deoxygenated sample, 90° – t pulse sequence, t = 1 s, scalar decoupled, 4096 transients accumulated; (c) deoxygenated sample, 90° – t pulse sequence, t = 1 s, dipolar decoupled, 2048 transients accumulated; (d) spectrum c minus spectrum b; (e) deoxygenated sample, proton enhanced, 1-ms Hartmann–Hahn matched contact, 2-s repetition time, dipolar decoupled, 2048 transients accumulated. Spectra have been normalized to compensate for differences in numbers of accumulations. Digital line broadenings of 20 Hz, in (a)–(c), and of 50 Hz, in (d) and (e), were employed to enhance sensitivity. The chemical shift scale is relative to external CS_2.

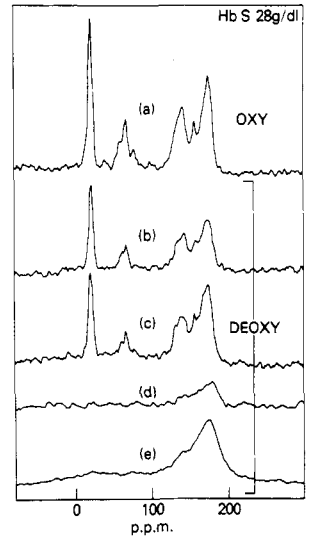

FIGURE 2: Comparison of spectra of a 28 g/dL preparation of hemoglobin S at 37 °C. (a) Oxygenated sample, 90° – t pulse sequence, t = 2 s, scalar decoupled; (b) deoxygenated sample, 90° – t pulse sequence, t = 2 s, scalar decoupled; (c) deoxygenated sample, 90° – t pulse sequence, t = 2 s, dipolar decoupled; (d) spectrum c minus spectrum b; (e) deoxygenated sample, proton-enhanced, 1-ms Hartmann–Hahn matched contract, 2-s repetition time, dipolar decoupled. Transients (4096) were accumulated in each case. Digital line broadenings of 20 Hz, in (a)–(c), and 50 Hz, in (d) and (e), were employed to enhance sensitivity. The chemical shift scale is relative to external CS_2.

S and deoxyhemoglobin S spectra (Figure 2a and 2b) showed that the signal intensity in the deoxy spectrum was only 0.58 of that measured in the oxy spectrum (i.e., 0.42 of the carbon did not contribute to the scalar-decoupled deoxy spectrum).

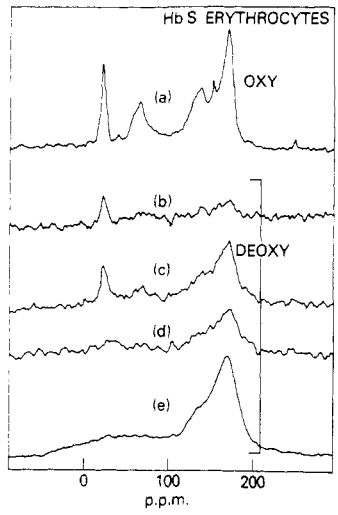

FIGURE 3: Comparison of spectra of sickle erythrocytes at 37 °C. (a) Oxygenated sample, 90° – t pulse sequence, t = 1 s, scalar decoupled, 8192 transients accumulated; (b) deoxygenated sample, 90° – t pulse sequence, t = 10 s, scalar decoupled, 2048 transients accumulated: (c) deoxygenated sample, 90° – t pulse sequence, t = 2 s, dipolar decoupled, 2048 transients accumulated; (d) spectrum c minus spectrum b; (e) deoxygenated sample, proton-enhanced, 1-ms Hartmann–Hahn matched contact, 2-s repetition time, dipolar decoupled, 8192 transients accumulated. Spectra have been normalized to compensate for differences in numbers of accumulations. Digital line broadenings of 20 Hz, in (a)–(c), and 50 Hz, in (d) and (e), were employed to enhance sensitivity. The chemical scale is relative to external CS_2.

Second, dipolar decoupling restores most of the aliphatic side-chain intensity missing from the scalar-decoupled deoxyhemoglobin S spectrum (Figure 2b). Signal intensity in the side-chain aliphatic region of the dipolar-decoupled deoxyhemoglobin S spectrum (Figure 2c) is 0.9 of the intensity observed in the scalar-decoupled oxyhemoglobin S spectrum (Figure 2a). Third, the difference spectrum (Figure 2d) of the dipolar- and scalar-decoupled deoxyhemoglobin S samples shows a signal in the aliphatic region. This spectrum represents material in the hemoglobin S gel that is not isotropically mobile and, hence, is not observed in the scalar-decoupled spectrum. Fourth, a strong proton-enhanced ^{13}C signal is obtained for the deoxyhemoglobin S (Figure 2e). In the aliphatic region, the proton-enhanced line shape is similar to the difference spectrum signal in Figure 2d. However, the signal intensity is ca. 2.5 times greater in the proton-enhanced spectrum. The proton-enhanced spectrum also shows a very broad featureless signal in the downfield region (–50 to 150 ppm) assigned to the carbonyl and aromatic carbons.

Spectral behavior qualitatively similar to that seen in Figure 2 was observed for hemoglobin S solutions having a concentration of 37 g/dL. However, significantly different quantitative results were obtained. At 37 g/dL the intensity observed in the scalar-decoupled deoxy spectrum was only 0.42 of the intensity observed in the scalar-decoupled oxyhemoglobin spectrum. In agreement with this result, the signal intensity of the difference spectrum (dipolar decoupled minus scalar decoupled) and the proton-enhanced deoxyhemoglobin S spectrum at 37 g/dL were each 1.6 times those observed for similar spectra at 28 g/dL (Figure 2d and 2e, respectively).

Intensity differences in the spectra of sickle erythrocytes (hemoglobin S concentrations ca. 37 g/dL) (Figure 3a–e) are also more pronounced than those observed in the 28 g/dL solution. A comparison of Figure 3b with Figure 3c shows that the intensity of the aliphatic signal in the dipolar-decoupled spectrum is ca. three times that in the scalar-decoupled

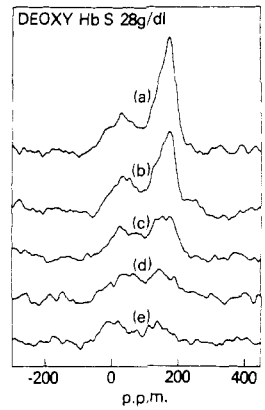

FIGURE 4: Partially relaxed, proton-enhanced dipolar-decoupled spectra of a 28 g/dL preparation of deoxygenated hemoglobin S at 37 °C. The pulse sequence by Torchia (1978) was used with delay times of (a) 0.05, (b) 0.25, (c) 0.75, (d) 2, and (e) 5 s. Transients (2048) were accumulated in each case, and a digital line broadening of 200 Hz was used. A 2-s delay was allowed for recovery of proton polarization in each case. The chemical shift scale is relative to external CS_2.

spectrum. This large difference in signal intensity is manifest in the intense signal observed in the difference spectrum (Figure 3d). As was found in the hemoglobin solutions, the proton-enhanced signal (Figure 3e) is over twice the signal observed in the difference spectrum (Figure 3d). The line shape of the proton-enhanced spectrum of the erythrocytes (Figure 3e) is like that observed in the cell-free preparation (Figure 2e).

T_1 Estimates. The T_1 values for rotationally restricted hemoglobin S were estimated from partially relaxed proton-enhanced spectra. The pulse sequence used to obtain the partially relaxed spectra (Figure 4a–e) has been described in detail elsewhere (Torchia, 1978). The decay of polarization depicted in Figure 4 is not exponential, since individual carbon signals are not resolved in the protein, and, in general, spin–lattice relaxation in solids is anisotropic (Gibby et al., 1972; Mehring, 1976). In spite of the complexity of the partially relaxed spectra, it is seen that the decay of polarization, as one expects, is most rapid in the region assigned to the side-chain aliphatic carbons and slowest for the region assigned to the backbone carbonyl carbons. The decay of polarization observed for hemoglobin in deoxygenated erythrocytes had the same time dependence shown in Figure 4 for the deoxygenated 28 g/dL cell-free preparation.

The intensities of the aliphatic carbon signals for oxyhemoglobin S and deoxyhemoglobin S in the scalar-decoupled spectra do not change when the time interval between successive $\pi/2$ pulses is lengthened from 2 to 10 s. This demonstrated that the "z" magnetization of the isotropically mobile hemoglobin S has essentially returned to equilibrium within a 2-s period.

Cross-Polarization Dynamics. Proton-enhanced spectra of a 28 g/dL deoxyhemoglobin S preparation are shown in Figure 5 as a function of the Hartmann–Hahn matched cross-polarization contact time. An examination of Figure 5 shows that the enhancement of the backbone C^{α} region is attained after ca. 50 μs of contact. The side-chain aliphatic and backbone carbonyl regions achieve their maximal enhancements after ca. 1 ms of contact. Proton-enhanced spectra of deoxygenated sickle erythrocytes showed the same time dependence depicted in Figure 5 for the 28 g/dL solutions.

Measurements at 4 °C. The spectra discussed so far were measured at 37 °C. At 4 °C the spectral properties of the 28 g/dL sample of deoxyhemoglobin S were like those of hemoglobin A and oxyhemoglobin S in that (a) the intensities

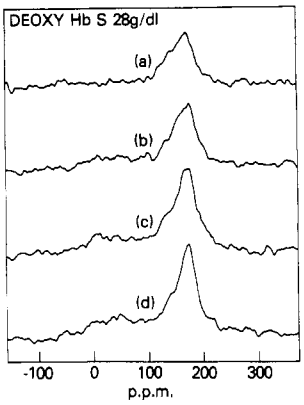

FIGURE 5: Proton-enhanced dipolar-decoupled spectra of a 28 g/dL preparation of deoxygenated hemoglobins S at 37 °C obtained with matched Hartmann–Hahn contacts of (a) 0.05, (b) 0.2, (c) 1, and (d) 3 ms. Transients (8192) were accumulated in each case with a repetition time of 2 s and a digital line broadening of 100 Hz. Samples were contained in 5-mm tubes as described under Materials and Methods. The chemical shift scale is relative to external CS_2.

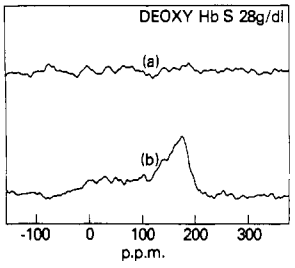

FIGURE 6: Comparison of proton-enhanced dipolar-decoupled spectra of a 28 g/dL preparation of deoxygenated hemoglobin S obtained at (a) 4 and (b) 37 °C. In each case a matched Hartmann–Hahn contact of 1 ms, a repetition time of 2 s, and digital filtering of 100 Hz were employed. Transients (8192) were accumulated on a sample in a 5-mm tube (see Materials and Methods). The chemical shift scale is relative to external CS_2.

of the dipolar- and scalar-decoupled spectra were the same and (b) a proton-enhanced spectrum was not observed (Figure 6a). On increasing the deoxyhemoglobin S sample temperature to 37 °C, a proton-enhanced spectrum was obtained (Figure 6b), and the general spectral behavior of the deoxyhemoglobin S sample was as shown in Figure 2. Cooling the sample again to 4 °C resulted in the loss of the proton-enhanced spectrum. The latter measurement was completed within 1 h after the temperature had been lowered.

The fully reversible changes in spectral properties with temperature, just described for the 28 g/dL deoxyhemoglobin S preparation, were not observed for the deoxygenated sickle erythrocytes. After incubation of the cells at 37 °C, the intensity in the dipolar-decoupled spectrum obtained at 4 °C was several times greater than the intensity measured in the scalar-decoupled spectrum. A proton-enhanced spectrum was also obtained at 4 °C which had the same line shape observed in the 37 °C spectrum (Figure 3e). These spectral characteristics remained essentially the same when the measurement was repeated after keeping the cells at 4 °C for 1 day.

Discussion

During the last several years much progress has been made in understanding the structure of the deoxyhemoglobin S gel, in cell-free preparations and in the cell. Electron diffraction results (Finch et al., 1973; Ohtsuki et al., 1977; Dykes et al., 1978), although differing in detail, suggest a microtubular structure for the paracrystalline gel. A crystal structure can also be obtained under certain conditions (Wishner et al., 1975; Pumphrey & Steinhardt, 1977). Progress has also been made in understanding the mechanism of gelation in concentrated solutions. Kinetic studies of the aggregation of deoxyhemoglobin S solutions (Malfa & Steinhardt, 1974; Moffat & Gibson, 1974; Hofrichter et al., 1974) have been interpreted in terms of a nucleation-controlled polymerization model (Hofrichter et al., 1975, 1976a,b). Thermodynamic analyses (Magdoff-Fairchild et al., 1976; Ross et al., 1977; Pumphrey & Steinhardt, 1977) confirm the earlier suggestion (Minton, 1974) that the polymerization may be considered as a phase transition with an insoluble polymer in chemical equilibrium with the concentrated monomeric deoxyhemoglobin S surrounding it.

The intracellular gelation of deoxyhemoglobin S presumably causes the deformation of the cell termed "sickling" (Dean & Schechter, 1978). Morphological (Rampling & Sirs, 1973; Zarkowsky & Hochmuth, 1975; Hahn et al., 1975) and rheological (Messer & Harris, 1970; Chein et al., 1975) measurements have been used to follow sickling. Magnetic resonance spectroscopy is a useful adjunct to these methods of studying the polymerization process. Proton nuclear resonance measurements so far have been used to identify surface histidine residues which differ between oxy- and deoxyhemoglobin S (Ho et al., 1975) and to study changes in water relaxation times upon gelation (Cottam et al., 1974; Lindstrom et al., 1976; Zipp et al., 1976). These methods, however, do not provide quantitative information about either the amount of polymer formed or the molecular dynamics in the polymer state. In contrast, the ^{13}C spectra reported here are sensitive to rotational motion of hemoglobin and thus directly probe molecular dynamics and relative hemoglobin content within solution and polymer phases. Neither chemically modified hemoglobin nor detailed spectral assignments are required; further, a particular form of the polymer need not be assumed.

Quantitative Analysis of NMR Data. The NMR measurements are sensitive to the existence of polymer as is demonstrated by two sets of observations made on deoxygenated hemoglobin S both in cells and in cell-free preparations at 37 °C. First, the proton-enhanced ^{13}C NMR spectra (Figures 2e and 3e) are similar to the spectrum (not shown) of dried polycrystalline hemoglobin. Second, the intensity of the dipolar-decoupled spectra of the aliphatic side-chain carbons (Figures 2c and 3c) is greater than that of the corresponding scalar-decoupled spectra (Figures 2b and 3b). On the other hand, in those cases (oxy- and deoxyhemoglobin A, in cells or cell-free solution, or oxyhemoglobin S in cells or cell-free solution) where previous experiments have shown that a polymer does not form, a proton-enhanced spectrum was not detected nor was an intensity difference measured between scalar- and dipolar-decoupled spectra (Figures 2a and 2b). These observations are consistent with the expectations that the cross-polarization procedure should detect *only* polymer, the scalar-decoupling procedure should measure *only* free monomer, and the dipolar-decoupling procedure should record the *sum* of monomer plus polymer.

From ^{13}C NMR spectra, the mass fraction of immobilized hemoglobin can be determined, provided certain conditions are fulfilled. We shall now discuss these conditions in terms of the two methods, A and B, applied.

Method A. In this procedure, aliphatic carbon signal intensity measured in the scalar-decoupled spectrum of oxy material is compared with aliphatic carbon signal intensity measured in the scalar-decoupled spectrum of an equal volume of deoxyhemoglobin S at the same concentration. Since only motionally free hemoglobin will give rise to scalar-decoupled

spectra, the intensity difference between the two spectra yields the mass fraction of immobilized material provided certain conditions are satisfied. First, the amount of immobilized material in the oxy form must be known. Both the dipolar-decoupled and proton-enhanced spectra indicate that there is no detectable immobilized material in the oxy state. Second, the effect of the deoxyhemoglobin paramagnetism on the spectra must be determined. A sizable portion of the hemoglobin signal could be lost due to dipolar broadening by the paramagnetic Fe^{2+} center, thereby leading to an overestimate of the amount of polymerized material. This is not the case since the absolute signal intensities of oxy- and deoxyhemoglobin A (both as cell-free preparations and as intact erythrocytes) are the same. Apparently, the electron T_1 is short enough to decouple the electron–nucleus interaction, thereby eliminating significant line broadening in the ^{13}C spectrum of monomeric deoxyhemoglobin S. An extremely short electron T_1 is consistent with the observation that deoxyhemoglobin A solutions do not exhibit an electron paramagnetic resonance spectrum (H. Kon, private communication).

A third type of error would arise if the relaxation times of rotationally free hemoglobin in the presence of polymer were sufficiently long as not to have substantially relaxed in 2 s, the time delay between successive $\pi/2$ pulses. As the intensity in the aliphatic region of scalar-decoupled hemoglobin spectra is the same when 2- and 10-s delays between pulses are used, it is apparent that the aliphatic region of the spectrum has relaxed completely within 2 s. The comparison of scalar-decoupled signal intensity in the aliphatic region should thus provide an accurate estimate of the mass fraction of immobilized material.

Method B. In this procedure the side-chain aliphatic carbon signal intensity measured in the dipolar-decoupled spectrum of deoxyhemoglobin S is compared with the corresponding signal intensity measured in the scalar-decoupled spectrum of the same sample. As with method A, several potential sources of error are present. First, the signal intensity in the dipolar-decoupled spectrum will reflect the sum of polymer and monomer only if the spin–lattice relaxation times of all the carbons in the polymer phase are short compared with the period between pulses (usually 2 s). As discussed, this condition is met for the protonated aliphatic carbons in the monomer. Comparison of the aliphatic signal intensity in Figure 4 shows that the side-chain aliphatic signal recovers 80–90% of its equilibrium value in 2 s. Hence, in the dipolar-decoupled spectrum, obtained with a 2-s delay, about 15% of the upfield aliphatic polymer signal is lost due to incomplete relaxation. Another potential loss of polymer signal can arise if severe line broadening results from the electron–nucleus interaction in the polymerized deoxy sample. Although we cannot precisely determine the magnitude of the signal loss due to paramagnetic broadening, the observation of almost equal intensities in the upfield aliphatic regions in Figure 2a,c indicates that it does not exceed 10%.

Degree of Polymerization in Deoxygenated Cell-Free Preparations and Erythrocytes at 37 °C. The fractions of hemoglobin S in the polymer phases in two preparations of deoxyhemoglobin S, as determined by methods A and B, are listed in Table I. In accordance with the considerations just discussed, method B gives an underestimate of the fraction polymerized, f_p, at each concentration. The ratio of f_p at 37 g/dL to f_p at 28 g/dL is 1.38 according to method A and 1.29 according to method B. Within experimental uncertainity, these results are in agreement with the ratio of the hemoglobin S masses (1.4) pelleted in centrifugation experiments (Ross et al., 1977) at the same concentrations. However, at each concentration the value of f_p calculated with method A (Table I) is only about 0.7 of the fraction of hemoglobin S found in the pellet in the centrifugation experiment. The reason for this large discrepancy is not clear but is currently under investigation.

For the erythrocyte preparations, method B was employed to determine the fraction of polymerized material (Table I). A pulse-repetition time of 10 s was employed to reduce losses due to saturation of the polymer magnetization. Although the nature of the physical state of high concentrations of hemoglobin S is difficult to characterize, the NMR results for the 37 g/dL cell-free preparation and the erythrocytes (37 ± 3 g/dL) confirm the results of Ross et al. (1977), showing that the amount of polymer formed is markedly dependent on concentration. We emphasize that the erythrocyte results are preliminary and that accurate determination of the fraction of polymer requires the use of well-defined cell populations and characterization of the NMR behavior of nonhemoglobin carbons in the cell. About 10% of the dry mass of the red cell is not hemoglobin (Harris & Kellermeyer, 1970).

Rotational Mobility of Free and Polymerized Hemoglobins. Using a ^{13}C NMR procedure described elsewhere (Lyerla & Levy, 1976) we have determined, from backbone carbon T_1 measurements, that the rotational correlation time, τ_R, of nongelling hemoglobin A at 37 °C is ca. 3×10^{-8} s. The broad, featureless signal in the downfield (carbonyl–aromatic) region of the spectrum of the hemoglobin S polymer (the observed line shape is, within uncertainty due to noise, indistinguishable from that of dried, polycrystalline hemoglobin S) indicates that chemical shift anisotropy and ^{14}N–^{13}C static dipolar coupling are not averaged by molecular motion in the polymer. Hence, the correlation times of the polymer backbone must exceed 10^{-4} s. The short time required to transfer polarization (50 μs) to the C^{α} carbons in the cross-polarization experiment (Figure 5) also indicates a rigid polymer backbone, on the 10^{-4} s time scale. Gelation, therefore, results in a reduction in the rate of molecular rotation by at least 4 orders of magnitude. It is interesting in this regard that the spectra of free hemoglobin is not substantially different in the presence and absence of polymerized material (cf. Figure 2b and 2a). This shows that the rotation of the monomeric hemoglobin is virtually unaffected by the presence of polymer (except possibly at very high hemoglobin concentrations). To a first approximation, the monomer and polymer exist as two separate phases, a model previously proposed by Minton (1974). Since rotational diffusion is relatively unaffected by the polymer, it follows that in this system the microscopic viscosity is independent of the macroscopic viscosity, which itself increases by orders of magnitude upon deoxygenation (Chien et al., 1976).

Experimental Limitations. Although the procedures discussed in this report should permit hitherto impossible measurements of the intracellular polymerization of hemoglobin S, a number of important limitations of these techniques should be noted. Foremost is the requirement, imposed by the low natural abundance of ^{13}C, of relatively long data accumulation times. This problem is partly circumvented by the sensitivity enhancement provided by cross-polarization. Even so, proton-enhanced spectra of the polymer require at least 15 min if a good signal/noise ratio (10:1) is to be achieved. Thus, in kinetic studies, experimental conditions must be chosen so as to permit measurements to be made over a period of hours. Signal to noise limitations will also produce ex-

perimental uncertainties of about 10% in the determination of the fraction of polymerized hemoglobin. This rules out the study of aggregated species present at low concentration as might occur during pregelation aggregation. While it is conceivable that careful T_1 measurements may someday permit distinctions to be made between polymers having different structures, such distinctions are not possible at present on the basis of spectral line shapes; polymerized, lyophilized, and crystalline [a gift from Professor J. Steinhart (Georgetown University, Washington, D.C.)] hemoglobin all manifest the same line shapes.

Potential for Further Studies. Notwithstanding the above-mentioned limitations, these double-resonance techniques possess considerable potential for the study of hemoglobin gelation in solution and in erythrocytes. The extent of polymerization can be investigated as a function of oxygen saturation, temperature, hemoglobin concentration, and ionic strength. Such studies can be carried out in the presence of inhibitors and in mixtures of varying hemoglobin composition.

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