crude acid was dissolved in 3 mL of dichloromethane and 2.2 mL (26 mmol) of oxalyl chloride was added dropwise rapidly at 25 °C. The mixture was stirred at 25 °C for 4 h and then distilled through a 3-in. Vigreux column. The fraction of bp 80–87 °C was collected, 1.69 g (59% yield) of colorless isobutyryl chloride-d_6. The acid chloride was added via syringe to a solution of 1.11 g (15 mmol) of dry *tert*-butyl alcohol and 2.0 mL (15 mmol) of *N,N*-dimethylaniline in 5 mL of ether. The resulting solid mass was allowed to stand at 25 °C for 24 h and was then partitioned between water and ether. From the ether was isolated, after simple distillation, a sample of **43**: bp 122–125 °C, 0.66 g, 29%. ^{1}H NMR ($CDCl_3$): δ 2.15–2.45 (br s, 1 H, –C*H*—CO), 1.44 (s, 9 H, CH_3). IR (neat): 3.29 (s), 3.33 (s), 4.42 (s), 4.62 (w), 4.73 (w), 5.75 (s), 7.29 (s), 7.39 (s), 7.79 (s) μm. Mass spectrum: no parent ion, *m/e* 135 (3.1%), 77 (48.3), 57 (100), 49 (40), 41 (22). Estimation of the percent deuterium was based on the *m/e* 135 fragment (loss of $-CH_3$ from the *tert*-butyl unit): 0.3% d_4, 2.4% d_5, 97.3% d_6.

Irradiation of *tert*-Butyl Lithioisobutyrate-d_6 (Lithium Enolate of 43) with *p*-Bromoanisole. With the external radiation apparatus, a mixture of *p*-bromoanisole (0.25 mL, 2.0 mmol) and the lithium enolate of **43** (prepared from 1.06 mL, 6.0 mmol, of **43** and 1.1 mol equiv of lithium diisopropylamide) in 40 mL of ammonia refluxes for 1.5 h. The products were isolated as before and analyzed by GC (6-ft × 0.25-in. column of 5% FFAP on Chromasorb W, programmed from 60 to 180 °C and with biphenyl as internal standard). The products observed were anisole (54% yield) and *tert*-butyl 3,3,3-trideuteriomethyl-2-(trideuteriomethyl)-2-(*p*-anisyl)propanoate (28% yield, retention time: 34.3 min). The mass spectrum of the anisole produced was compared with the mass spectrum of commercial anisole, under precisely the same conditions. Standard anisole: *m/e* 109 (7.4), 108 (87.4). Anisole from irradiation experiment: *m/e* 109 (53), 108 (82). The data are consistent with a mixture of anisole-d_1 (*m/e* 109, 35%) and anisole-d_0 (*m/e* 108, 65%).

Acknowledgment. We wish to thank the Public Health Service (NIH Grant AI-08687) for generous support of this work, including an NIH traineeship to T.M.B. through the Cornell Chemistry Department. We are grateful to Mr. Jeffrey Hayes for the experiments with compound **1c**.

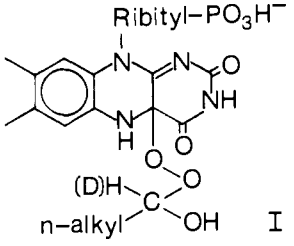
Supplementary Material Available: The preparation of starting materials (**1a–c**, **3a–c**, **4a–c**, **5a–c**, **6a–c**, **10**, **15**, and **21**) and comparison compounds (**6d**, **6e**, **16**, **18**, and **19**) is reported in detail, including six references (16 pages). Ordering information is given on any current masthead page.

Communications to the Editor

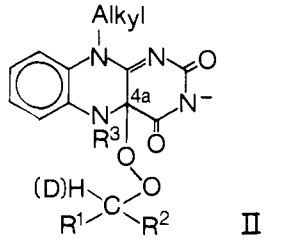
Formation of a Nonchemiluminescent Excited-State Species in the Decomposition of 4a-(Alkylperoxy)flavins

Sir:

Bacterial luciferase chemiluminescence is proposed to arise from chemical transformations of enzyme-bound I.[1] Previous model



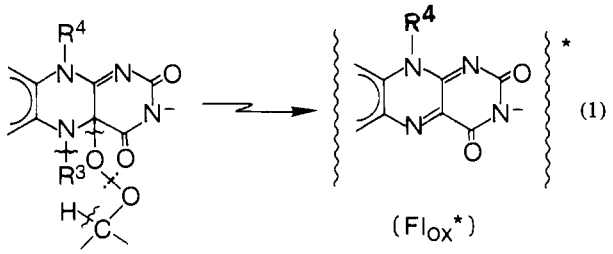
I



II

studies[2] have employed N^5-alkyl-4a-(alkylperoxy)flavins (II). The minimal requirements for chemiluminescence (CL) in the model reactions requires R^1 = alkyl or aryl, R^2 = OH or H, and R^3 = alkyl.[2] In the DMF solvent employed in the present study the substitution of OH for H as R^2 alters the kinetics of the peroxide decomposition but not the quantum yield (Φ) of CL. CL arises from a minor reaction path for the disappearance of II.[2] For this reason [II] is controlled by a non-CL reaction(s), and $-d[II]/dt$ and the exponential decay in light emission share a common rate constant.[3] We now describe experiments which support the formation of two excited species on decomposition of II (R^1 = $C_6H_4CH_3$-*p*; R^2 = H (D), OH; R^3 = C_2H_5).[4]

CL, in the absence of added fluorescer, is due to excited flavin (Fl_{ox}*) (eq 1; II = **1–6**) as shown by the fact that chemilumi-



(1)

II

1, R^3 = Et; R^4 = Me
2, R^3 = *m*-$CH_3OC_6H_4CH_2CH_2$; R^4 = Me
3, R^3 = Et; R^4 = *m*-$HOC_6H_4CH_2CH_2$
4, R^3 = Et; R^4 = *m*-$CH_3OC_6H_4CH_2CH_2$
5, R^3 = Et; R^4 = C_6H_5
6, R^3 = Et; R^4 = 2′,6′-$(CH_3)_2C_6H_3$

nescent emissions are identical with the fluorescence emissions of Fl_{ox}.[5] Particularly convincing evidence for this is the identity of the chemiluminescent emission spectra obtained on decomposition of **1** and **2**. Though these two 4a-(alkylperoxy)flavins possess different structures, they provide the same Fl_{ox}. Also when R^4 is aromatic (**5** and **6**), there is very little chemiluminescent emission

(1) (a) Hastings, J. W.; Balny, C.; LePeuch, C.; Douzou, P. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3468. (b) Hastings, J. W.; Balny, C. *J. Biol. Chem.* **1975**, *250*, 7288. (c) Balny, C.; Hastings, J. W. *Biochemistry* **1975**, *14*, 4719. (d) Hastings, J. W.; Gibson, Q. H.; Friedland, J.; Spudich, J. In "Bioluminescence in Progress"; Johnson, F. H., Haneda, Y., Eds.; Princeton University Press: Princeton, NJ, 1966; p 151.

(2) (a) Kemal, C.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 995. (b) Kemal, C.; Chan, T. W.; Bruice, T. C. *Ibid.* **1977**, *74*, 405. (c) Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99*, 7066.

(3) Maskiewicz, R.; Sogah, D.; Bruice, T. C. *J. Am. Chem. Soc.* **1979**, *101*, 5347.

(4) For experimental procedures, see ref 1b,c. (In this study the solvent has been dry DMF unless stated otherwise, 30 °C.) Quantum yields are based on initial concentrations of compounds II which are formed quantitatively on mixing excess (10^{-2} M) *p*-methylbenzyl hydroperoxide or 1′-(*p*-methylbenzyl) hydroxyperoxide with the appropriate N^5-ethylflavinium cation (10^{-4} M). The presence of alkyl peroxides in excess of that required to generate II does not influence the time course or Φ. All reactions are carried out under dry and anaerobic conditions. Quantum yields for chemiluminescent reactions were determined from the areas under plots of c.p.s. vs. time. Measurements were made with a PAR quantum photometer Model 1140A calibrated by use of the standard luminol reaction [Lee, J.; Wesley, A. S.; Ferguson, J. F.; Seliger, H. H. In ref 1d, pp 35–43]. Chemiluminescence emission spectra and fluorescence emission spectra (corrected) were recorded on a Perkin–Elmer MPF-3 spectrofluorophotometer. The fluorescence quantum efficiency of Rhodamine-B in *t*-BuOH was determined by employing the reported value in EtOH [Parker, C. A.; Rees, W. T. *J. Chem. Soc.* **1960**, 596] as standard.

(5) The fluorescent emissions of compounds Fl_{ox} are as follows: R^4 = Me (518 nm), R^4 = *m*-$HOC_6H_4CH_2CH_2$ (526 nm), R^4 = *m*-$CH_3OC_6H_4CH_2CH_2$ (512 nm).

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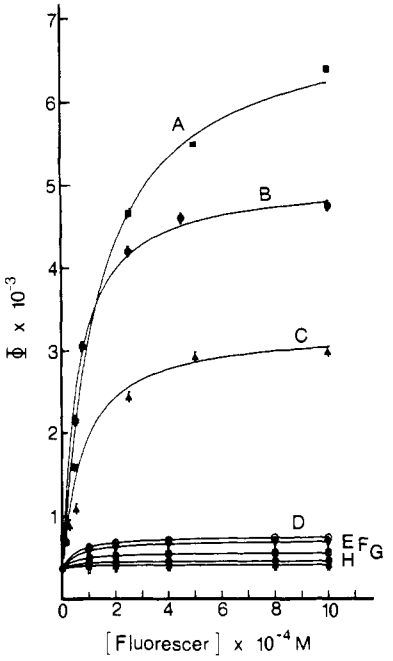
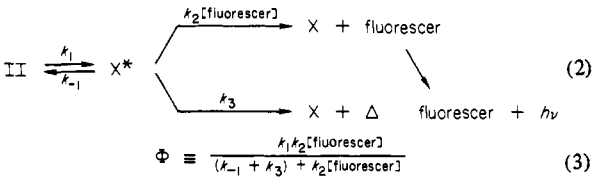


Figure 1. Plot of the quantum yield (Φ) vs. fluorescer concentration (A = Rhodamine-B, B = 3-methyllumiflavin, C = 6,7,8-trimethyllumazine, D = rubrene, E = perylene, F = pyrene, G = coronene, and H = 9,10-diphenylanthracene). All reactions were carried out at 30 °C with the initial reactant concentrations being [$Fl_{ox}Et$] = 10^{-4} M and [*p*-$CH_3C_6H_4CH_2OOH$] = 10^{-2} M. The solvent employed with A was 1:40 acetonitrile–*t*-BuOH and for B–H, absolute DMF.

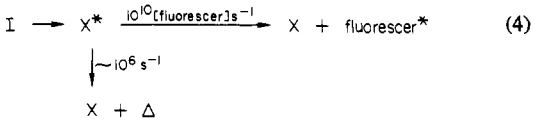
which is in accord with our finding that isoalloxazines substituted with phenyl substituents at N^{10} are only feebly fluorescent. Excited flavin may be formed directly from II (as in eq 1). Alternatively, excited flavin may be formed by the decomposition of II to an excited species which can then transfer its energy to Fl_{ox} which is present as an impurity in II. (Fl_{ox} impurity in **1**–**4** can be shown by fluorescence spectrometry to amount to less than 1%.) The former situation (eq 1) is most likely since a tenfold dilution of **1** does not result in a decrease in Φ and the efficiency of a bimolecular energy transfer from an excited product of II to Fl_{ox} impurity should decrease upon decrease in [II] and consequently decrease in [Fl_{ox} impurity]. Exchange of H for D in II results in an isotope effect on the quantum yield (Φ^H/Φ^D) of 1.7–2.0 (see also ref 2). The bonds that must be broken (⁓) to form Fl_{ox}* are shown in eq 1; the reaction is probably initiated by cleavage (···) of the O–O bond.

If, however, Fl_{ox} (50–1000-fold > [Fl_{ox} impurity] in **1**) or other fluorescers possessing λ_{max} for excitation >400 nm are added to the reaction solution, there is an increase in Φ which becomes constant at high [fluorescer] (Figure 1). In each case the emitting species is excited fluorescer.[6] These results cannot be attributed to energy transfer from chemiexcited Fl_{ox}* generated from II to added fluorescer, since energy transfer from Fl_{ox}* to Fl_{ox} (when this is the added fluorescer) could hardly increase Φ. The increased CL observed on addition of fluorescer is attributed to energy transfer to fluorescer by a nonfluorescent excited species X*. Apparently, energy transfer from X* to Fl_{ox} impurity in **1** does not occur due to the latter's low concentration. Thus, II breaks down to give mainly dark products and, in minor reactions, both Fl_{ox}* and X*. The presence of fluorescers increases neither the rate constant for the disappearance of II from solution nor the rate constant for the decay of light emission. Also, the Φ_{max} for each fluorescer is not at all related to the latter's E° for one-electron release. These findings argue against the occurrence of fluorescer molecule catalysis of the consumption of II by chemically induced electron exchange luminescence (CIEEL).[8]

The minor reaction path leading to the formation of X* from II and energy transfer from X* to fluorescer can be described by eq 2 and 3. The values of $(k_{-1} + k_3)/k_2$, calculated from the



[fluorescer] required for $^1/_2\Phi_{max}$ in a plot of Φ vs. [fluorescer] (Figure 1), were found to be essentially independent of the nature of the fluorescer [6–8 × 10^{-5} M in DMF and 1.4 × 10^{-4} M in anhydrous *t*-BuOH].[9] Since k_3 and k_{-1} must be independent of fluorescer, the observation that $(k_3 + k_{-1})/k_2$ is virtually independent of fluorescer dictates that k_2 is also independent of fluorescer. The value of k_2 may only be independent of the nature of the fluorescer if it represented a diffusion-controlled energy transfer ($\sim 10^{10}$ s^{-1}). The varied spectral characteristics of the fluorescers speak strongly against a constant rate for dipole–dipole energy transfer.[10] Ignoring k_{-1} (reversible formation of X* is improbable), eq 4 is obtained. A value of $\sim 10^6$ s^{-1} for the rate



constant for X* → X + Δ would require X* to be unusually long-lived for an excited singlet state.[10] Generation of the excited species as a triplet followed by crossover to the first singlet excited state (i.e., $^3X^* \rightarrow {}^1X^*$) prior to energy transfer is unlikely. Thus, the presence of $^3O_2$ has no influence upon CL associated with formation of Fl_{ox}* or X*. It follows from eq 4 that the value of Φ_{max} should be a function of the efficiency of energy transfer from X* to fluorescer molecule and the efficiency of fluorescence emission by fluorescer*. A reasonably linear plot is obtained (slope ≪ 1) when Φ_{max} is plotted against the relative integrated intensity of fluorescer fluorescence. Only 9,10-diphenylanthracene has a low excitation wavelength (395 nm);[6] hence high excitation energy deviates from linearity.

Rhodamine-B is the most efficient fluorescer employed. When Φ_{max} for this fluorescer (7×10^{-3})[9] is corrected for its fluorescence efficiency in *t*-BuOH (0.6)[4] a corrected value for Φ_{max} (~0.01) is obtained which may be compared to that previously determined[1] for bacterial luciferase (~0.1). Koka and Lee have suggested[7] that the blue CL of the bacterial luciferase of photobacterium

(6) The λ_{max} for chemiluminescent emission (λ_{CL}) and fluorescent emission (λ_{fl}) when excited at y_{nm} follow: Rhodamine-B (*t*-BuOH), $\lambda_{CL} = \lambda_{fl}$ = 585 nm, y = 530 nm; 3-methyllumiflavin (DMF), $\lambda_{CL} = \lambda_{fl}$ = 518, y = 467 nm; 6,7,8-trimethyllumazine (DMF), $\lambda_{CL} = \lambda_{fl}$ = 490 nm, y = 430 nm; rubrene, $\lambda_{CL} = \lambda_{fl}$ = 565 nm, y = 467 nm; perylene (DMF), $\lambda_{CL} = \lambda_{fl}$ = 470 nm, y = 450 nm; pyrene (DMF), $\lambda_{CL} = \lambda_{fl}$ = 510 nm, y = 470 nm; coronene (DMF) $\lambda_{CL} = \lambda_{fl}$ = 485 nm, y = 438 nm; 9,10-diphenylanthracene (DMF), λ_{CL} = 408 nm, λ_{fl} = 410 nm, y = 393 nm.

(7) Koka, P.; Lee, J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 3068.

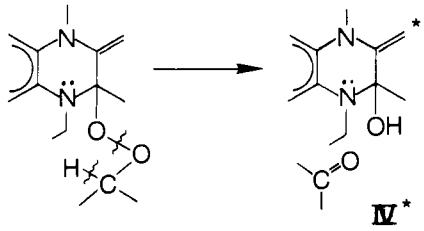
(8) (a) Dixon, B. G.; Schuster, G. B. *J. Am. Chem. Soc.* **1979**, *101*, 3116. (b) Koo, J.; Schuster, G. B. *Ibid.* **1978**, *100*, 4496. (c) Schmidt, S. P.; Schuster, G. B. *Ibid.* **1978**, *100*, 1966. (d) Smith, J. P.; Schuster, G. B. *Ibid.* **1978**, *100*, 2564. (e) Koo, J.; Schuster, G. B. *Ibid.* **1977**, *99*, 6107. (f) Schuster, G. B. *Acc. Chem. Res.* **1979**, *12*, 366.

(9) Φ_{max} and molarity of fluorescer at $^1/_2\Phi_{max}$: Rhodamine-B (*t*-BuOH), $\Phi_{max} = 7 \times 10^{-3}$, $M = 1.4 \times 10^{-4}$ M; 3-methyllumiflavin (DMF), $\Phi_{max} = 4.8 \times 10^{-3}$, $M = 6 \times 10^{-5}$ M; 6,7,8-trimethyllumazine (DMF), $\Phi_{max} = 2.9 \times 10^{-3}$, $M = 8 \times 10^{-5}$ M; 6,7,8-trimethyllumazine (*t*-BuOH), $\Phi_{max} = 2.5 \times 10^{-3}$, $M = 1.4 \times 10^{-4}$ M; rubrene (DMF) Φ_{max} 6.9×10^{-4}, $M = 7 \times 10^{-5}$ M; perylene (DMF), $\Phi_{max} = 6.7 \times 10^{-4}$, $M = 7 \times 10^{-5}$ M; pyrene (DMF), $\Phi_{max} = 5.1 \times 10^{-4}$, $M = 6 \times 10^{-5}$ M; coronene (DMF), $\Phi_{max} = 4.65 \times 10^{-4}$, $M = 8 \times 10^{-5}$ M; 9,10-diphenylanthracene (DMF), $\Phi_{max} = 4.1 \times 10^{-4}$, $M = 7.0 \times 10^{-5}$ M. These values were determined by computer fitting of Φ values vs. [fluorescer] employing eq 3 (see Figure 1).

(10) Turro, N. J. "Modern Molecular Photochemistry"; Benjamin/Cummings: Menlo Park, CA, **1978**; Chapter 5.

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).

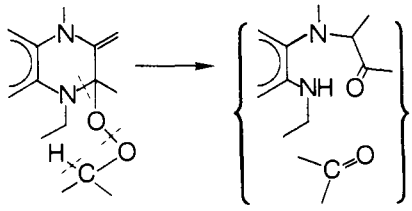
Replacement of H by D in II results in a decrease in Φ_{max} (for fluorescer rubrene, $\Phi^H/\Phi^D = 1.7$; for fluorescer Fl_{ox}, $\Phi^H/\Phi^D = 1.8$), but there is no change in the first-order rate constants for either the disappearance of II or the diminution in the intensity of light emission. 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)

IV*

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–π* states). VI may ring close to form IV, one



VI* (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.

(12) Hastings, J. W.; Nealson, K. H. *Annu. Rev. Microbiol.* **1977**, *31*, 549.

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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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(2) Klevan, L.; Armitage, I. M.; Crothers, D. M. *Nucleic Acids Res.* **1979**, *6*, 1607.

(3) Hogan, M. E.; Jardetzky, O. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6341.

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(5) Rill, R. L.; Hilliard, P. R., Jr.; Bailey, J. T.; Levy, G. C. *J. Am. Chem. Soc.* **1980**, *102*, 418.

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(9) (a) Patel, D. J. *Acc. Chem. Res.* **1979**, *12*, 18. (b) Reinhardt, C. G.; Krugh, T. R. *Biochemistry* **1977**, *16*, 2890. (c) Krugh, T. R.; Nuss, M. E. "Biological Applications of Magnetic Resonance"; Academic Press: New York, 1979.

(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 ϕX 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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