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Polynucleotide Melting in Heavy and Light Water†

R. D. Blake

ABSTRACT: The effect on the melting temperature, t_m, of transferring the helix–coil (h → c) transition from H_2O to D_2O has been examined for a wide variety of nucleic acid helixes consisting of either two or three strands twisted into either the A- or B-like secondary structure. Very high precision in measuring the t_m was achieved by monitoring the transition by the absorbance difference approximation method with as many as six different helical species mixed together in the same solution. The average standard deviation in the t_m is ±0.08 °C and ±0.11 °C for the δt_m between solvents. In all cases the melting temperature in D_2O is higher than in H_2O. The δt_m decreases with increasing Na^+ counterion concentration in approximate proportion to the increase in unit transition enthalpy; therefore, most studies were concentrated on the effect of transfer in the moderately low ionic condition of 0.018 M Na^+. At this $[Na^+]$, δt_m for transfer ranged from −0.51 °C for the G·C base pair in DNA to −2.05 °C for the melting of the three-stranded dA·2rU helix and showed a strong dependence on the conformation of the helix. The δt_m for helixes with A-like structures is twice that for B structures. We find no evidence for lattice support of the helix by "ice-like" water nor any effect on δt_m by the chemical nature of the base or pentose. It is proposed that the solvent effect is due to differences in dielectric saturation of Na^+ between the two solvents resulting in a significantly higher dielectric constant in D_2O than in H_2O in the neighborhood of the nucleic acid, where the concentration of Na^+ may exceed 3 M. The difference in free energy for transferring the h → c transition from H_2O to D_2O increases linearly with the difference in $[Na^+]$ condensed to the helix and coil for the different helixes examined in this study. This variation is 40% greater for the A family than for the B family of helixes, reflecting the difference in charge density between these two conformations.

The nature of the interaction of deoxyribonucleic acid (DNA)[1] with water is important to the study of the structure of DNA, since water is necessary in preserving the integrity of the helix. The problem is complex, however, and there still is not a good description of interactions at the molecular level. Certainly some water is firmly bound through hydration–solvation (Tunis & Hearst, 1968). It is less certain how water

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[1] Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; bp, base pair; EDTA, ethylenediaminetetraacetic acid.

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molecules are involved in other processes that have been proposed such as hydrophobic or solvophobic bonds and water bridges [e.g., Lewin (1974)]. However intimate their involvement may be with the nucleic acid, it is clear that the properties of a significant amount of water in the neighborhood of the helix are different from those of bulk solution (Falk et al., 1962; Falk, 1966).

Changes in free energy associated with specific changes in the interaction of water with nucleic acids during the transition from helix to coil (h → c) are quite small, perhaps less than 1–10% of the changes associated with the loss of intramolecular stacking forces, and so are difficult to measure. One approach that has been used in a number of studies is to examine the effect on the h → c transition of exchanging heavy for light water. Previous results have indicated the effect of transfer is nil or small (Miles, 1960; Lewin, 1974; Blake, 1975). While nothing substantial has resulted from these earlier studies, there are good reasons why they should be continued. These include newer evidence for trace contamination of most commercial D_2O by multivalent cations, the recent development of a comprehensive description of counterion condensation as a fundamental physical property of the polynucleotide chain, and, finally, the recent development of greatly improved sensitivity in following the h → c transition. Manning's explanation for the expectation of extraordinarily high concentrations of territorially bound counterion (Manning, 1978, 1979) raises the possibility that small energy fluctuations associated with differences in local dielectric arising from perturbations of the electrostricted inner hydration layers of the nucleic acid salt may be detected in a transfer experiment and, moreover, show a dependence on the difference in charge density between helix and coil, that is to say, the conformation of the helix. The results reported below are consistent with this expectation.

We have sought to enhance experimental sensitivity to very small changes in energy of bound water through examination of the relative effects of D_2O and H_2O on the melting temperatures, t_m's, of different helical forms mixed in the same solution. The high-resolution difference approximation method in use in this laboratory for following h → c transitions (Blake & Lefoley, 1978; Yen & Blake, 1980) has the capability of distinguishing δt_m's ($t_m^{H_2O} - t_m^{D_2O}$) differing by only 0.10 °C for the transfer of a polynucleotide transition from light to heavy water. The energy associated with an increase or decrease in t_m of such magnitude is determined with the familiar relationship

$$T_m = \Delta H/\Delta S \quad (1)$$

where ΔH and ΔS are the (mean) unit enthalpy and entropy per base pair (bp) for the h → c transition. From this we see that

$$\delta t_m/T_m = \frac{\delta\Delta H}{\Delta H} - \frac{T_m\delta\Delta S}{T_m\Delta S} \quad (2a)$$

$$= \delta\Delta G_{tr}/\Delta H \quad (2b)$$

Assuming a t_m and ΔH of, say, 50 °C and 8 kcal·(mol of bp)$^{-1}$, we see the difference approximation method for determining δt_m's corresponds to an apparent sensitivity to free energy changes of only 3 cal·(mol of bp)$^{-1}$ and is therefore well suited for detecting subtle effects of water on the stability of polynucleotide helixes.

Experimental Procedures

Materials

Polynucleotides. Synthetic and natural polydeoxyribo- and polyribonucleotides were purchased from commercial supply houses. With the minor exception of the melting of poly(dA·dT)s prepared by DNA polymerase and by terminal transferase, no significant differences in melting profiles could be detected among samples from different sources.

D_2O. Deuterium oxide, 99.96–99.996 atom % D, was purchased in 100-g lots from commercial sources in the United States, W. Germany, and Canada. The specific conductance (25 °C) was generally ~1.5 $\mu\Omega^{-1}$, approximately the same as our double-distilled H_2O. Atomic absorption analysis indicated the trace presence in all commercial D_2O of submicromolar quantities of several divalent cations, particularly Mg, Mn, and Ca; consequently, 0.1 mM EDTA was added to all buffers. Both D_2O and H_2O were (re)distilled in glass by a high-yield vacuum sublimation process that resulted in a more than a 10-fold reduction in conductivity. While experiments in undistilled D_2O led to results at low ionic strengths that clearly reflected the trace contamination of multivalent cations, such effects were eliminated by distillation or the addition of EDTA. Poly(U), a sensitive indicator of metal ion catalyzed hydrolysis, was incubated in a 100-fold enriched D_2O distillation residue for 18 h at room temperature. No degradation could be detected in analytical ultracentrifugation profiles over time indicating trace metal contamination of commercial D_2O apparently does not include Pb(II) (Farkas, 1968), Zn(II) (Eichhorn & Butzow, 1965), and La(III) (R. D. Blake and J. R. Fresco, unpublished experiments). Due to the hygroscopic nature of D_2O special precautions were always taken to minimize exposure to the atmosphere.

Methods

Melting Curves. Derivative melting curves were obtained as previously described (Blake & Lefoley, 1978; Yen & Blake, 1980). The rate of heating was 6.750 °C·h^{-1}. The total polynucleotide concentration in any single experiment varied between 10 and 100 μmol of nucleotide residues/L (0.1–0.8 A_{260} unit) for synthetic homo- and copolymer mixtures and approximately twice those concentrations for natural DNAs. T_m's were determined as described below or from fifth derivative profiles as previously described (Yen & Blake, 1980).

H_2O/D_2O Transfer. Matching polynucleotide solutions in H_2O and D_2O were obtained by redissolution of identical lyophylized preparations in H_2O and D_2O. A stock solution of mixed polynucleotides was first prepared with dialysis against the appropriate buffer in triple-distilled water. Precise aliquots of 1.00 mL of this stock were then delivered with the same single-volume micropipet into 10–16 tubes and shell frozen. After complete lyophilization each sample was redissolved with the addition of 1.00 mL of redistilled H_2O or D_2O with the same micropipet and then incubated for at least 18 h to ensure complete proton exchange (Englander & von Hippel, 1972). The relative pipetting error appears to be below the error in measuring t_m as may be seen from examination in Table I. This table lists results of six replicate experiments on a mixture of five synthetic helixes (Figure 1) in H_2O and D_2O. The standard error in the determination of t_m by repetitive melting of a helical complex in the same tube (±0.08 °C) is the same as that by replicate melting of the complex from each of the several tubes (±0.06–0.11 °C, Table I).

Results

Melting of Polynucleotides in H_2O and D_2O at Low Ionic Strength. (1) Melting of dA·2rU, rA·2rU, rA·rU, rA·dT, and dA·dT. High sensitivity to small differences in energy associated with the melting of different helixes was made possbile by preparing several together in the same solution, generally with the rA·rU complex present as an internal secondary standard since we have examined it most extensively. The

Table I: Melting Temperatures (°C) of Some Polynucleotide Helices in H_2O and D_2O with 0.018 M Na^+

solvent[a]	expt		helix (transition) dA·2rU (3 → 1)	rA·2rU (3 → 2)	rA·rU (2 → 1)	rA·dT (2 → 1)	dA·dT (2 → 1)
H_2O	1		22.76	27.71	42.26	49.46	52.08
	3		22.86	27.73	42.33	49.56	52.08
	5		22.81	27.80	42.29	49.62	52.14
	7		22.91	27.88	42.11	49.49	52.01
	9		22.78	27.80	42.34	49.40	51.93
	11		22.66	27.74	42.36	49.60	52.12
		mean:	22.79	27.78	42.28	49.54	52.08
		SD:	±0.086	±0.063	±0.092	±0.090	±0.091
D_2O	2		24.71	29.05	43.67	50.99	52.81
	4		24.88	29.08	43.61	50.86	52.69
	6		24.93	28.95	43.61	50.91	52.75
	8		24.96	28.98	43.56	50.96	52.70
	10		24.80	29.13	43.61	51.03	52.84
	12		25.00	29.10	43.67	51.01	52.82
		mean:	24.88	29.05	43.63	50.96	52.78
		SD:	±0.108	±0.084	±0.049	±0.064	±0.066
δt_m (mean difference)			−2.09	−1.27	−1.35	−1.42	−0.70
SD of differences			±0.105	±0.121	±0.087	±0.126	±0.108

[a] Containing 5.0 mM sodium cacodylate, 0.2 mM NaEDTA, 15.0 mM NaCl, and $[Na^+]$ = 0.0179 M, pH(D) 6.8.

example of the melting of the five complexes, dA·2rU, a three-standard helix which dissociates to single strands by a single transition (3 → 1) (Riley et al., 1966), rA·2rU (3 → 2) (Blake & Fresco, 1966), rA·rU (2 → 1) (Stevens & Felsenfeld, 1964), rA·dT (2 → 1) (Riley et al., 1966), and dA·dT (2 → 1) (Sigler et al., 1962), is shown in the upper half of Figure 1. This collection of helixes was prepared by adding a 3 μM (nucleotide residue) stock solution of rA·rU in 0.0180 M Na^+ to an equal amount of dA·dT in the same buffer and incubating the mixture at 55 °C, slightly above their melting temperatures for 5′, followed by refrigeration (4 °C) overnight. When cooled from 55 °C the complementary polydeoxyribo and polyribonucleotide strands react at random with one another to form the five complexes in proportion to the areas under the corresponding melting transitions in Figure 1. Additional complexes either do not form at all or melt below 10 °C, the starting temperature for the curve in Figure 1. The assignment of the transitions in this figure was established from continuous variation experiments (mixing curves) at different temperatures, from melting curves produced from each complex separately, and from examination of the [Na] dependence of t_m for each transition.

A better appreciation for the level of sensitivity that can be achieved by the direct difference approximation method of obtaining melting curves may be had from examination of an enlargement of the rA·rU transition (2 → 1), as shown in the lower half of Figure 1. A precise determination of melting temperature can be made simply from the line bifurcating the profile at the peak. Usually, however, t_m's are determined by computer where $d^2A/dT^2 = 0$, as well as from the principal maxima in fifth derivative profiles (Yen & Blake, 1980). The effects of $H_2O \rightarrow D_2O$ transfer are not large; therefore, an appreciation of the level of error is essential. The t_m results of six different experiments in both H_2O and D_2O are given in Table I, for which the average standard error in the t_m of any transition is only ±0.08 °C. The next to the bottom row in this table lists differences in melting temperatures, δt_m, for the five complexes in H_2O and D_2O. Values range from −0.70 to −2.09 °C for differences between the means of two normally distributed populations of melting temperatures, with standard deviations near ±0.11 °C for a population of six observations in H_2O and six in D_2O by t-statistic evaluation. This level of

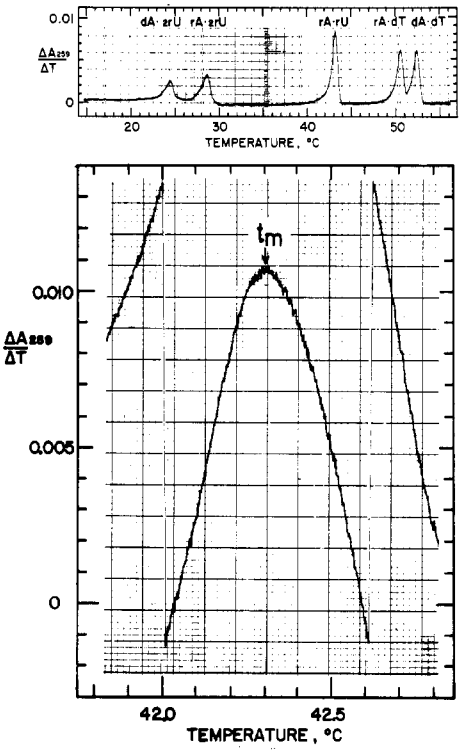

FIGURE 1: Differential absorbance melting curve of dA·2rU (3 → 1), rA·2rU (3 → 2), rA·rU (2 → 1), rA·dT (2 → 1), and dA·dT (2 → 1) in 0.018 M Na^+ at a constant rate of 6.75 °C/h and Δt = 0.2 °C. These five helixes were prepared from a reannealed mixture of rA·rU and dA·dT (see text). (Top) Full melting curve showing all transitions. (Bottom) Enlargement of the rA·rU transition at 5 times greater sensitivity with a folded $\Delta A/\Delta T$ scale for the center section.

error is characteristic of all transfer experiments below and is usually well below differences in δt_m's for different helical transitions.

Table II summarizes results from a large number of measurements with different sources of D_2O and polynucleotides. Two very different values for δt_m are listed for the melting of rA·2rU (3 → 2). The larger value, −2.13 °C, is for a preparation that was permitted to anneal for >72 h at 4 °C in the absence of competing complexes, while the smaller value, −1.30 °C, is for a preparation in the presence of competing mixtures

Table II: Melting in Heavy and Light Water, with 0.018 M Na^+, and Structural Properties of Polynucleotides

helix	δt_m[a] (°C)	$\delta\Delta G_{tr,obsd}$[b] [cal·(mol of bp)$^{-1}$]	transition	helix family[c]	$\Delta\xi^{-1}$[d]	$\delta\Delta G_{tr,el}$[e] [cal·(mol of phosphate)$^{-1}$]	$\Delta[Na^+]^{loc}$[f] (mol)
dA·2rU	−2.05 (19)	−92	3 → 1	A	0.433	+12.6	3.08
rA·2rU	−2.13 (6)[g]	−34	3 → 2	A			
	−1.30 (12)[h]	−20	3 → 2	A			
rA·dT	−1.49 (12)	−37	2 → 1	(A)	0.324	+5.2	1.16
rA·rU	−1.47 (33)	−36	2 → 1	A	0.327	+4.9	1.17
d(A-T)·d(T-A)	−0.95 (5)	−25	2 → 1	B	0.364	+3.5	1.22
(dA·dT)	−0.86[i]	−21	2 → 1	B	0.312	+2.6	0.95
dA·dT	−0.74 (23)	−18	2 → 1	B	0.313	+2.0	1.03
(dG·dC)	−0.51[i]	−13	2 → 1	B	0.175	+4.5	0.73

[a] $\delta t_m = t_m^{H_2O} - t_m^{D_2O}$ (number of paired experiments). [b] From eq 2b where ΔH is the calorimetric enthalpy. [c] Standard designation for the form of the helix observed by fiber diffraction. The more subtle distinctions among helixes, such as between A-DNa and RNA-11 or B-DNA and C-DNA, are not recognized. [d] From eq 12. [e] From eq 9. [f] From eq 11. [g] From annealed preparations containing only rA and rU and free of other complexes. [h] From annealed preparations that include the presence of dA and dT. [i] From extrapolation.

of dA, rA, dT, and rU as described above. These differences are quite reproducible and obviously reflect a difference in the structures of rA·2rU prepared under the two conditions, for which, at the present time, we have no tenable explanation. The dependences of t_m on $[Na^+]$ for both rA·2rU preparations are nearly identical at 30.5 °C per decade change in [Na]. Also, the transition for a mixture of rA·2rU prepared by the two methods is identical with those for the separate preparations. It should be stressed that no such duplexity of δt_m was observed for the other four helixes in this series, as can be readily verified by comparing results in Table I with those in Table II.

The largest number of paired measurements, 33, have been made on rA·rU (2 → 1). The average δt_m for this complex, −1.47 ± 0.10 °C, is identical with that for the 2 → 1 transition of the hybrid rA·dT, −1.49 °C, but exactly twice that for the 2 → 1 transition of dA·dT, −0.74 °C. The only apparent reason for this difference is the difference in their conformations, the former two helixes assume the A-like RNA-11 conformation (Arnott et al., 1968) while the latter is in the B structure (Arnott & Selsing, 1974). To determine whether differences could also be detected in the various equilibria among the intermediate states during melting in the two solvents, we conducted an evaluation of the several parameters describing the melting curves by established statistical thermodynamical analysis (Applequist, 1969). The evaluation of these parameters involved computer fits to the observed profiles over the entire temperature range of melting (>2.5 °C) in both H_2O and D_2O because the full shape of the profile is quite sensitive to values for the parametric constants used to compute the theoretical profiles. A value of 0.022 was obtained for the stacking parameter (assuming mismatching degrees of freedom during melting) (Blake, 1973) and 2.22 for the loop closure exponent for the melting of both rA·rU and rA·dT in both H_2O and D_2O. A small difference in unit enthalpy was all that was necessary to bring them into register on the temperature scale. In other words, all four profiles are identical when superimposed on the same temperature scale. These values are identical with those found earlier in two independent studies of the melting of rA·rU (Blake, 1973; Freire & Biltonen, 1978). Only small adjustments in the parametric constants are necessary to describe the melting of dA·dT, yet again no differences could be detected between profiles in H_2O and in D_2O. These results indicate that within the limits of such analysis to detect small shifts in local equilibria induced by transferring the transition from H_2O to D_2O, none could be observed.

We examined this question from a different vantage point by measuring the variation in δt_m with the mole fraction of D_2O, X_{D_2O}. The results are shown in Figure 2, where it can be seen that the effect on t_m of transferring the transitions for dA·2rU (3 → 1), rA·rU (2 → 1), rA·dT (2 → 1), and dA·dT (2 → 1) from H_2O to D_2O is that t_m varies linearly with the proportion of D_2O, with no significant attraction by one or the other solvent with either helix or coil. The underlying assumption is, of course, that any compensation by opposing effects is insufficient to obscure interactions that might involve the solvent. Even if such compensation were present, one would expect to see some variation from linearity with at least one or more of these helixes.

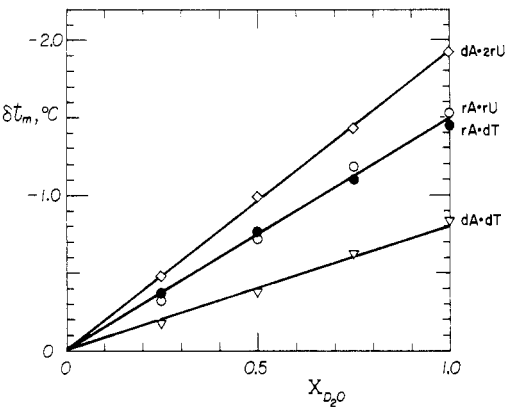

FIGURE 2: Variation of δt_m for the difference in melting temperature in H_2O and D_2O with mole fraction of D_2O, X_{D_2O}, for dA·2rU (3 → 1), rA·rU (2 → 1), rA·dT (2 → 1), and dA·dT (2 → 1).

Thus, we conclude that factors associated with the particular conformation of the helix are principally responsible for variations in the increase in melting temperature on transferring the h → c transition for polynucleotides from H_2O to D_2O. The exchange of solvent water molecules that interact with the pentose directly cannot be responsible for the transfer result since the hybrid rA·dT behaves identically with rA·rU.

(2) *Melting of d(A-T)·(T-A).* The δt_m for this alternating copolymer was determined with both rA·rU and dA·dT present for direct comparison. An average value of −0.95 °C for five replicate experiments was obtained, which is reasonably close to, but not identical with, that for the homopolymer dA·dT, −0.74 °C, with which it is most related by structure (Arnott et al., 1974).

(3) *Melting of λ, E. coli, and Calf Thymus DNAs.* Thus far the polynucleotide complexes examined have consisted of just A-T or A-U base pairs. Transfer of natural DNAs from H_2O to D_2O offers the opportunity to determine the effects of G-C base pairs. Because it is stronger than A-T the random incorporation of G-C among A-T base pairs in a helix leads

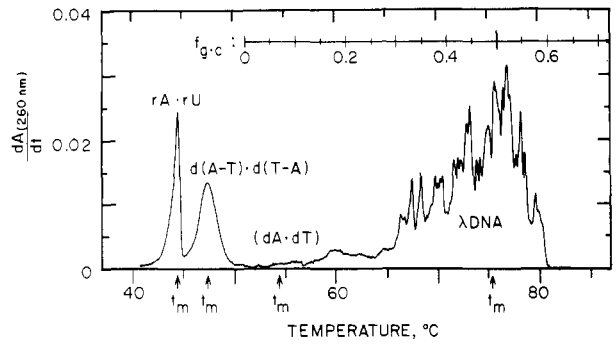

FIGURE 3: Differential absorbance melting curve of λ DNA with rA·rU and d(A-T)·d(T-A) markers in 0.018 M Na^+ at a constant rate of 6.75 °C/h and Δt = 0.23 °C. Melting temperatures of the synthetic helixes were determined as described in the text, while that for λ DNA was determined from the temperature corresponding to half the integrated area of the profile. The inset Marmur–Doty (Marmur & Doty, 1962) scale for the variation of t_m with the fractional G-C base composition of each subtransition, $f_{G\cdot C,i}$, corresponds to the expression $t_{m,i} = t_{A\cdot T} + \Delta t f_{G\cdot C,i}$, where $t_{A\cdot T}$ is the mean t_m for d(A-T)·d(T-A) and dA·dT (not shown in the figure), and Δt = 48.98 °C, representing the increase in melting temperature for the incorporation of the stronger G-C base pair into DNA.

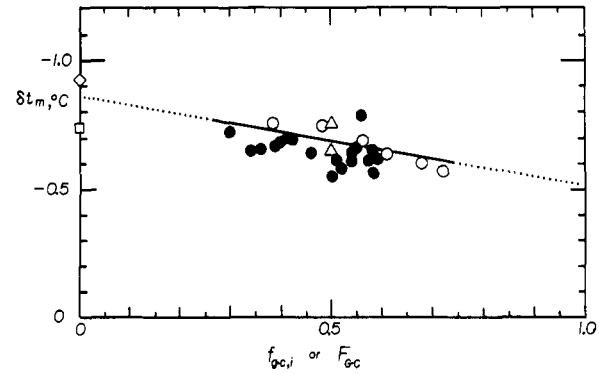

FIGURE 4: Variation of δt_m with the fractional base composition, $f_{G\cdot C}$, of the DNA in transition from helix to coil. The $f_{G\cdot C,i}$ of each domain contributing to a resolvable subtransition in the melting profiles of λ DNA (●) and the five principal satellite components of calf thymus DNA (○) were determined by a two-wavelength spectral decomposition (Blake & Lefoley, 1978; Blake & Haydock, 1979). Other data include the δt_m for *E. coli* (△) where $F_{G\cdot C}$ = 0.50, d(A-T)·d(T-A) (◇), and dA·dT (□).

to regions that behave as what may best be described as phase boundaries between helical and coil regions during melting (Azbel, 1973, 1979, 1980; Yen & Blake, 1981). Rather than a single sharp transition as seen with synthetic homopolynucleotide systems (e.g., Figure 1), natural DNAs melt as a collection of discrete domains. Resolution of the individual subtransitions under the fine structure in such a melting curve depends on the distribution of local G-C contents for the entire collection of domains. In λ DNA this distribution is exceptionally broad so that despite its large size (48 000 bp) the melting curve is characterized by a large number of subtransitions, of which ~30 can be readily detected without special deconvolution analysis. Figure 3 shows a melting curve of DNA in 0.018M Na^+, including the internal standards rA·rU and d(A-T)·d(T-A). At least 26 distinct peaks can be counted in this profile. The fractional G-C base composition, $f_{G\cdot C,i}$, of each domain contributing to a distinguishable peak was taken as previously determined by spectral decomposition (Blake & Haydock, 1979) since the variation of $t_{m,i}$ for the ith domain with $f_{G\cdot C,i}$ is not quantitatively accounted for by the familiar Marmur–Doty relationship (Marmur & Doty, 1962). The latter best describes the melting behavior (t_m) of a mixed population of DNAs of different overall base content, $F_{G\cdot C}$. A linear scale representing the Marmur–Doty relationship was included in Figure 3 simply to provide a qualitative appreciation for the range of base composition in domains of λ DNA. This scale was established from the mean value for the t_m's of d(A-T)·d(T-A) and dA·dT (excluded in this particular experiment) and the overall t_m of λ DNA, for which $F_{G\cdot C}$ = 0.49. The averages of four paired determinations of $\delta t_{m,i}$ for the transfer of the subtransition for each domain into D_2O are plotted in Figure 4 against the base composition of the domain. Values for $\delta t_{m,i}$ vary from −0.56 to −0.80 °C. While some of the scatter is probably real, the general trend in the relationship between $\delta t_{m,i}$ and $f_{G\cdot C}$ is slightly negative. This trend is clear when the δt_m's for d(A-T)·d(T-A) and dA·dT are considered.

The melting of *Escherichia coli* DNA ($F_{G\cdot C}$ = 0.50) involves a normal distribution of a very large number of subtransitions overlapping in temperature and so results in a rather smooth bell-shaped profile (Blake & Lefoley, 1978). The δt_m's for the variation in the maxima of such curves plotted for two paired experiments bracket the results of domain melting of λ DNA. Also plotted in Figure 4 are the δt_m results of six replicate transfer experiments on calf thymus DNA. Values for the melting behavior of the five principal satellite components of differing repetitive sequence and $F_{G\cdot C}$, plus the main band for the melting of unique sequences, are included. Correspondence with the λ DNA results is quite good, again indicating a negative variation between δt_m and $F_{G\cdot C}$.

Taken collectively, these results show that the magnitude of δt_m for natural DNAs corresponds quite well with that for synthetic helixes known to assume a B-like conformation. Further, δt_m varies with the fractional G-C base composition of the DNA, with a linear slope of −0.35 °C and with extrapolated values of −0.86 °C for the A-T base pair and −0.51 °C for the G-C base pair (Table II). This variation is mainly due to the unit transition enthalpy, which is quite different for the A-T and G-C base pairs, having an inverse effect on δt_m as prescribed by eq 2. The unit enthalpy can be estimated for DNAs of different fractional G-C content with good precision by

$$\Delta H = 11250 + (2.6 - F_{G\cdot C})(450 \log [Na^+] - 580)\ \text{cal}\cdot(\text{mol of bp})^{-1} \quad (3)$$

obtained by fitting a large number of independent values for the calorimetric transition enthalpy from the literature (Blake & Haydock, 1979). Although the decrease in δt_m with G-C content is accompanied by an increase in transition enthalpy, compenstion is inadequate to ensure a constant value for $\delta\Delta G_{tr}$. The free energy for transfer of a G-C base pair is 40% less than that for an A-T base pair (cf. Table II). Evidently the exchange of deuterium for hydrogen bonds between bases in the helical state does not increase the strength of the bond over that for the same exchange between bases in the coil state and the aqueous solvent, since the extra H bond in the G-C base pair does not lead to a higher $\delta\Delta G_{tr}$. In fact, the opposite appears to be the case. The explanation is that G and C coil residues have greater residual structure than A and T (Blake & Haydock, 1979), so that the net energetic effect of changing the solvent is greater for the A-T base pair transition. The solvent transfer effect, then, does not directly involve the chemical nature of the base pair or, as demonstrated above, the sugar. Rather it seems to arise from differences in charge density associated with different conformations of helix.

Melting of Polynucleotides in H_2O and D_2O at Different [Na^+]. Melting of rA·rU. The h → c transition of rA·rU was examined over a wide range of [Na^+] in both H_2O and D_2O,

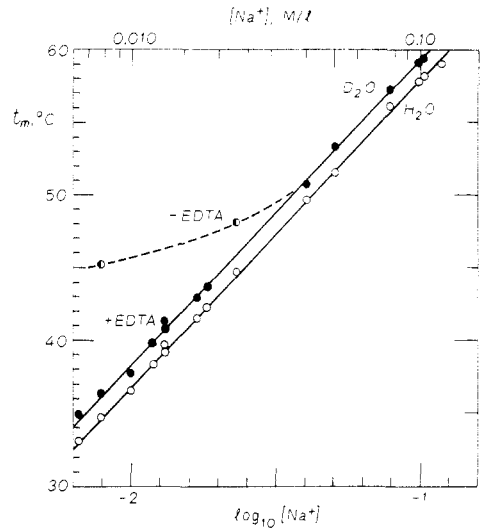

FIGURE 5: Variation of t_m for rA·rU with [Na^+] in H_2O (○) and D_2O (●). The buffer consists of 2 mM sodium cacodylate and NaCl prepared as described by Blake & Haydock (1979), where 0.2 mM NaEDTA is present in those experiments connected by solid lines, and absent in those connected by the broken line (◐) in D_2O. The variation of t_m in H_2O in the presence and absence of EDTA are coincident over the full range of [Na^+] in this figure.

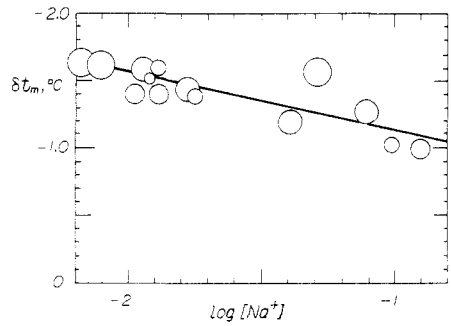

FIGURE 6: Variation of δt_m with [Na^+] for the melting of rA·rU in H_2O and D_2O. Buffer conditions described in the legend to Figure 5.

and the results were plotted in Figure 5. Above 0.03 M Na^+, the t_m for rA·rU increases linearly with log [Na^+] in both H_2O and D_2O in almost parallel fashion. However, below 0.03 M Na^+ and in the absence of EDTA, the t_m of this complex in undistilled D_2O deviates sharply from that in H_2O. The t_m levels off at ~45 °C with decreasing [Na^+], giving the impression of a striking solvent isotope effect at low [Na^+] (Lewin, 1966). That this effect is artifactual and due to the contamination of unredistilled commerical D_2O by submicromolar amounts of multivalent cations is indicated by the total elimination of the deviation in t_m in redistilled D_2O (see Materials) or by the addition of 0.2 mM EDTA. The plateau value of t_m in undistilled D_2O does not appear to vary much with the commercial source of the D_2O.

The dependence on [Na^+] of δt_m for the transfer of the h → c transition of rA·rU from H_2O to D_2O is illustrated in Figure 6. The variation is negative, with δt_m decreasing from a value of −1.7 °C at 6 mM Na^+ to a value of only −1.0 °C at 0.15 M Na^+. The slope for the variation of δt_m with [Na^+] for calf thymus repetitive DNAs is almost 50% greater than this. Above 0.4 M Na^+ the solvent isotope effect is nil, and $\delta t_m \rightarrow 0$ for calf thymus DNA and rA·2rU (3 → 1), two helixes that can be examined conveniently at such high salt concentrations. Most of this variation in δt_m with [Na^+] is due to the increase in transition enthalpy (cf. eq 2) with increasing [Na^+]. From a compendium of published calorimetric studies, the variation in unit transition enthalpy with [Na^+] for rA·rU has been represented by the linear expression (Blake, 1973)

$$\Delta\bar{H} = 9333 + 1175 \log [Na^+] \quad (4)$$

with good precision. Compensation of the decrease in δt_m with [Na^+] by the increase in $\Delta\bar{H}$ is not entirely complete, and $\delta\Delta G_{tr}$ decreases by ~8 cal·(mol·bp)$^{-1}$ per decade increase in [Na^+]. The explanation is presumably experimental error or that residual structure and compactness in the coils, probably of rA, increases very slightly with increasing ionic strengh.

Discussion

Results show the solvent isotope effect to be strongly dependent upon the difference in conformation between helix and coil. Subtle chemical differences such as the nature of the sugar or base pair do not contribute directly to the effect even though these same chemical features, certainly the sugar, determine the conformation of the helix. Two possibilities exist to produce a solvent effect. First, water molecules may interact with themselves to form a quasi-crystalline lattice or buttress work through hydrophobic, surface tension, or bridging bonds favoring the helix in D_2O over that in H_2O. Alternatively, the solvent effect arises from energetic differences between H_2O and D_2O molecules that interact directly with the polynucleotide, e.g., through hydration–solvation. Results summarized in Table II do not support the concept of lattice-structured water making a major contribution to the effect, particularly when the behavior of rA·rU, rA·dT, and dA·dT are considered. These three helixes differ inconsequentially considering the magnitude of the observed solvent effect. It cannot be said categorically, of course, that hydrophobic or surface tension bonds do not play a small role in supporting the helix but rather that the energy of transfer ($H_2O \rightarrow D_2O$) is small or zero. Results of an extensive tabulation by Arnett & McKelvey (1969) indicate the free energy of transfer of "hydrophobic" nonelectrolytes is small and variable in sign, especially when compared to the ΔG_{tr} for salts. Nevertheless, any suggestion by these studies of a lack of structured water around DNA is consistent with the results of Falk et al. (1963, 1970) from infrared spectral studies indicating a total absence of any regions of ordered "ice-like" water in the hydration shell. Numerous NMR [e.g., Kuntz et al. (1969)] and dielectric studies indicate the translational and rotational behavior of water molecules in intimate contact with the helix are reduced relative to the bulk solvent but show no exceptional latticed order. In fact, they are incapable of crystallizing into the ice lattice at very low temperatures (Kuntz et al., 1969).

The results in Table II also do not seem to favor the alternative that the effect arises from direct interactions with the polynucleotide since sugar and base moieties do not appear to be involved while the phosphate and counterion are qualitatively indistinguishable in different conformational states. They are not, however, quantitatively indistinguishable. It will be appreciated that the fractional charge on the phosphate or, more specifically, the extent of counterion association with the polynucleotide is very sensitive to conformation so that the solvent effect could be due to differences in dielectric properties or differences in the free energy of counterion solvation between the two solvents. Results in Table II qualitatively support the notion that the solvent effect is somehow associated with differences in charge density among helixes. Thus, the transfer of the 3 → 1 transition for the highly charged dA·2rU complex from H_2O to D_2O yields the largest value for δt_m. Following this are the differences in δt_m for the 2 → 1 transitions between rA·rU and rA·dT on the one hand and dA·dT

on the other. Because of its more extended structure, dA·dT has a greater distance between anionic centers on opposing strands of the helix and accordingly gives a smaller value for δt_m than do rA·rU and rA·dT.

Changes in t_m for solvent transfer are proportional to differences in free energy for transferring the h → c transition from one solvent to the other as prescribed by eq 2. As noted, an apparent correlation exists between the magnitude of the solvent effect and the charge density of the helix; therefore, as a first approximation, we assume $\delta\Delta G_{tr,obsd}$ to be primarily of electrostatic origin. The dielectric constants of heavy and light water differ by only 0.5% at 25 °C; nevertheless, it is instructive to examine the electrostatic consequences of this difference. The repulsive electrostatic free energy per mole of anionic point charge q arranged in a linear array at distances b (centimeters) from one another on the polynucleotide (Manning, 1978) is

$$G_{el} = -RT[q^2/(\epsilon kTb)] \ln (\kappa b) \quad (5)$$

where ϵ is the bulk dielectric constant, k is Boltzmann's constant, and κ is the Debye screening parameter

$$\kappa^2 = \frac{8\pi N_0}{1000}[q^2/(\epsilon kT)][Na^+]^{1/2} \quad (6)$$

Terms of order κ are neglected in eq 5 because the $[Na^+]$ is only 0.018 M in these experiments and $\kappa b \ll 1$. According to Manning (1978, 1979) polynucleotides, like all polyelectrolytes, have a saturated charge fraction less than unity due to territorial condensation by countercations. The fraction, f, of sodium counterion condensed in the proximity of each phosphate is

$$f = q_{net}/q = \xi^{-1} \quad (7)$$

where

$$\xi \equiv q^2/(\epsilon kTb) \quad (8)$$

is a charge density parameter governing the condensation process. Substitution can be made in eq 5 of q by q_{net}. Of special interest is the change in electrostatic free energy associated with the h → c transition. Allowing also for those contributions to the free energy from the counterion diffusion potential, the tendency of delocalized bound Na^+ to diffuse away from the immediate vicinity of the nucleic acid, leads to the expression (Manning, 1978)

$$\Delta G_{el}/(RT) = \Delta[(1 - \xi^{-1}) \ln ([Na^+]^{loc}/[Na^+]) - \xi^{-1} \ln (\kappa b)] \quad (9)$$

where $[Na^+]^{loc}$ is the concentration of "bound" Na^+ constrained by long range electrostatic forces to a local volume V_p (cubic centimeters per mole of anionic phosphate)

$$V_p = 41.1(\xi - 1)b^3 \quad (10)$$

while the local concentration of delocalized bound Na^+ is

$$[Na^+]^{loc} = 10^3(1 - \xi^{-1})V_p^{-1} \quad (11)$$

in units of molarity when b is expressed in angstroms. $[Na^+]^{loc}$ is a constant for a given polynucleotide conformation and independent of the bulk $[Na^+]$. The change in the phosphate charge fraction in eq 9 due to the h → c transition is

$$\Delta\xi^{-1} = \xi_c^{-1} - \xi_h^{-1} \quad (12)$$

Since more than one "coil" is produced during the transition, we must write

$$\xi_c^{-1} = \sum f_i \xi_{c,i}^{-1} \quad (13a)$$

Table III: Mean Contour Axis Univalent Charge Spacing in Polynucleotide Helixes and Coils

polynucleotide	b, measured value (Å)	method[a]	ref	b, value used (Å)
rA·2rU	1.01	diff	*b*	1.01
dA·2rU	1.02	diff	*b*	1.02
d(A-T)·d(T-A)	1.52	diff	*c*	1.52
rA·rU	1.55	diff	*d*	1.55
rA·dT	1.55	melt	*e*	1.55
dA·dT	1.63	diff	*f*	1.63
DNA_h	1.69	diff	*g*	1.69
DNA_c ($F_{G\cdot C} = 1.0$)	3.10	melt	*h*	3.1
rC	3.11	diff	*i*	
rA	3.8	scatt	*j*	3.4
	3.2	hydro	*k*	
	3.1	melt	*l*	
	3.51	melt	*e*	
dA	3.3	melt	*m*	3.4
	3.43	calc	*n*	
	3.46	melt	*e, h*	
DNA_c ($F_{G\cdot C} = 0.5$)	3.87	calc	*o*	
	3.74	melt	*h*	
DNA_c ($F_{G\cdot C} = 0.34$)	4.3	melt	*l*	
	3.88	melt	*h*	
DNA_c ($F_{G\cdot C} = 0$)	3.98	melt	*e, h*	4.0
$d(A\text{-}T)_c$	4.25	melt	*e*	4.2
rU	4.3	hydro	*p*	4.5
	4.37	calc	*q*	
	4.5	melt	*l*	
	4.5	calc	*o*	
	4.60	melt	*e*	
dT	4.46	melt	*e*	4.5
	4.9	melt	*m*	

[a] Methods of measuring the charge spacing, b, are as follows: diff, from fiber diffraction analysis; melt, from analysis of the cation dependence of melting [e.g., Record et al. (1978), pp 144-145]; calc, from nonbonded, torsional, and electrostatic potential energy minimization; hydro, from hydrodynamic measurements of the unperturbed end-to-end distance of monodisperse specimens; scatt, from low-angle X-ray scattering. [b] Arnott & Bond, 1973. [c] Arnott et al., 1974. [d] Arnott et al., 1968. [e] This work. [f] Arnott & Selsing, 1974. [g] Arnott & Hukins, 1972. [h] Blake & Haydock, 1979. [i] Arnott et al., 1976. [j] Gulick et al., 1970. [k] Stannard & Felsenfeld, 1975. [l] Record et al., 1976, 1978. [m] Pless & Ts'o, 1977. [n] Hingerty & Broyde, 1978. [o] Olson & Manning, 1976; Manning, 1976. [p] Inners & Felsenfeld, 1970. [q] Broyde & Hingerty, 1980; S. Broyde, personal communication.

where f_i is the fraction of the ith coil product of the transition. Similarly

$$b_c = \sum f_i b_{c,i} \quad (13b)$$

Equation 9 shows explicitly the relationship between electrostatic free energy and conformation of the nucleic acid. There is a strong dependence of ΔG_{el} on the change, during the h → c transition, of the charge spacing, b, the fundamental structural parameter of the nucleic acid. Values for charge spacings of the different nucleic acids examined in this study are given in Table III. Those for helixes were computed in a straightforward manner from fiber diffraction results, while those for coils represent the most self-consistent values from analyses of the cation dependence of melting (Record et al., 1976; Blake & Haydock, 1979), from potential energy minimization studies (Olson & Manning, 1976; Hingerty & Broyde, 1978; Broyde & Hingerty, 1980), and from hydrodynamic and low-angle X-ray scattering analysis (Stannard & Felsenfeld, 1975; Gulick et al., 1970). With selected values for the charge spacing from Table III and accurate values for the bulk dielectric constants of H_2O and D_2O as a function of temperature from the careful measurements of Malmberg

(Malmberg & Maryott, 1956; Malmberg, 1958), we have calculated the electrostatic free energy of transfer, $\delta\Delta G_{tr,el}$ for h → c transitions of helixes examined in this study and listed in Table II. Changes in phosphate charge fraction during the h → c transition are tabulated in column 6 of this table, while column 7 lists the calculated values of $\delta\Delta G_{tr,el}$ for the expected electrostatic consequences of transferring the h → c transition from H_2O to D_2O. The variation in phosphate charge fraction with $\delta\Delta G_{tr,el}$ is somewhat irregular, while the apparent electrostatic energy seems to vary as $\delta\Delta G_{tr,el} \simeq -0.15\delta\Delta G_{tr,obsd}$ for all helixes. Interestingly, this relationship holds fairly well for both conformational families of helix. However, calculated electrostatic free energies are all ~7-fold smaller than observed values and have the wrong sign, indicating helixes should be slightly destabilized in D_2O, contrary to what is observed. This is a consequence of the slightly lower dielectric constant of D_2O having a greater effect on the counterion diffusion potential represented by the first term on the right-hand side of eq 9.

We have considered the possibility that the solvent isotope effect arises in part from the difference in Na^+ hydration energy between H_2O and D_2O. The assumption would be that a small number of Na^+ liberated during the h → c transition are site bound to the helix. The difference in total Na^+ condensed to helix and coil, calculated by eq 11, exceeds 1 M for DNA and 3 M for dA·2rU (Table II, column 8), so it seems not improbable that a small number of Na^+ might be tightly site bound with partial or total dehydration and in numbers below detectability by most probes. The energetic effect of Na^+ hydration is quite large, close to −90 kcal·mol^{-1} (Robinson & Stokes, 1959) while the free energy of transfer is only +95 cal·mol^{-1} (Arnett & McKelvey, 1969); thus

$$\begin{aligned} Na^+_{gas} &\rightarrow Na^+_{H_2O} \quad \Delta G_{hy}^{H} = -89.7 \text{ kcal·mol}^{-1} \\ Na^+_{gas} &\rightarrow Na^+_{D_2O} \quad \Delta G_{hy}^{D} = -89.6 \text{ kcal·mol}^{-1} \qquad (14) \\ \hline Na^+_{H_2O} &\rightarrow Na^+_{D_2O} \quad \Delta G_{tr} = +95 \text{ cal·mol}^{-1} \end{aligned}$$

Since melting is accompanied by release of Na^+ and the energy of transfer is positive, the helix would be stabilized in D_2O slightly over that in H_2O, as observed. A plot of $\Delta[Na^+]^{loc}$ against $\delta\Delta G_{tr,obsd}$ shows the relationship to be linear and vary with the type of helix (Figure 7). The slope for A-like helixes is −30.0 cal·(mol of bp)$^{-1}$·(mol of Na^+)$^{-1}$ and −20.0 for B structures. These values are quite large, and would require that fully one-quarter to one-third of all Na^+ condensed to the helix be site-bound, clearly outside the realm of possibility as cause for the observed effect. We are reminded by Manning (1978) that the evidence for site-binding is nil, indeed, the NMR results of Reuben et al. (1975) indicate little if any loss of hydration.

The basis for an attractive alternative explanation of the solvent isotope effect can be found in a recent discussion by Manning (1981 and personal communication) of reasons for the effects of ionic strength and cation specificity on the helix angle of twist and axial extension of DNA. It is pointed out that the dielectric constant of a 1 M aqueous solution of NaCl at 25 °C is only 66.7 (Pottel, 1973), 15% lower than pure water, and that such a value may be more appropriate for the local environment of the DNA molecule where the cation concentration exceeds 1 M. The electrostatic potential gradient is exceedingly intense near the surface of a cation, causing extreme orientational polarization of adjacent water molecules. It has long been recognized that the macroscopic value for the dielectric constant of an aqueous electrolyte applies only beyond the coordination sphere of the cation and is a poor approximation of effects at distances on the order of molecular dimensions [e.g., Stokes (1964)]. As has been noted, the free energy of Na^+ hydration in D_2O is less by ~95 cal than that in H_2O, which may indicate a dielectric saturation in the neighborhood of D_2O solvated cations that is something less than in H_2O. If the dielectric constant of a concentrated Na^+ solution in D_2O is significantly greater than that of a corresponding H_2O solution, the positive free energy associated with intrastrand phosphate repulsion will be lower in D_2O, resulting in a more stable helix over that in H_2O, as observed. How much the helix is stabilized in D_2O over that in H_2O by this "salt effect" is difficult to estimate at the present time. The electrostatic free energy, given by eq 9, accounts only for long-range interactions and, as such, is governed more by the dielectric constant of the bulk solution. From an experimental point of view, it remains to be demonstrated that the solvent isotope effect shows the "expected" dependence on the cation species.

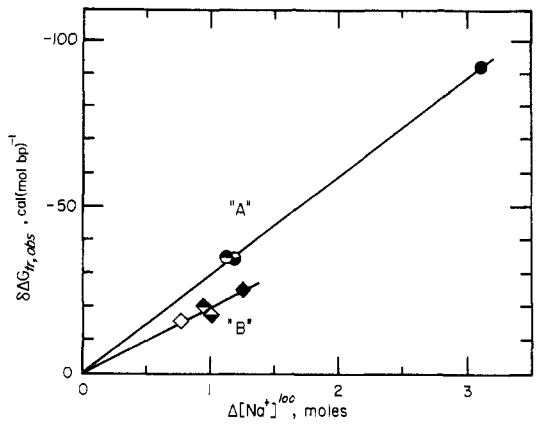

FIGURE 7: Variation of the free energy of transfer, $\delta\Delta G_{tr,obsd}$, with the difference in local Na^+ concentration, $\Delta[Na^+]^{loc}$, between that delocalized bound to the helix and to the coil. The different helical systems represented include dA·2rU (●), rA·rU (⊖) and rA·dT (⊜) with A-like helical structures, and d(A-T)·d(T-A) (♦), dA·dT (♦), (dA·dT) (♦) and (dG·dC) (◊) with B structures. The latter two were determined from extrapolated values for δt_m for the A-T and G-C base pair in Figure 4. The solid lines represent the least-squares fit to results for helixes conforming to either the B or A structure, where the origin is weighted as one point.

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