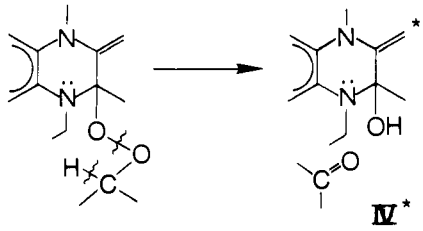
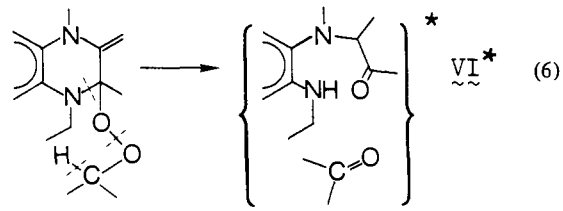
is due to energy transfer to protein-bound 6,7-dimethyl-8-(1′-D-ribityl)lumazine. It is, therefore, of interest that 6,7,8-trimethyllumazine behaves well as a fluorescer in the present system (curve C in Figure 1).

Replacement of H by D in II results in a decrease in $\Phi_{max}$ (for fluorescer rubrene, $\Phi^H/\Phi^D = 1.7$; for fluorescer $Fl_{ox}$, $\Phi^H/\Phi^D = 1.8$), but there is no change in the first-order rate constants for either the disappearance of II or the diminution in the intensity of light emission. This finding establishes that the C–H (D) bond in II is broken on generation of X* and that the formation of X* (like the formation of $Fl_{ox}$*) is not a major pathway in the decomposition of II. A number of possible candidates for X* have been considered. The $N^5$-ethylflavin pseudobase (IV), $Fl_{ox}$, and $N^5$-ethyl-10a-spirohyrantoin (V) are the major decomposition products of II.[11] Flavin pseudobase (as in IV) was proposed by



(5)

Hastings and Nealson to be the emitter for bacterial luciferase.[12] The $N^5$-ethylflavin pseudobases are nonfluorescent in solution and frozen solution[2] and have an emission maximum at too long a wavelength to be candidates for X*. Nor does X* represent excited V* (there is no energy transfer to added fluorescers when solutions of either pure $p$-$CH_3C_6H_4CHO$, V, or IV are photoexcited at their excitation maximum). Photoexcitation of the spent reaction solution (once CL is completed) at wavelengths between 250 and 450 nm in the presence of Rhodamine-B does not result in an enhanced fluorescence of the latter. Thus, compound X may represent a thermodynamically unstable molecule, although it is also possible that X* is quenched by a component formed in the spent reaction mixture after CL. Possible candidates for X* are the proposed[2c] Russell fragmentation products (VI*, or >C=O*, as n–$\pi$* states). VI may ring close to form IV, one



(6)

of the known products[11] at the end of the CL reaction. Our current experimental concerns are with the mechanisms by which the two excited species ($Fl_{ox}$* and X*) are formed and the identity of X.

**Acknowledgment.** This work was supported by a grant from the National Science Foundation. We thank Professors Therese Wilson, Cemal Kemal, and Peter J. Wagner for their comments and Professor Gerhard W. E. Plaut for a generous gift of 6,7,8-trimethyllumazine.

(11) Products were separated by high-performance LC, using a silica column (Lichrosorb S1-60) and eluting with acetonitrile (flow rate 1 mL/min, 17.3 psi): $N^5$-ethylflavin-10a-spirohydantoin, 83%, $R_t$ = 10.59 min; $N^5$-ethylflavin-4a-pseudobase, 14%, $R_t$ = 3.59 min; $Fl_{ox}$, 3%, $R_t$ = 7.30 min.

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**Peter T. Shepherd, Thomas C. Bruice***

*Department of Chemistry*
*University of California at Santa Barbara*
*Santa Barbara, California 93106*

*Received June 2, 1980*

## Effect of Intercalating Ligands on the $^{31}P$ Chemical Shift of DNA

*Sir:*

Recently, extensive $^{31}P$, $^1H$, and $^{13}C$ NMR studies of native double-stranded DNA have been reported for biopolymer samples with molecular weight reduced by sonication or nuclease digestion of chromatin.[1-5] The $^{31}P$ experiments have shown promise of providing conformational information about the structure of the double helix in both the solution[1-4] and solid states.[6] Gorenstein and co-workers[7] have proposed that $^{31}P$ chemical shifts in phosphodiesters such as DNA should be especially sensitive to the P–O torsional angles in the polynucleotide chain. $^{31}P$ NMR should then be an excellent method for solution conformational analysis of DNA. Somewhat surprisingly $^{31}P$ studies of DNA in nucleosome particles[4] and T4 bacteriophage[8] do not reveal any significant chemical shift changes relative to the purified DNA samples. An analysis of the factors which influence the $^{31}P$ chemical shift in DNA complexes is obviously of importance in interpreting NMR results on important biopolymer aggregates such as chromatin. For this reason we have prepared complexes of sonicated DNA with three well-characterized but quite different intercalating ligands, ethidium, quinacrine, and daunorubicin, and have characterized these complexes by $^{31}P$ NMR. Previous studies on intercalating ligand–nucleic acid complexes have utilized short self-complementary deoxyribo- and ribonucleotide segments.[9] These studies have not been conclusive about the magnitude of the $^{31}P$ chemical shift change induced by the ligands or even whether upfield or downfield shifts are expected.

The preparation and characterization of calf thymus DNA for $^{31}P$ NMR experiments involves extensive sonication with a high-power sonicator.[10] Treatment of this sample with S1 nuclease to remove any single-strand regions had no effect on the NMR spectra. A saturation complex of ethidium bromide (approximately 0.5 mol of ethidium bound per mol of base pair) was prepared,[11] and the $^{31}P$ NMR spectrum of this complex is shown

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(10) Calf thymus DNA was purchased from Worthington Biochemicals and prepared (extensive sonication) as previously described.[4] The final solution was dialyzed against PIPES buffer (0.01 M piperazine-$N,N'$-bis[2-ethanesulfonic acid]; 0.001 M EDTA; pH 7.0) and stored at 4 °C. For higher ionic strength experiments solid NaCl was added directly to this sample. The DNA was also extensively characterized by polyacrylamide gel electrophoresis (Maniatis, T.; Jeffrey, A.; van de Sande, H. *Biochemistry* **1975**, *14*, 3787). Electrophoresis in 5% polyacrylamide gels with TBE buffer (0.09 M Tris-borate, 2.5 mM EDTA, pH 8.3), using Hae III restriction fragments of $\phi X$ 174 DNA as markers, gave a band centered around 180 base pairs with approximately two-thirds of the DNA between 140 and 220 base pairs, in agreement with previous results.[4] Stopping the sonication earlier gave higher molecular weight DNA with a broader distribution of molecular weights. As the DNA reaches a limiting number of base pairs (~200 under our sonication conditions), it becomes more homogeneous around this limit. Electrophoresis on 7% urea denaturing polyacrylamide gels gave a band centered around 180 nucleotides, indicating that the DNA contains no significant amount of single-stranded or nicked regions.

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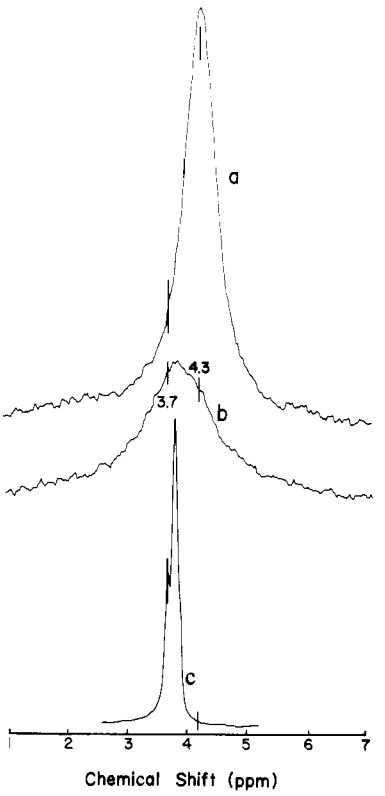


**Figure 1.** $^{31}P$ NMR spectra referenced to internal trimethyl phosphate of (a) native DNA at 30 °C (5000 transients), (b) a saturated ethidium–DNA complex at 30 °C (5000 transients), and (c) denatured DNA at 85 °C (1000 transients). The DNA concentration in all cases was approximately 0.05 M P/L and instrumental conditions were as specified in ref 11.

**Table I.** $^{31}P$ NMR Results for DNA in Different Conformational States

| | chemical shift, ppm | $T_1$,[a] s | line width at half-height, Hz |
|---|---|---|---|
| native DNA | 4.3 | 2.2 | 15 |
| denatured[b] DNA | 3.7–3.9 | 4–6 | ~2 |
| saturated Et–DNA complex | 3.7 | 2.1 | 45 |

[a] Determined by the inversion recovery method. [b] The spectrum for denatured DNA consists of several overlapping peaks and the values represent the range obtained for the denatured forms at 85 °C.

in Figure 1 along with similar spectra for uncomplexed native and denatured DNA. A summary of the results produced by ethidium binding and by denaturation of DNA is given in Table I. Both ethidium binding and denaturation produce downfield shifts of similar magnitude in the DNA $^{31}P$ NMR spectrum but other changes are distinctly different: (i) denaturation produces several narrow peaks while the ethidium–DNA complex has a single peak which is broader than the peak for native DNA; (ii) $T_1$ values for DNA increase markedly on denaturation while they are not significantly changed on ethidium binding; (iii) NOE values approach the full theoretical value on denaturation while they do not significantly change on complexation with ethidium. Current theories[1-6,12] of NMR suggest that, as a result of denaturation, the torsional angles in the sugar phosphate backbone undergo a change, resulting in a downfield shift, and the molecular mobility increases significantly, resulting in increased $T_1$ and NOE values. On complexation, it would seem that the torsional angles also change, resulting in a downfield shift, but that the molecular mobility remains similar to that of native DNA. This is consistent with denaturation models for the double helix which lead to a more flexible single-stranded species[13] and with intercalation models which predict unwinding of the double helix while maintaining its rigidity and hydrogen bonding.[13,14]

These results suggest that unwinding of the sugar phosphate chain of DNA may be a primary factor in the $^{31}P$ chemical shift changes induced as a result of ethidium binding. To test this hypothesis, we prepared saturation complexes of DNA with the intercalating acridine quinacrine and the intercalating anthracycline daunorubicin. We have quantitatively determined unwinding angles for these drugs, using the Vinograd method with ethidium as a standard.[15] The unwinding angles, which cover the range from the smallest to the largest known values for single intercalating molecules, are 26° for ethidium, 17° for quinacrine, and 10° for daunorubicin.[15] The $^{31}P$ chemical shift changes in ppm induced by these three compounds in their saturated intercalation complexes are 0.62 for ethidium, 0.40 for quinacrine, and 0.16 for daunorubicin. There is thus a strong correlation between the relative unwinding angles for these three compounds and the extent of the $^{31}P$ chemical shift of their DNA complexes. All three complexes give a single broad peak in their $^{31}P$ spectra with an increase in line width at half-height of approximately threefold for all three. Since $T_1$ values do not significantly change from native DNA to the ethidium complex (Table I), this broadening seems too large to be accounted for by changes in $T_2$.

There are several possible explanations for the increase in line width: (i) ethidium-induced chemical shift heterogeneity such as the heterogeneity seen with the pure poly(dA·dT), with poly(dA·dT) in nucleosomes, and in the actinomycin complex with small self-complimentary nucleotide segments;[17] (ii) an increase in a slower correlation time without a significant change in an internal motion correlation time[1] due to ethidium binding; (iii) an intermediate exchange rate for complexed and uncomplexed phosphate groups (as indicated above for ethidium, all of the three intercalating ligands studied in this work have maximum binding ratios of 0.5 ligands/DNA base pair or less[1,18-20] so that even at saturation at least half of the phosphates are uncomplexed at any time).

Ethidium and quinacrine both saturate DNA at one molecule of ligand per two base pairs.[18,19] Daunorubicin has not been rigorously analyzed by nearest-neighbor analysis, but Scatchard plots suggest a maximum binding ratio of around 0.4 mol of ligand per base pair at low ionic strength.[20] For this reason the ethidium and daunorubicin complexes were also compared at 0.35 mol of ligand per base pair and the chemical shift changes were 0.30 for ethidium and 0.12 for daunorubicin, indicating that the chemical shift differences are not accounted for by differences in the number of binding sites for these ligands. Competition between intercalation and outside stacked ligands might also affect the results. To test this point, we added NaCl to the basic buffer system to a concentration of 0.2 M and recorded the spectra for DNA and

(11) Fourier transform spectra were obtained at 24.15 MHz in 10-mm tubes on a JEOL FX60Q spectrometer with quadurature detection. The temperature of the samples was maintained with a JEOL NM5471 variable-temperature controller. Samples were prepared by lyophilizing the DNA stock solution (15 mg/mL) and redissolving it in 99.8% $D_2O$ (Aldrich). Spectra were accumulated with the use of a 90° pulse angle, 8K data points, a spectral width of 1000 Hz, and a repetition time of $5T_1$ s or greater. Nonselective proton decoupling was used in all cases and a 0.5-Hz broadening function (exponential filter) was applied to each free induction decay before Fourier transformation.

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ethidium-DNA complexes. At equivalent binding ratios there were no significant differences for chemical shifts between the two buffer systems. Since it is known that outside binding is eliminated for ethidium in high ionic strength buffers,[21] external interactions do not have any significant effect on the $^{31}P$ chemical shifts reported here. The results of this paper then indicate that the exchange-averaged $^{31}P$ chemical shift in DNA complexes with intercalating ligands depends strongly on the structure of the ligand. The factor which correlates most strongly with the magnitude of the chemical shift change from native DNA is the ligand unwinding angle.

The $^{31}P$ chemical shifts obtained for these three complexes are not sufficient for derivation of any functional dependence of $^{31}P$ chemical shifts on unwinding angles and such an analysis will require experiments with numerous intercalating molecules for which quantitative unwinding angles are known.

**Acknowledgment.** This research was supported by National Institutes of Health Grant CA 24454 and by the donors of the Petroleum Research Fund, administered by the American Chemical Society.

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(22) After submission of this paper, Hogan and Jardetzky (*Biochemistry* **1980**, *19*, 2079) indicated that the $^{31}P$ NMR signal is totally lost for a 300 base pair preparation of calf thymus DNA when complexed with ethidium. The molecular weight average of our DNA is only about 60% of that of Hogan and Jardetzky and this may account for the difference between their findings and ours. We find about a 30% loss of area in our $^{31}P$ signal at saturation for ethidium and this could be due to the loss of area for DNA at the upper molecular weight end of our sample. We have shown, however, that natural double-helical DNA gives measurable $^{31}P$ NMR signals when complexed with intercalating ligands, and this technique should prove to be quite a valuable probe of intercalation, provided DNA of sufficiently low molecular weight is used.

**Robert L. Jones, W. David Wilson***

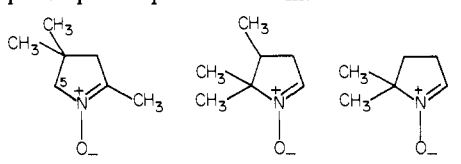
*Department of Chemistry*
*Georgia State University*
*Atlanta, Georgia 30303*

*Received March 14, 1980*

## Singlet Oxygen and Spin Trapping with Nitrones

*Sir:*

A major problem in the study of certain biological and aqueous systems is the identification and differentiation of reactive oxygen intermediates. These include the hydroxyl radical (·OH), the superoxide anion ($O_2^-$), and singlet oxygen ($^1O_2$). It has previously been demonstrated that nitrone spin traps can be used for the identification of both ·OH and $O_2^-$ radicals in water,[1] and this has resulted in their use as probes in a variety of systems.[2] However, the interaction of $^1O_2$ with nitrone spin traps has been ignored. Ching and Foote[3] in 1975 demonstrated that cyclic nitrones can quench $^1O_2$ in $CDCl_3$. When the methylene hydrogens were at the C5 position (II) only physical quenching of $^1O_2$ was observed. Compound III (DMPO) is very close in structure to II and is widely used as a "spin trap".[4] This communication focuses on the reactivity of $^1O_2$ with DMPO and other nitrone spin traps in aqueous medium.



I II III

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(2) For a listing of applications see: Janzen, E. G.; Wang, Y. Y. *J. Phys. Chem.* **1979**, *83*, 894-6.

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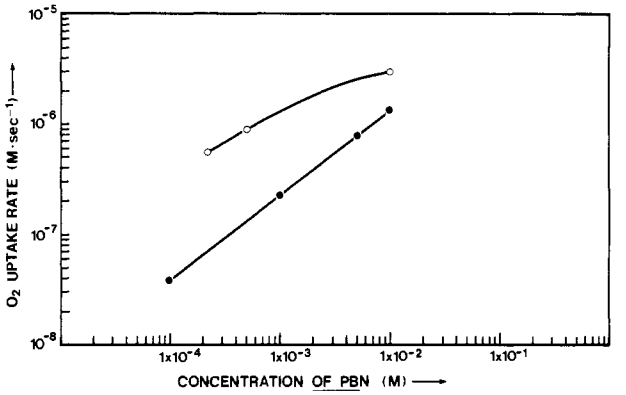


**Figure 1.** Methylene blue sensitized photooxidation of PBN in $H_2O$ (●) and $D_2O$ (○).

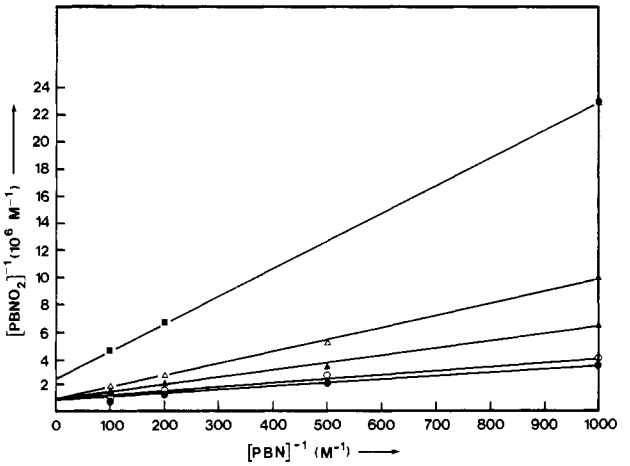


**Figure 2.** Azide quenching of the methylene blue sensitized photooxidation of PBN in $H_2O$. $[N_3^-]$: (●) 0.0 M; (○) $1 \times 10^{-4}$ M; (▲) $5 \times 10^{-4}$ M; (△) $1 \times 10^{-3}$ M; (■) $4 \times 10^{-3}$ M. $PBNO_2$ is the unknown reaction product between PBN and $^1O_2$.

We have monitored the reactivity of $^1O_2$ with nitrones by measuring oxygen uptake from solution, using an oxygen electrode[5] and illuminating a $1 \times 10^{-4}$ M aqueous solution of the $^1O_2$ sensitizer methylene blue. Figure 1 shows the rate of oxygen uptake as a function of the concentration of the spin trap $\alpha$-phenyl *N*-*tert*-butylnitrone[6] (PBN). Since the lifetime of $^1O_2$ increases from 2 $\mu$s in $H_2O$ to 20 $\mu$s in $D_2O$,[7] there should exist a region in the PBN concentration where this lifetime difference is manifest in relative rates of $O_2$ uptake for $D_2O$ vs. $H_2O$ (if $^1O_2$ is involved). Figure 1 shows that indeed the rate is increased when $H_2O$ is replaced with $D_2O$ and that a value of (rate $D_2O$)/(rate $H_2O$) of 10 is approached. This is consistent with the participation of $^1O_2$ in a chemical reaction wiith PBN.

Another method of demonstrating the participation of $^1O_2$ in a reaction is to determine whether or not the rate of the reaction is reduced upon the addition of a physical quencher of $^1O_2$. Sodium azide ($N_3^-$) was selected for these experiments and, when introduced to this system, resulted in a reduction in $O_2$ uptake. Foote[8] has shown that a constant intercept in a plot of $[AO_2]^{-1}$ vs. $[A]^{-1}$ (where A in this case is PBN) at various $N_3^-$ concentrations can be interpreted as proof that no triplet quenching by the $N_3^-$ occurs. In our experiments (Figure 2) this was true up to an $[N_3^-]$ of $1 \times 10^{-3}$M. However, at an $N_3^-$ concentration of $4 \times 10^{-3}$ M a slightly higher intercept was observed. Nevertheless, using results obtained for the constant-intercept concentration range allowed the calculation of the rate constants for both azide

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