Nucleic Acids: A Nuclear Magnetic Resonance Study

Abstract. *Single-stranded nucleic acids in aqueous media exhibit proton magnetic resonance spectra with acceptable signal-noise ratios if multiple scans of the spectra are stored in and subsequently read out of a computer of average transients. Partial ordering of the adenine bases of polyadenylic acid in solution is reflected in contributions to the chemical shifts that are removed on "melting." More significantly, secondary structure such as occurs in complexes of polyA and polyU and in double-stranded DNA is manifested in proton magnetic resonance spectra below the "melting" temperature by severe resonance line broadening which results from anisotropic nuclear dipole-dipole interactions. The proton resonances are narrowed in and above the "melting" region, and measurement of integrated resonance intensities can give insight into quantitative aspects of hybridization, "melting," and other nucleic acid interactions.*

The property of hypochromism of the electronic transition at 2600 Å of nucleic acids has been used to detect and study "melting" of secondary structure of such species in solution. In spite of the success of this approach, alternative means of investigating these phenomena might provide further insight into the molecular processes involved. Nuclear magnetic resonance (NMR) has provided detailed information regarding structures, interactions, and motions of molecules but has lacked sensitivity when compared to other spectroscopic techniques and, consequently, has been of limited value in biological research. A major improvement in the effective sensitivity of NMR spectrometers (*1*) has resulted from coupling of a computer of average transients to the output of an NMR spectrometer (*2*). Having used this technique, we now present representative proton magnetic resonance (PMR) spectra of nucleic acids in the random-coil form and show the value of NMR in quantitative studies of interactions of nucleic acids.

Solutions for NMR studies were prepared from weighed quantities of nucleic acids (*3*) in D_2O or D_2O containing NaCl, and concentrations are expressed here as the molarities of the component purine and pyrimidine bases. Direct measurement of *p*D values of these very concentrated nucleic acid solutions were considered unreliable but *p*D values of more dilute solutions of the same materials ranged from 6.5 to 7.5. The actual nucleic acid content of commercial preparations of polyA and polyU were determined by comparison of the intensities of PMR spectra of solutions of these substances with the intensities of known concentrations of hydroquinone in the same solutions. The molarity of the polyU calculated from these measurements agreed with the molarity by weight, but the molarity of the polyA indicated by PMR intensities was 12.5 percent less than the weight molarity. Consequently, in all solutions containing polyA the weight molarity was adjusted by this factor. Synthetic polyribonucleic acids prepared by use of polynucleotide phosphorylase are polydisperse (*4*). Sedimentation coefficients for the polyA and polyU used were 2.1 and 4.2, respectively (*5*). The sedimentation patterns indicated that the bulk of the nucleic acid molecules had molecular weights in the range of 10^4 to 10^5. Both nucleic acids contained a lower molecular weight component, about 20 percent in the case of polyA and less for polyU. The amount of monomer or small oligonucleotides incapable of becoming part of a higher molecular weight complex at room temperature appeared negligible since no residual PMR intensity was observed when equivalent quantities of polyA and polyU were mixed. Independent experiments have shown that PMR intensities of uridylic acid and high molecular weight polyA are not affected when these species are present in the same solution. Sedimentation analysis of the calf thymus DNA (*3*) showed that this material was also somewhat polydisperse but possessed a median $S_{20,w}$ of 13.8 (*5*).

Proton magnetic resonance spectra of the nucleic acid solutions were obtained with a high-resolution NMR spectrometer (*3*) operated at 60 Mcy/sec. Temperatures of the samples were controlled and measured to ± 0.25°C. The signal-noise ratio of the spectrometer output was improved by repeatedly storing selected field regions of a spectrum in a computer of average transients (*3*). The solvent HDO proton resonance was used as the initial field position for each spectrum. Resonances were calibrated relative to that of HDO at 25°C by the side band modulation technique and by interpolation from charted spectra. Measurements of resonance displacements from that of HDO at temperatures other than 25°C were corrected for the independently determined shift of the HDO resonance to higher fields with increasing temperature. The PMR intensities were obtained by area integration of charted resonance absorptions. The NMR spectrometer response was constant throughout a day's experimentation within the reproducibility of the intensity measurements. Changes in spectrometer response from day to day were evaluated by periodic examination of PMR intensities of a standard sample of polyuridylic acid in D_2O.

Selected regions of the PMR spectra of polyA, polyU, polyI, polyC, and of random-coil calf thymus DNA are shown in Figs. 1 and 2. Resonance displacements to low and to high field relative to that of HDO at 25°C are expressed, respectively, as negative and positive frequency displacements (cy/sec). Resonance assignments, based on analyses of PMR spectra of nucleosides (*6*) and on intensity relationships, are shown in parentheses below the resonance positions. Resonances from other hydrogen atoms of the ribose moieties have also been observed, but they are for the most part obscured at least partially by the HDO resonance and have not been included in Figs. 1 and 2. Protons bound to oxygen or nitrogen atoms exchange rapidly with D_2O and are only observed as an undifferentiated contribution to the intensity of the HDO resonance. The PMR spectra of previously undenatured, double-stranded DNA were not detected until the nucleic acid was heated to the "melting" temperature (above 75°C). The spectrum of DNA in Fig. 2 was obtained at 95°C and arises, therefore, from single-stranded, random-coil DNA. The resonances at +140 cy/sec and +175 cy/sec are assigned to the 2′deoxyribose protons and to the protons of the methyl group of thymine, respectively. There is a splitting of 7 cy/sec of the methyl proton resonance of DNA (Fig. 2*c*) that is not present in thymidylic acid. This splitting is indicative of two nonequivalent chemical environments for thymine in single-stranded calf thymus DNA. Chemical shifts of methyl groups of thymine components of

single-stranded DNA would be expected to be differentially influenced by the nature of the two nearest neighbor bases through, for example, ring current effects. Precedent for sensitivity of methyl resonances in polymers to nearest neighbor influences has been established by Bovey and Tiers (*7*) who noted differences in the α-methyl chemical shifts of up to 18 cy/sec (at 60 Mcy/sec) for polymethylmethacrylate in the isotactic, syndiotactic, and atactic forms. Other explanations for the appearance of two methyl resonances for thymine in single-stranded calf thymus DNA are possible and we are currently investigating this point with DNA from other sources.

The proton resonances of polyA shown in Fig. 1 shifted progressively 15 to 25 cy/sec to lower fields as the temperature was increased from 25°C to 60°C but were not further shifted above 60°C. These temperature dependences of chemical shifts of polyA probably result from the conversion of a partially ordered configuration to a random-coil configuration. Ring currents which exist in conjugated systems produce well-characterized contributions to chemical shifts (*8*) and have been shown to cause high-field shifts of proton resonances of porphyrin molecules stacked in solutions (*9*). Thus, a partial ordering of the bases of polyA at room temperature may cause a similar high-field shift of the adenine resonances (*10*). This shift is removed as the partially ordered nucleic acid is converted to a random-coil configuration by heating. There is a considerable narrowing of the lowest field resonance of polyA through this same temperature range. The resonance frequencies of the PMR spectra of the other nucleic acids of Fig. 1 did not shift by more than a few cycles per second as the temperature was increased from 25°C to 90°C although some narrowing of the resonances was observed. In particular, the resonances of polyI were broad at 25°C but had narrowed appreciably by 60°C indicating increased motion of the polymer molecules in the solution at higher temperatures.

The remainder of this report is concerned primarily with the observation that for helical nucleic acids such as the complexes of polyA and polyU or double-stranded DNA, line widths are too great to permit detection of nuclear resonances under high resolution conditions. Line widths in NMR will not be dealt with in any detail here since they have been covered in a number of reviews (*11*). A major source of line broadening in NMR does arise from anisotropic nuclear dipole-dipole interactions that depend in sensitive fashion on inter- and intramolecular motions of the molecules concerned (*12*). These motions of helical nucleic acids are sufficiently slow (long correlation times) that PMR line widths are too great for detection with a high resolution NMR spectrometer. Upon "melting," rotational modes of single strands and partially separated double strands of nucleic acids are activated so that line-width manifestations of dipolar interactions are almost completely averaged to zero and the resonances become observable under high-resolution conditions.

For example, when a solution containing equal concentrations of polyA and polyU in D_2O at *p*H 7.0 was examined, no PMR spectrum was detected other than that for HDO. As the

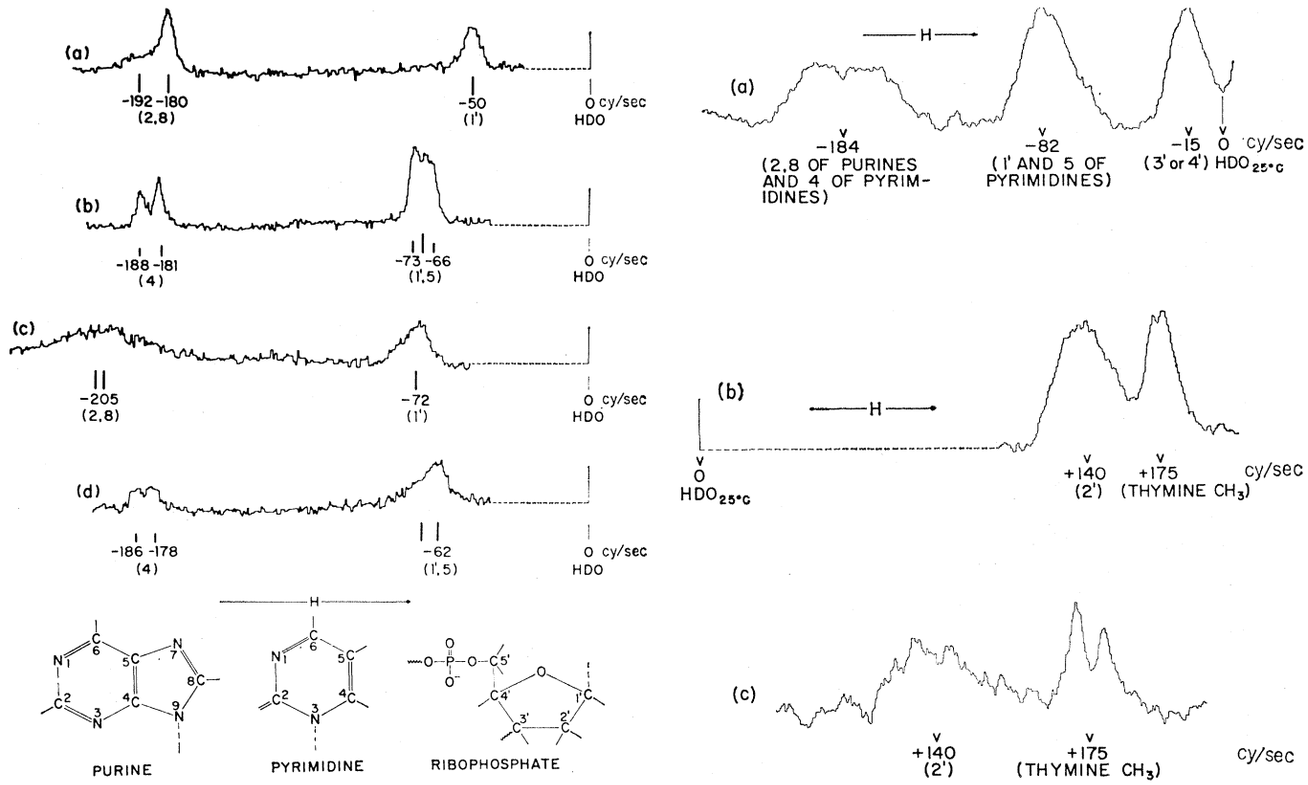

Fig. 1 (left). *a*, polyA, 32.0 mg/ml in D_2O, 35.5°C, 8 traces stored; *b*, polyU, 31.8 mg/ml in D_2O, 35°C, 8 traces stored; *c*, polyI, 20.2 mg/ml in D_2O, 75°C, 10 traces stored; *d*, polyC, 21.0 mg/ml in D_2O, 60°C, 10 traces stored. The numbering system used is indicated at the bottom of the figure. Fig. 2 (right). Calf thymus DNA in D_2O. *a*, 22.1 mg/ml, 16 traces stored at 95°C; *b*, 22.1 mg/ml, 14 traces stored at 95°C; *c*, 10.5 mg/ml, 24 traces stored at 85°C. Spectrometer scan rates were identical for *a* and *b*, but slower for *c*.

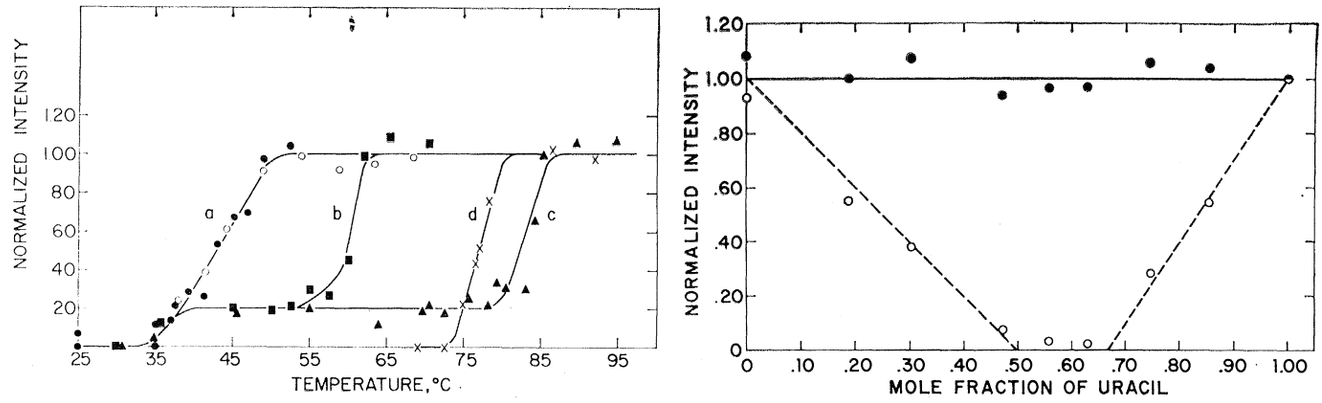

Fig. 3 (left). *a*, PolyA 15.8 mg/ml, polyU 16.2 mg/ml in D_2O; calculated for A, 3.75 $\times$ $10^{-2}M$; for U, 4.70 $\times$ $10^{-2}M$ or 55.7 percent U; solid circles: temperature increasing; open circles: temperature decreasing. *b*, Solid squares: PolyA 16.5 mg/ml, polyU 16.5 mg/ml in 0.10*M* NaCl in D_2O; calculated for A, 3.92 $\times$ $10^{-2}M$; for U, 4.79 $\times$ $10^{-2}M$ or 55.0 percent U. *c*, Solid triangles: PolyA 16.0 mg/ml, polyU 16.1 mg/ml in 1.00*M* NaCl in D_2O; calculated for A, 3.80 $\times$ $10^{-2}M$; for U, 4.67 $\times$ $10^{-2}M$ or 55.1 percent U. *d*, Calf thymus DNA 29.6 mg/ml in D_2O. Fig. 4 (right). Solid circles: normalized intensity at 65°C. Open circles: normalized intensity at 25°C. Solid line: expected intensity for no complex formation of A and U. Dotted line: expected intensity if poly(A + U) is formed at U mole fractions less than 0.50, and poly(A + 2U) is formed at U mole fractions greater than 0.66. All solutions contained approximately 32 mg of polyribonucleic acid per ml of D_2O.

solution was heated above 35°C proton resonances were observed in the field range −30 cy/sec to −80 cy/sec (region 1) and −180 cy/sec to −200 cy/sec (region 2). These regions correspond to the low-field resonances of single-stranded polyA and polyU. It is apparent, therefore, that when polyA and polyU form the well-known helical poly(A + U) and poly(A + 2U) complexes (*13*) the motions of protons of the complexes are sufficiently restricted so that the resonances are broadened and are not detected by a high-resolution NMR spectrometer. In contrast, the PMR spectra of single-stranded polyA and polyU are rather sharp because of rapid local motion of the polymer segments. As an illustration, it is seen from Fig. 1*b* that the 7 cy/sec nuclear spin-spin doublet of the 4-proton of polyU is clearly resolved.

The formation of poly(A + U) and poly(A + 2U) from polyA and polyU thus can be followed quantitatively from the intensity of the PMR spectra of solutions containing these species. For example, a "melting" curve for a solution containing approximately equal amounts of polyA and polyU is shown in Fig. 3*a*. The observed intensities in regions 1 and 2 at a given temperature are expressed as fractions of the expected intensities of polyA and polyU if these were in the random-coil configuration, with due consideration to the concentration of protons contributing to each region and the inverse dependence of all PMR intensities on absolute temperature. Data from regions 1 and 2 were averaged to obtain each point in Fig. 3*a*. The expected intensity in region 1 or 2 for random-coil nucleic acid was determined from the intensity of a solution of known concentration of polyU at *p*H 7. By PMR or other techniques (*4*) polyU shows no evidence of forming complexes with itself above 25°C. Other experiments showed PMR intensities of solutions containing polyA or polyU alone to be independent of temperature from 25°C to 80°C when corrected for the inverse dependence of PMR intensities on temperature. Equilibrium appeared to be attained in these solutions in a few minutes, a time short compared to that required for an intensity measurement. The complexing and "melting" of polyA and polyU were completely reversible (see Fig. 3*a*). The "melting" temperature at *p*H 7 without added electrolyte did not vary with the polyA-polyU ratio in our solutions. Addition of NaCl to solutions of polyA and polyU raised the "melting" temperature of the complex (Fig. 3, *b* and *c*) as had been shown previously by optical-density measurements (*14*). A somewhat unexpected feature of the "melting" curves of Fig. 3, *b* and *c*, is the occurrence of an initial "melting" at 36.5°C which accounts for about 20 percent of the final PMR intensity developed on complete "melting." The cause of this behavior in the solutions 0.1*M* and 1.0*M* in sodium ion is not fully understood but the most likely interpretation is that it represents "melting" by complexes or complex regions containing the lower molecular weight polyA as a component. As indicated earlier, the polyA used in these studies contained about 20 percent of relatively low molecular weight material.

In Fig. 4, the resonance intensities of solutions containing polyA and polyU in various ratios at 25° and 65°C (that is, below and above the "melting" temperature) are shown, again expressed as fractions of intensities expected if no complexes are formed. Evaluation of integrated intensities of weak PMR lines even when enhanced by a computer of average transients is difficult as may be seen from the scatter of points in Fig. 3, where each point represents data from a single computer-enhanced spectrum. The points in Fig. 4 for the most part represent average values from two or more spectra.

The results shown in Fig. 4 confirm that no appreciable complex formation occurs in these solutions at 65°C and suggests that at 25°C poly(A + U) is formed when the mole fraction of U is less than 0.50 and that poly(A + 2U) is formed when the mole fraction of U is greater than 0.66. Solutions at 25°C in which the mole fraction of U is between 0.50 and 0.66 probably contain a mixture of these complexes. Fresco (*15*) and Felsenfeld and Rich (*16*) have shown that the relative stabilities of poly(A + U) and poly(A + 2U) depend critically on temperature and on the concentration of monovalent cations. Our solutions are about 8.5 $\times$ $10^{-2}M$ in monovalent cation from the potassium counter ion introduced with the nucleic acids. Al-

though our experimental conditions are not strictly comparable with those of the earlier workers (*15, 16*) since we use a much higher ratio of nucleic acid to solvent, our results are consistent with their conclusion that either poly(A + U) or poly(A + 2U) can be the predominant complex at cation concentrations below 0.1*M* and a temperature of 25°C. In our experiments, the predominant species clearly depends on the ratio of polyA to polyU with the poly(A + 2U) complex being favored when the ratio is low.

A "melting" curve for double-stranded calf thymus DNA based on PMR intensities of spectral regions 1 and 2 is shown in Fig. 2*d*. Again the intensities are expressed as fractions of intensities expected if all the protons of region 1 or 2 contributed to the intensity, now taking into consideration the base composition of the DNA (*17*).

Thus, it appears that intensities of PMR spectra can be used in the way optical-density measurements are used to elucidate secondary structure and "melting" of synthetic and natural nucleic acids. The PMR technique presently requires higher concentrations of nucleic acids than the optical-density technique but has the compensating advantage of permitting absolute determination of the degree of strand complexing (optical density changes give a relative measure of changes in secondary structure). In addition, observation of behavior of individual PMR lines can give detailed insight into changes in molecular structure and motion during complex formation or "melting." For example, as a polyA-polyU complex is "melted," sharp resonances characteristic of single-stranded polyA and polyU emerge from the broad background resonance of the complex. The resonances do not gradually sharpen as the complex is "melted," which would be expected if during "melting" there existed a fast equilibrium between the portions that are and are not "melted" within individual complexes. If the latter were true, the resonances would be expected to narrow gradually over the "melting" range. This result may be taken as further evidence for the all-or-nothing, cooperative nature of "melting" in the polyA-polyU complexes. On the other hand, individual resonances of the DNA spectra exhibit a considerable decrease in width as well as an increase of integrated intensity as the temperature is increased through the "melting" range. This NMR "melting" behavior of DNA suggests the existence of discrete structural regions in individual DNA double strands that exhibit a range of "melting" temperatures. This tentative interpretation is consistent with the nonuniformity of strengths of base pair interactions over the length of DNA double strands (guanine-cytosine as opposed to adenine-thymine interactions).

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References and Notes

1. Abbreviations: NMR, nuclear magnetic resonance; PMR, proton magnetic resonance; polyA, polyadenylic acid; polyU, polyuridylic acid; polyC, polycytidylic acid; polyI, polyinosinic acid; DNA, deoxyribonucleic acid; poly(A + U), double-stranded helical complex of polyA and polyU; and poly(A + 2U), triple-stranded helical complex of polyA and polyU.
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* Contribution No. 939.

26 February 1964

Hemoglobin Polymorphism: Its Relation to Sickling of Erythrocytes in White-Tailed Deer

Abstract. *Hemoglobins with different electrophoretic mobilities are associated with bizarre shapes seen during ordinary light microscopy of erythrocytes from the white-tailed deer,* Odocoileus virginianus; *nonsickling is contingent upon a specific hemoglobin. Sickling in vitro, which is produced to a maximum degree in the presence of oxygen and elevated* p*H, is not associated with hematological abnormalities or disease despite marked differences in physical characteristics of sickled and nonsickled cells.*

Although the sickling of deer erythrocytes was reported more than a century ago by Gulliver (*1*), the phenomenon has not previously been related to the presence of variant hemoglobins. Both Gulliver and Mandel described peculiar shapes of certain mammalian erythrocytes, especially in the families *Camelidae* and *Cervidae*, 70 years before the discovery of sickling in the human erythrocyte. Various bizarre shapes were found for different animals, but the red cells of all deer which were examined became sickle-shaped regardless of species.

The discovery of an abnormal hemoglobin in sickle cell anemia by Pauling *et al.* (*2*) prompted numerous investigations which have revealed many genetically controlled abnormal hemoglobins in man. Some of these have been directly associated with morphological changes in the red blood cell. As a consequence, investigators of the sickled and crescent-shaped erythrocytes in several species of deer have attempted to incriminate hemoglobin in the sickling phenomenon, but they have offered no evidence for the occurrence of more than a single hemoglobin within each species (*3*). In previous reports, the red blood cells of all the