in Figure 1b (see below), a Gaussian resonance in the absence of motion and with a second moment $\Delta\omega_0^2$ is narrowed by any motions which modulate the anisotropic broadening interactions. The result is a Lorentzian line with a half-intensity half-width δ given by

$$\delta = \Delta\omega_0^2\tau_c/2\pi \text{ (in Hz)} \quad (1)$$

where τ_c is the correlation time of the motion.

For a rigid solid such as HAP, the relevant correlation time τ_c is that for rotational reorientation of the solid particle in a colloidal suspension.

The Stokes-Einstein equation for a sphere of radius a immersed in a continuous medium of viscosity η can be used to estimate this rotational correlation time:

$$\tau_c = 4\pi\eta a^3/3kT \quad (2)$$

Assuming values of a = 250 Å (for a 500-Å-long particle) and η = 1 cP (for water at 20 °C)[7] yields a value for τ_c at 20 °C of 1.6×10^{-5} s. Since this estimate results in $(\Delta\omega_0^2)^{1/2}\tau_c = 0.08 \ll 1$, the criterion for motional narrowing is met,[3] and eq 1 is applicable.

Substituting the estimated τ_c and the measured $\Delta\omega_0^2$ into eq 1 yields δ = 61 Hz, compared to the observed δ = 36 Hz. The calculated line width would become smaller if the shorter correlation time for motion around the long (500 Å) axis were taken into account, but the quantitative expression for δ would become more complicated. In view of the possibility of incomplete disaggregation and particle size dispersion, we consider the observed agreement in line widths satisfactory. Further sharpening should be achievable by lowering η, either by raising the temperature or by using a lower viscosity liquid.

The measured isotropic chemical shift of HAP, 2.8 ppm, agrees with the value measured for other hydroxyapatite samples by ^{31}P magic-angle sample spinning methods;[8] it differs somewhat from the value of ca. 6 ppm observed[6] for aqueous PO_4^{3-}.

The use of colloidal suspensions serves to sharpen the NMR signals of surface-adsorbed species as well as of the colloidal solid itself. The peak at 19 ppm in Figure 1c arises from EHDP molecules chemisorbed on the HAP surface. The ^{31}P NMR spectrum of this sample *before* ultracentrifugation and resuspension (Figure 1b) exhibits a sharp component at 19.1 ppm superimposed upon a broad component. The sharp component arises from free EHDP molecules in solution and the broader component from chemisorbed EHDP that is not in rapid exchange with free molecues in solution. Apparently chemisorption on the HAP surface does not result in a detectable change in the ^{31}P chemical shift of EHDP.

The assignment of the broader component at 19 ppm to chemisorbed EHDP is supported by the ^{31}P NMR spectrum of another sample of colloidal HAP in which the EHDP resonance showed only the broader component. The amount of EHDP added to this sample and the measured surface area of the HAP implied that about 36 Å^2 of HAP surface was available for each EHDP molecule (incomplete suspension of all the HAP introduces some error). This is a plausible value for a monolayer coverage and suggests that the broader component arises from all of the chemisorbed EHDP.

We have obtained spectra with better signal/noise at 121.5 MHz on a Bruker CXP-300 in which the bound EHDP has a half-height width of about 660 Hz. This line width is plausible in view of the expected larger second moment of solid EHDP compared to that of HAP. The colloidal HAP line width is also greater than that observed at lower field because of a measurable phosphorus chemical shift anisotropy.[8]

As shown here, the use of colloidal suspensions offers a means of obtaining high-resolution NMR spectra of selected solids and surface-adsorbed species using conventional high-resolution NMR equipment.

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Effect of DNA Molecular Weight, Temperature, and Magnetic Field Strength on the ^{31}P NMR Results of DNA Complexed with Ethidium

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Because of its sensitivity to phosphate bond and tortional angles,[1] ^{31}P NMR spectroscopy should be widely applicable in conformational studies of nucleic acids. Analysis of smaller molecules such as tRNA and synthetic complementary deoxynucleotide segments has yielded information on the structure of these molecules and how their conformation changes with factors such as temperature, pH, and bound ligands.[1a,2] Recently ^{31}P NMR spectroscopy has been applied to higher molecular weight DNA samples such as synthetic double helical deoxypolynucleotides,[3] nucleosomes,[4] viruses,[5] and low[6] and high molecular weight[7] DNA fragments. Application of ^{31}P NMR spectroscopy to the analysis of the interaction of DNA with intercalating ligands has produced somewhat conflicting results.[8,9] Hogan and Jardetsky found that the ^{31}P chemical shift of DNA did not change, and the area was totally lost as the DNA was titrated to saturation with ethidium.[9] Jones and Wilson studied ethidium and several other intercalating ligands and found downfield shifts for the ^{31}P resonance of DNA in the presence of these compounds and no significant area loss.[8] The downfield shifts were best correlated with the unwinding angle produced by the intercalating ligand.[8]

In an effort to explain the apparently different results with the important intercalating drug, ethidium, we sonicated DNA for different time periods and fractionated the samples by gel exclusion chromatography.[10] We have obtained for the first time ^{31}P spectral results for a high molecular weight (1400 base pairs) DNA complex with an intercalating ligand. These results are reported here, and are compared to a low molecular weight DNA sample (190 base pairs), as a function of ionic strength, temperature, magnetic field strength, and ethidium to DNA ratio.[11] The chemical shifts, T_1 values, and line widths at 24.15 MHz for the low and high molecular weight fractionated DNA samples as a function of added ethidium are collected in Table I. As can

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Table I. ^{31}P NMR Results for DNA Samples of Different Molecular Weight

ν[a]	chemical shift, ppm[b]	line width, Hz[c]	T_1, s[d]
	Low Molecular Weight DNA		
0	4.30	16	2.2
0.16	4.20	26	
0.30	4.07	32	
0.45	3.76	39	
0.60	3.73	45	2.3
0.72	3.69	50	2.3
	High Molecular Weight DNA		
0	4.3	40	2.2
0.15	4.2	70	
0.29	3.9	80	
0.45	3.8	95	2.3
0.60	3.7	120	
0.72	3.7	140	2.3

[a] The ratio of moles of ethidium added per mole of DNA base pairs. [b] Chemical shifts are measured relative to internal trimethyl phosphate and all shifts reported in this table are upfield from trimethyl phosphate. All results are at 24.15 M Hz, 30 °C, and in PIPES 10 buffer. [c] Line widths at half-peak-height. [d] Determined by the inversion recovery method. Because of the time required, T_1 values were not determined at each ratio.

be seen, the chemical shift changes and T_1 values are similar for the two DNA samples but the line widths for the higher molecular weight sample are greater. The fractionated DNA samples were also analyzed at higher magnetic field strength, and the results for the low molecular weight sample are shown in Table II. The chemical shift changes are quite similar for the two field strengths but the line widths and T_1 values are greater at higher field as expected.[6d,7a] Because of the extensive broadening of the ^{31}P signal at high molecular weight and high field strength in the presence of ethidium, it is difficult to obtain accurate results at high field strength, and only results from the low molecular weight sample are included in Table II. Changing ionic strength over a range 0.02–0.2 by adding sodium chloride had a negligible effect on these results. Gorenstein[1a] has studied the interaction of ethidium with Poly A·oligoU and finds, in agreement with our results, that ethidium causes downfield shifts of the ^{31}P signals with this double helical nucleic acid. Gorenstein[1a] has also pointed out that downfield shifts of 0.5–1.0 ppm in the DNA ^{31}P signal are expected on the basis of the predicted changes in the double helix tortional angles produced by intercalating drugs like ethidium.

Table II. ^{31}P Results for Low Molecular Weight DNA at 101.2 MHz

ν[a]	chemical shift, ppm	line width, Hz	T_1, s
0	4.25	53	3.7
0.2	4.08	99	3.6
0.4	3.82	123	3.8
0.7	3.67	143	3.6

[a] All quantities and experimental conditions not listed are as in Table I. These results are for unfractionated DNA. Samples analyzed with fractionated low molecular weight DNA gave similar results.

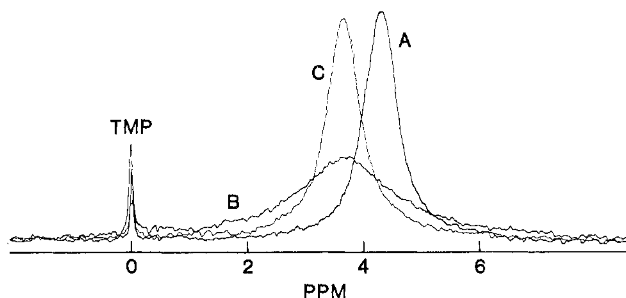

Figure 1. ^{31}P spectral results at 24.15 MHz in PIPES 10, 0.01 M DNA base pairs, and 10-mm NMR tubes are illustrated. The spectra are referenced to trimethyl phosphate (TMP): (A) DNA alone at 30 °C, (B) DNA with a ratio of 0.7 mol of ethidium per mole of DNA base pairs at 30 °C, and (C) the same ethidium–DNA sample at 60 °C. The terminal phosphate signal is near 0 ppm and is separated from the TMP line at 60 °C. Spectra were obtained after 4000 scans with fast Fourier transformation of 8192 time domain points, a 90° pulse, 13-s delay time, broad band proton decoupling, and 0.5-Hz line broadening.

Both the high and the low molecular weight samples were examined at several temperatures in the presence of saturating concentrations of ethidium bromide. At low field the chemical shift of the high molecular weight DNA sample did not change significantly, T_1 decreased from 2.3 to 1.8 s, and the line width decreased from 140 to 41 Hz as the temperature was increased from 30 to 60 °C. Over the same temperature range the chemical shift and T_1 of the low molecular weight DNA behaved as with the high molecular weight sample, and the line width decreased from 50 to 16 Hz. These line width and chemical shift changes are illustrated in Figure 1 with spectra for low molecular weight DNA at 30 °C and a saturated ethidium–DNA complex at 30 and 60 °C. Temperature experiments at high field produced changes of similar magnitude. For example, an ethidium–low molecular weight DNA sample with a ν of 0.4 had a line width of 120 Hz at 30 °C, 74 Hz at 60 °C, and a chemical shift of 3.8 ppm across the range 30–60 °C. Provided that the signal to noise was high enough to clearly establish the ^{31}P signal base line, as in Figure 1, there was no significant area loss in any of these experiments with added ethidium. For this ionic strength and DNA concentration, the T_m of this DNA is over 80 °C and the T_m of the DNA–ethidium complex is over 90 °C. When DNA begins to melt, T_1 increases by several seconds and the line width decreases to less than 5 Hz,[6a] confirming that all of the results presented here are below the melting region of this DNA.

These results illustrate that the ^{31}P line width in DNA increases with decreasing temperature, increasing field, increasing molecular weight, and increasing molar ratio of ethidium bromide. The area of a ^{31}P peak will apparently decrease as the line becomes so broad as to decrease the signal intensity to near the noise level. Hogan and Jardetsky[9] were at higher field, lower temperature, and higher

(10) All experiments were conducted in PIPES buffer (0.01 M piperazine-N,N'-bis(2-ethanesulfonic acid); 0.001 M EDTA; pH 7.0 with NaCl added to the desired ionic strength). For sonication 1 g of calf thymus DNA (Worthington Biochemical Corp.) was dissolved in 100 mL of PIPES buffer with 0.5 M NaCl. Sonication was conducted under N_2 at 0–3 °C by using a Heat Systems 375W sonicator with a high gain Q horn, full power, and a 10% pulse cycle to prevent heating. Initially the samples were sonicated twice for 15 min (1.5 min sonication time) followed by 1 h of stirring to facilitate soluton of the DNA. A high molecular weight DNA sample was prepared with a 60-min sonication time (6 min total sonication) and a low molecular weight sample with a 600-min sonication time (60 min total sonication). Both samples were then filtered through a 0.45-μm Millipore filter, precipitated with one volume of ethanol at 0 °C, collected by centrifugation, dissolved in PIPES buffer with 0.1 M NaCl (PIPES 10), and dialyzed against the same buffer. The DNA concentration at this point was near 0.05 M DNA phosphate/l. Two to three milliliters of both DNA samples were applied to a Sepharose CL-4B column (2.5 × 90 cm) and eluted with PIPES 10. Five milliliter fractions were collected and analyzed by polyacrylamide gel electrophoresis (Maniatis, T.; Jeffrey A.; van de Sande, H., *Biochemistry* **1975**, *14*, 3787) against ΦX174-Hae III and λ-Hind III restriction enzyme fragments from BRL Inc. As expected, the low molecular weight sample which is near the sonication limit was much more homogeneous. The center peak tubes (approximately 1/2 of the low molecular weight and 1/3 of the high molecular weight DNA sample) were pooled, made 0.3 M in sodium acetate, precipitated with two volumes of ethanol at 0 °C, collected by centrifugation, and dissolved and dialyzed against PIPES 10. These samples were characterized by polyacrylamide (7 and 10%) and 1.2% agarose gel electrophoresis against both ϕX and λ fragments. Two-thirds of the high molecular weight material was between 1200 and 1700 base pairs, with the peak at 1400 base pairs. Two-thirds of the low molecular weight sample was between 170 and 210 base pairs with the peak at 190 base pairs in excellent agreement with our previous results.[8] The concentration of both samples was near 0.01 M base pairs in PIPES 10 buffer. For NMR experiments they were lyophilized and redissolved in the same volume of 99.8% D_2O containing 0.01% trimethyl phosphate as an internal reference.

(11) NMR spectra were obtained in 10-mm NMR tubes (Wilmad) on either a JEOL FX60Q (24.15 MHz for ^{31}P) or a Bruker WM250 (101.2 MHz for ^{31}P) NMR spectrometer with quadrature detection. Other conditions were as previously reported.[8]

molecular weight than Jones and Wilson,[8] and this might explain why they incorrectly observed an apparent total area loss while Jones and Wilson did not. The erroneous area observation led Hogan and Jardetsky[9] to incorrectly propose that the internal motion of the DNA double helix backbone is essentially stopped when ethidium intercalates. The results reported here and earlier[8] for the DNA ^{31}P resonance in the DNA–ethidium complex are consistent with significant motion of the phosphate in the complex and with intermediate or fast exchange (depending on interpretation of the line widths) of ethidium among sites.

The ^{31}P NMR linewidth in DNA samples such as those used in these studies depends on correlation time through both dipole–dipole and chemical shift anisotrophy relaxation.[6b,6d,7a] There is probably also a contribution from chemical shift heterogeneity[3c,6d,7a] which could increase with added ethidium. Addition of ethidium could cause lifetime broadening of DNA ^{31}P NMR signals which, along with changes in correlation time, might account for the large observed changes in line width with temperature. Detailed studies to evaluate the importance of all of these factors with a broad range of DNA–ligand complexes are in progress. We have illustrated, however, that ^{31}P spectra can be obtained, even at high molecular weights, for DNA and its intercalation complexes in solution. This technique should prove to be a valuable tool in studying DNA–ligand interactions.

Acknowledgment. This research was supported by National Institutes of Health Grant CA 24454 and RR 09201.

A Hexanuclear Iron–Sulfide–Thiolate Cluster: Assembly and Properties of $[Fe_6S_9(S\text{-}t\text{-}C_4H_9)_2]^{4-}$ Containing Three Types of Bridging Sulfur Atoms

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Received June 1, 1981
Revised Manuscript Received August 19, 1981

The synthetic and biological chemistry of iron–sulfide–thiolate clusters, until lately, has been confined to bi- and tetranuclear species having planar $[2Fe\text{-}2S]^{2+,+}$ and cubane-type $[4Fe\text{-}4S]^{3+,2+,+}$ core units, respectively, capable of existence in multiple oxidation levels.[1-4] Variations on these structural themes, viz., core S/Se atom exchange[5] and terminal thiolate ligand substitution with nonsulfur ligands,[6] have been accomplished with the synthetic clusters $[Fe_nS_n(SR)_4]^{2-}$ (n = 2, 4). However, only upon the very recent spectroscopic detection of protein 3-Fe sites,[7] and crystallographic demonstration of the cyclic [3Fe–3S] core structure

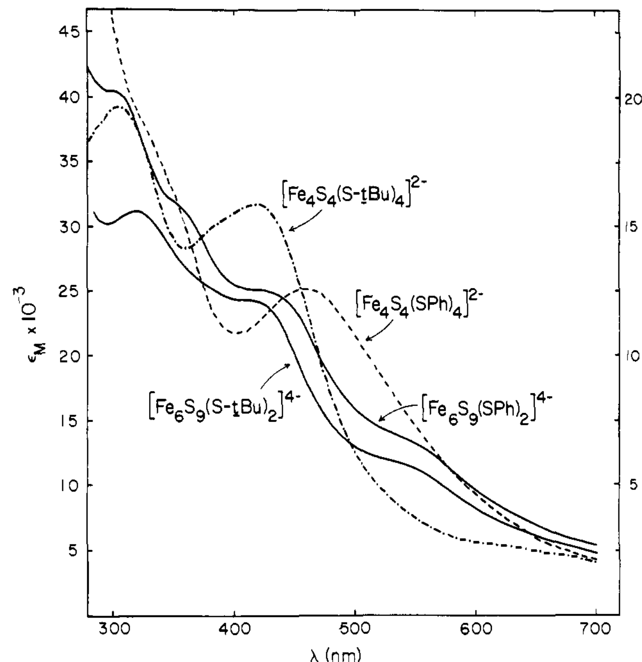


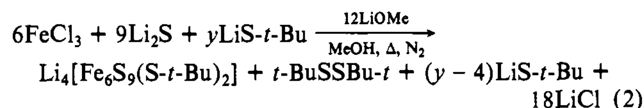
Figure 1. Absorption spectra in Me_2SO solutions: (a) authentic $[Fe_4S_4(S\text{-}t\text{-}Bu)_4]^{2-}$, right-hand ϵ_M scale; (b) the product of reaction 2, $[Fe_6S_9(S\text{-}t\text{-}Bu)_2]^{4-}$, after isolation as its $Me_3NCH_2Ph^+$ salt; (c) solution b + 2.0 equiv of PhSH, $[Fe_6S_9(SPh)_2]^{4-}$; (d) solution b + 27.5 equiv of PhSH, $[Fe_4S_4(SPh)_4]^{2-}$. Spectra b–d refer to left-hand ϵ_M scale, with 1.5 × ϵ_M plotted for spectrum d.

in *A. vinelandii* ferredoxin (Fd) I,[8] has a fundamentally different Fe–S core structure emerged. These findings together with the possible occurrence of "unconventional" clusters $[Fe_nS_m(S\text{-}Cys)_x]$ (n, $m \neq$ 2, 4 and/or $x < n$) in other proteins (e.g., *D. africanus* Fd III[9] and Mo–Fe–S protein,[10] certain hydrogenases[11]) have directed our attention toward the generation of heretofore unrecognized Fe/S/SR clusters.

While investigating the effect of reactant mole ratio variation on the standard cluster assembly system (eq 1),[12] it was observed

$$4FeCl_3 + 4Li_2S + 6LiSR \xrightarrow[N_2]{MeOH} Li_2[Fe_4S_4(SR)_4] + RSSR + 12LiCl \quad (1)$$

that in the presence of excess LiOMe an intensely colored filtrate persisted after precipitation of the sparingly soluble $n\text{-}Bu_4N^+$ salt of the R = t-Bu cluster. From this filtrate a solid of composition $(Me_3NCH_2Ph)_4[Fe_6S_9(S\text{-}t\text{-}Bu)_2]\cdot MeOH$ (**1**), confirmed by crystallography, was isolated. Thereafter the reaction system (eq 2) was devised in order to improve yields by repressing tetramer formation. When conducted with the indicated stoichiometry



$$6FeCl_3 + 9Li_2S + yLiS\text{-}t\text{-}Bu \xrightarrow[MeOH,\ \Delta,\ N_2]{12LiOMe} Li_4[Fe_6S_9(S\text{-}t\text{-}Bu)_2] + t\text{-}BuSSBu\text{-}t + (y - 4)LiS\text{-}t\text{-}Bu + 18LiCl \quad (2)$$

(y = 4–8 equiv) a deep orange-brown solution is rapidly generated, which turns dark red brown upon stirring at ~55 °C for 1–2 h. Precipitation of the product with $(Me_3NCH_2Ph)Br$ followed by recrystallization from Me_2SO/MeOH yields black prisms of **1** (55–70%), whose absorption spectrum (Figure 1) distinguishes

†U.K. Science Research Council/NATO Postdoctoral Fellow, 1980–1981.

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