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# Thermal stability of poly (A) and poly (U) complexes in $H_2O$ and $D_2O$: isotopic effects on critical temperatures and transition widths

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## ABSTRACT

This is a study of the effect of total and partial deuteration of solvent on critical temperatures and profiles of all four reactions occurring in poly(A)•n poly(U) (n = 1 or 2) aqueous systems. The study was done at observational times not longer than hydrogen exchange times at base pairs in helically ordered structures, and it was extended to a wide range of salt concentrations at neutral pH.

The dependence of stability of polymer helical order on hydrogen mass does not appear to be merely attributable to the stronger intrahelical deuterium bonding. Substituting Deuterium for Hydrogen implies a probably predominant modulation of the entropy term of polymer-solvent interaction.

Effects of deuteration on the width of the 2{poly(A)•poly (U)} → poly(A)•2poly(U)+poly(A) interconversion reaction were also observed. They bear on the role of polymer-solvent interaction on pattern recognition leading to formation of ordered structures. They also bear on the role of the same interaction on the "breathing" of ordered structures of this type.

## INTRODUCTION

The fundamental genetic implications of conformational stability in native nucleic acids and in model polynucleotide systems have stimulated in the past a great amount of work[2-4]. Deuteration studies of nucleic acids in non aqueous solvents date back to two decades ago[5,6]. The thermodynamics of base transfer from organic to aqueous solvents was also studied[7,8]. Conclusions concerning base-solvent interaction appear conflicting[5,9].

Poly(A) and poly(U) have proved particularly useful model systems. Upon mixing, they are able to form two well defined helical complexes: the double stranded poly(A)•poly(U) with 1:1 monomer ratio and the triple stranded poly(A)•2poly(U) with 1:2 monomer ratio[10-14]. The following four reactions and the relative stability diagram are well known (see e.g. refs. 11, 15 and 16a)

$$\text{poly(A)}\cdot\text{2poly(U)} \rightleftarrows \text{poly(A)}\cdot\text{poly(U)}+\text{poly(U)} \tag{1}$$

$$\text{poly(A)}\cdot\text{poly(U)} \rightleftarrows \text{poly(A)}+\text{poly(U)} \tag{2}$$

$$\text{poly(A)}\cdot\text{2poly(U)} \rightleftarrows \text{poly(A)}+\text{2poly(U)} \tag{3}$$

$$2\left[\text{poly(A)}\cdot\text{poly(U)}\right] \rightleftarrows \text{poly(A)}\cdot\text{2poly(U)}+\text{poly(A)} \tag{4}$$

Read from left to right these equations correspond to cooperative transitions whose sharpness along the temperature scale (particularly marked in this case of homopolymers)is one of the physical indications of the known structural and functional stability of nucleic acids.

In the present work we have studied the H-D isotope shifts of critical temperatures corresponding to reactions (1) to (4) above and the effects of the same isotopic substitution (total and partial) on trasition profiles. This type of study must be distinguished from that of isotope alterations of chemical kinetics[17,18] and is here particularly meaningful, since timescales of hydrogen exchange at base pairs in these helically ordered structures are longer than experimental observation times[19-21]. Under these circumstances,much can be learned on the nature of phase transitions from shifts of critical temperatures due to total and partial deuteration[22-25].

## MATERIALS AND METHODS

Polyriboadenylic acid, potassium salt (poly(A)) and polyribouridylic acid, potassium salt (poly(U)) were purchased from Sigma (London). Deuterium oxide (99.8%)was obtained from BOC Ltd. (Prochem), London. All other chemicals were reagent grade. Possible contamination with ribonucleases was carefully avoided, by adding a chelating agent.

Separate stock solutions of poly(A) and poly(U) were prepared, each approximately $5\cdot10^{-3}$M (polymer bases) in $5\cdot10^{-1}$M potassium phosphate buffer (pH=7.0)+$10^{-2}$M EDTA (potassium salt) both in $H_2O$ and $D_2O$. Polymer concentrations were measured spectrophotometrically: $\varepsilon_{257}= 10.0\ cm^{-1}/mM$ for poly(A) and $\varepsilon_{261}= 9.5\ cm^{-1}/mM$ for poly(U)[13]. Stock solutions of poly(A) and poly(U) were then mixed in the appropriate monomer molar ratio and diluted in water or heavy water containing appropriate amounts of salt, to obtain the desired polymer and salt concentrations. Various $D_2O/H_2O$ proportions were obtained by mixing samples previously prepared in $H_2O$ and in $D_2O$. Samples prepared in this way were stored at 4°C for at least 3 days prior to use[13]. Results on reactions (2) and (3) obtained with systems prepared by mixing poly(A) and poly(U) solutions in 1:1 monomer molar ratio were identical to those obtained with samples having 1:2 monomer molar ratio.

The pH of the solutions was measured with a Radiometer PHM 72 digital pH-meter and a type E 5021 microelectrode. Unless otherwise stated, pH meter reading was 7.0 for all samples, including those containing $D_2O$. Independence of critical temperatures upon slight pH changes is discussed in the next section.

Absorbancy-temperature profiles were determined on a Cary 118C spectrophotometer equipped with a precisely thermostatted cell-holder. Thermal influence from the variable temperature sample holder on the detector was avoided by independently thermostatting the detector compartment. This assured extreme baseline stability. The cuvette temperature was scanned uniformly at a rate slow enough to allow both thermal and kinetic equilibrium to be attained at each temperature. Heating rate was about 0.25°C/min in most experiments. Transition profiles determined at this rate were identical in shape to those determined at doubled or halved rates and to those taken at equilibrium points. Temperatures were measured with a Leeds-Northrup precision bridge and a platinum thermometer calibrated against a similar, NBS certificated, one.

Transition profiles were not corrected against thermal expansion of solutions. Critical temperatures, indicated by $T_m$, are defined as the temperature values at which absorbancy changes are at 50% of their total. Absorbancy-temperature profiles for reactions (1) and (2) were measured at 258 nm, those for reaction (3) at 258 nm or at 280 nm and those for reaction (4) at 280 nm[13]. Critical temperatures and transition profiles did not depend upon the particular wavelength chosen.

A special procedure was required in the determination of $T_m$ values at Log $[K^+] \sim -1.1$ where triple and double helix melting profiles partially overlap. It was preliminarly checked that the effect of salt concentration was merely a shift of melting temperature, and that shapes and amplitudes of melting profiles remai ned unaltered. Then, the resulting experimental profile was computer fitted as a sum of the two distinct and invariant transition profiles relative to reactions (1) and (2) respectively. Transition temperatures only were allowed to vary for optimization and values giving the best fitting were then taken as the "experimental" transition temperatures. This procedure did allow working at high salt concentrations. The overall accuracy in the region of profile overlap may have been impaired.

Phase diagrams such as those shown in Fig.1 were computer analyzed following the method of Massoulié[15,26]. A rather high accuracy of this type of fitting was desired, in order to account for our isotope effects. In view of the large number (six) of independent parameters, we expected the existence of a multiplicity of fitting error minima, close to each other, which in fact we found. Thus, we attempted with a smaller number of parameters (two and four) and, also, we used a refinement procedure. In this procedure, we started from the values of parameters obtained by Massoulié's method. These values where then slightly altered in order to obtain, this time, minimal r.m.s. deviations of critical temperatures through the entire set of experimental data, relative to all four reactions (1), (2), (3) and (4).

## RESULTS

### i) Isotope shifts of transition temperatures

The effect of deuteration at different salt concentrations and neutral pH on reactions (1) to (4) are shown in the "phase diagram" of Fig.1. Differences between Fig.1, a) to d), concern fitting curves only (see Discussion), while experimental points are identical. Reaction (3) is not measurably affected by deuteration. Critical temperatures of the remaining reactions appear instead to be shifted upwards by deuterations (Tab.I).

The stabilizing effect of isotopic substitution as measured by $\delta T_{mi} = T^{d}_{mi} - T^{h}_{mi}$ (where d and h stand for deuterium and hydrogen respectively, and i = 1, 2 or 4 refers to case (1), (2), (4) respectively), decreases with increasing $[K^+]$. The value of the $\delta T_{m1}/\delta T_{m2}$ ratio, however, is not far from unity except at the highest salt concentrations.

Possible differences by up to 0.4 pH units between pD values

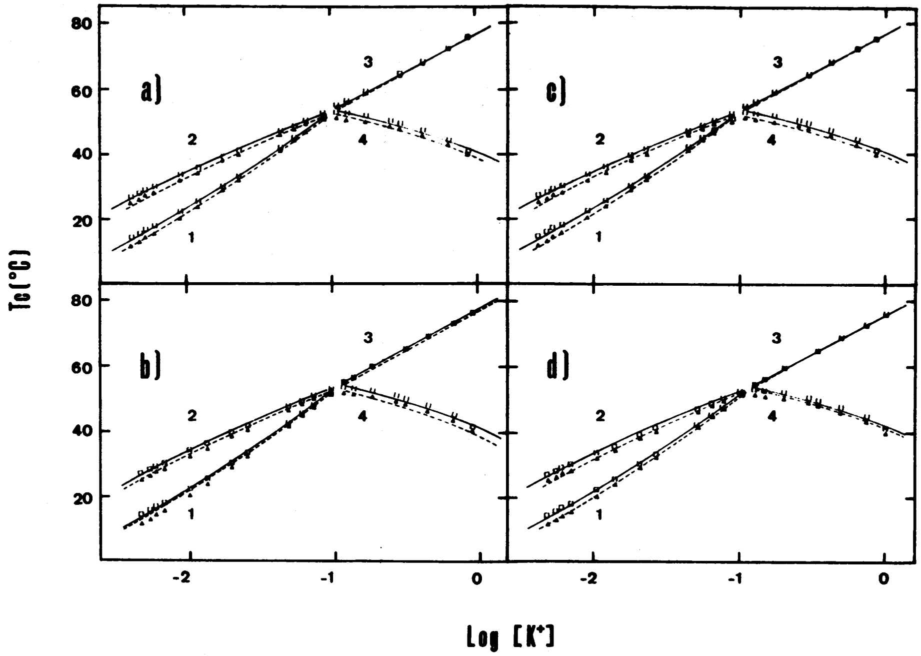

Figure 1: "Phase diagrams" showing the effect of deuteration on critical temperatures ($T_m$) at neutral pH. Branches (1) to (4) in the figure correspond with reactions (1) to (4) respectively. Experimental data: full triangles refer to the $H_2O$ case and are fitted by broken lines; open squares refer to the $D_2O$ case and are fitted by full lines. Polymer concentration: $2.5 \cdot 10^{-5}$M (in base pairs). Each point is the average of at least 3 measurements Cases a), b), c) and d) refer to different ways of fitting the same experimental data (see Discussion).

and actual meter readings[27,28] were considered as a possible origin of artifacts. The dependence of $T_m$ upon pH near neutrality is shown in Tab. II. Changes by up to 0.8 pH units induced in cases (1) and (2) changes of $T_m$ not larger than 0.5°C (to be compared with a $\delta T_m \geq 1.7$°C at identical pH-meter readings). The dependence in case (4) is slightly more marked, yet small enough to show that the isotopic effects we are dealing with, are not the result or artifacts of this type.

The dependence of $T_m$ and $\delta T_m$ values upon polymer concentration is reported in Tab. III. Slightly higher melting temperatures were only observed at high polymer and low salt concentra

Table I. Dependence of isotopic shift $\delta T_{mi}$ of critical temperatures of reactions (1), (2) and (4) in the text upon salt concentration. Polymer concentration: $2.5 \cdot 10^{-5}$M (in base pairs) for reactions (1) and (2), $10^{-4}$M (in base pairs) for reaction (4).

| Log $[K^+]$ | $\delta T_{m1}$(reac.1) | $\delta T_{m2}$(reac.2) | $\delta T_{m1}/\delta T_{m2}$ | $\delta T_{m4}$(reac.4) |
|---|---|---|---|---|
| -2.38 | 2.6°C | 2.0°C | 1.3 | --- |
| -2.05 | 2.0°C | 1.7°C | 1.2 | --- |
| -1.92 | 1.6°C | 1.6°C | 1.0 | --- |
| -1.74 | 1.0°C | 1.1°C | 0.9 | --- |
| -1.35 | 0.7°C | 0.9°C | 0.8 | --- |
| -1.18 | 0.3°C | 0.7°C | 0.4 | --- |
| -0.90 | --- | --- | --- | 1.9°C |
| -0.60 | --- | --- | --- | 1.6°C |
| -0.38 | --- | --- | --- | 1.5°C |
| -0.20 | --- | --- | --- | 1.3°C |
| -0.07 | --- | --- | --- | 1.0°C |

tions (reactions (1) and (2)). This is probably due to the amount of $[K^+]$ introduced together with polymers, not negligible in this low-salt region. No similar dependence was found in the case of reaction (4). Isotopic shifts of critical temperatures were also found to be independent upon polymer concentration.

### ii) Isotope effects on the width of transition profiles

Transition profiles were studied in totally and in partially deuterated systems. Profile of transition (3) was found to be in

Table II. Dependence of critical temperatures for reactions (1), (2) and (4) in the text upon pH near neutrality. Polymer concentration: $2.5 \cdot 10^{-5}$M (in base pairs) for reactions (1) and (2); $10^{-4}$M for reaction (4). $[K^+]$: $10^{-2}$M for reactions (1) and (2); $4.2 \cdot 10^{-1}$M for reaction (4). The estimated experimental error is ± .2°C for reactions (1) and (4) and ± .15°C for reaction (2). The slightly higher experimental error for reaction (1) and (4) is a consequence of the greater transition width.

| pH | $T_{m1}$(reac.1) | $T_{m2}$(reac.2) | $T_{m4}$(reac.4) |
|---|---|---|---|
| 6.85 | 22.2°C | 33.4°C | 46.3°C |
| 7.25 | 22.0°C | 33.2°C | 46.7°C |
| 7.65 | 21.7°C | 33.0°C | 47.0°C |

TABLE III. Critical temperatures and isotopic effect for reactions (1) to (4) in the text, at various polymer concentrations. [K⁺]: $10^{-2}$M for reactions (1) and (2); $10^{-1}$M for reaction (3); $8.4 \cdot 10^{-1}$M for reaction (4). The estimated experimental error is ± .2°C for reactions (1) and (4) and ± .15°C for reactions (2) and (3). The slightly higher experimental error for reactions (1) and (4) is due to the greater transition width. As in the text, superscript h and d stand for hydrogen and deuterium respectively.

| Polymer concentration (in base pairs) | Reaction 1 | | | Reaction 2 | | | Reaction 3 | | Reaction 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $T^h_{m1}$ °C | $T^d_{m1}$ °C | $\delta T_{m1}$ °C | $T^h_{m2}$ °C | $T^d_{m2}$ °C | $\delta T_{m2}$ °C | $T^h_{m3}$ °C | $T^d_{m3}$ °C | $T^h_{m4}$ °C | $T^d_{m4}$ °C | $\delta T_{m4}$ °C |
| $2.5 \cdot 10^{-5}$M | 22.0 | 24.0 | 2.0 | 33.2 | 34.9 | 1.7 | 54.4 | 54.5 | 40.4 | 41.5 | 1.1 |
| $2.5 \cdot 10^{-4}$M | 22.0 | 24.0 | 2.0 | 33.1 | 34.7 | 1.6 | 54.4 | 54.5 | 40.4 | 41.5 | 1.1 |
| $1.2 \cdot 10^{-3}$M | 22.6 | 24.5 | 1.9 | 33.9 | 35.4 | 1.5 | 54.6 | 54.9 | 40.3 | 41.3 | 1.0 |
| $2.5 \cdot 10^{-3}$M | 23.1 | 25.1 | 2.0 | 34.6 | 36.1 | 1.5 | 55.0 | 55.1 | 40.2 | 41.4 | 1.2 |

dependent of deuteration (along with the critical temperature of this transition). Concerning transitions 1 and 2, progressive deuterations caused progressive increases of $T_{m1}$ and $T_{m2}$ values, but again the shape of profiles was left unchanged (Fig.2). This is true in the entire range of salt concentrations studied. Also, no dependence upon polymer concentration was found.

At variance with these results, the shape of the profile of transition (4) was found to depend upon deuteration and the polymer concentration at which double helices were formed. When double helices were formed at high enough polymer concentration, transitions in $H_2O$ appeared sharper than those in $D_2O$ (Fig.3).As an example, when polymer concentration of solutions mixed to form double helices was $2.5 \cdot 10^{-3}$M, the width of transition (4) $\Delta T_4$ (measured between 75% and 25%) was $\Delta T_4 \sim 3.7$°C in $D_2O$ and $\Delta T_4 \sim 3.2$°C in $H_2O$,whilst,when polymer concentration was $1.0 \cdot 10^{-4}$M, the width of transition (4) was $\Delta T_4 \sim 3.2$°C both in $H_2O$ and $D_2O$. In other words, the presence of deuterium affects $\Delta T_4$ only if the

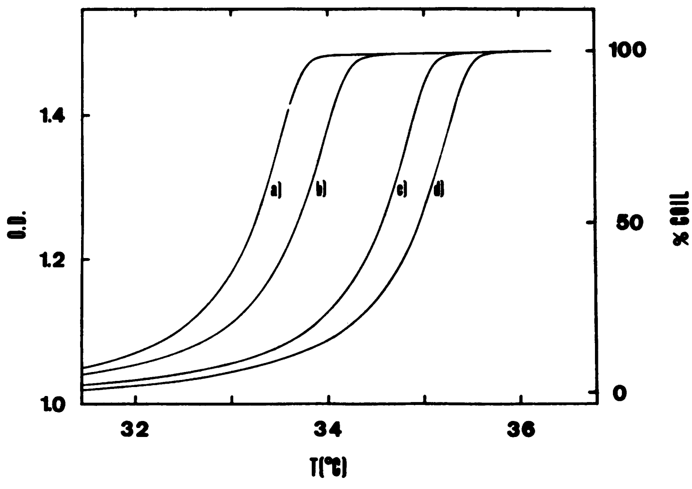

Figure 2: Melting curves (reaction (2) in the text) of $\sim 1.5 \cdot 10^{-4}$M (in base pairs) poly(A) • poly(U) in $5 \cdot 10^{-3}$M phosphate buffer, pH = 7.0+$10^{-4}$M EDTA, potassium salt. a) $H_2O$; b) 25% $D_2O$ in $H_2O$; c) 75% $D_2O$ in $H_2O$; d) 100% $D_2O$. Curves have identical shapes, being only shifted on the temperature scale. The same results were obtained at various polymer concentrations, and also in the case of reaction (1). In the case of reaction (3) melting profiles as well as melting temperatures, were unaffected by partial or total deuteration. For reaction (4) see text.

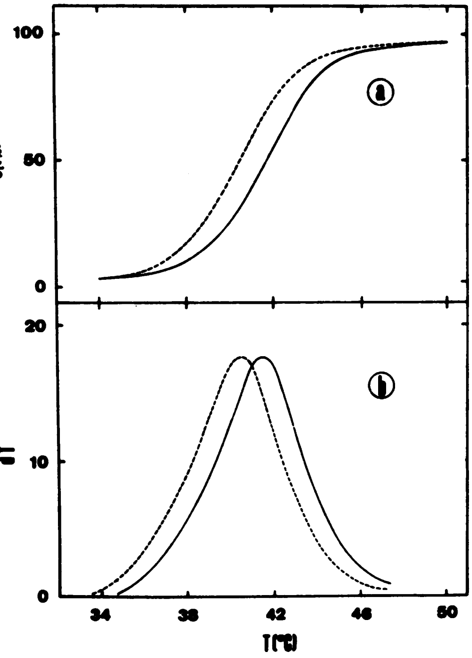

Figure 3: a) interconversion transition profiles (reaction (4) in the text). Dashed line: sample formed and diluted in $H_2O$; solid line: sample formed and diluted in $D_2O$. Polymer concentration: $10^{-4}$M (in base pairs) $[K^+] = 8.4 \cdot 10^{-1}$M. b) First derivatives of curves shown in a).

concentration at which helices are formed is sufficiently high. Although rather small, this effect was systematically and reproducibly found within ±0.1°C. Its independence upon temperature scanning rate ruled out the possibility of artifacts due to a difference in transition kinetics between the $H_2O$ and $D_2O$ cases. The effect was also independent upon final polymer concentration (Table IV) and incubation time, up to 100 hrs. The search for a possible dependence upon pH-pD differences[27,28] was also extended to this specific feature. In the pH range 6.5 to 7.5, $\Delta T_4$ values were strictly constant. No similar dependence was observed for either $T_{m4}$ or its isotope shift $\delta T_{m4}$ nor for $T_m$ and $\delta T_m$ of transitions (1),(2) and (3). To answer the question whether the isotope effect on $\Delta T_4$ depended solely on the final H/D atomic proportion or also on the conditions in which double helices had been allowed to form, sample were studied in which double stranded helices had been formed in different conditions of solvent isotopic composition, at $2.5 \cdot 10^{-3}$M polymer concentration, as mentioned above.

TABLE IV. Dependence of interconversion transition width $\Delta T_4$, at neutral pH, upon polymer concentration, when double helices, both in $H_2O$ and $D_2O$, are formed at $2.5 \cdot 10^{-3}$M (in base pairs) polymer concentration and then diluted to the concentrations listed in the Table. $[K^+] = 8.4 \cdot 10^{-1}$M.

| Polymer concentration (in base pairs) | $\Delta T(H_2O)$ | $\Delta T(D_2O)$ |
|---|---|---|
| $5.0 \cdot 10^{-5}$M | 3.3 ±.1 | 3.8 ±.1 |
| $1.0 \cdot 10^{-4}$M | 3.2 ±.1 | 3.7 ±.1 |
| $2.5 \cdot 10^{-4}$M | 3.3 ±.1 | 3.7 ±.1 |
| $6.2 \cdot 10^{-4}$M | 3.2 ±.1 | 3.8 ±.1 |
| $2.5 \cdot 10^{-3}$M | 3.2 ±.1 | 3.7 ±.1 |

A set of experiments was performed using double stranded helices formed in $H_2O$ and then diluted in $D_2O$ (to a final 96% $D_2O$ concentration) and viceversa. Results in Table VI and Fig.4 show that: i) the effect on $T_{m4}$ is the same in the two cases and ii) the effect on $\Delta T_4$ vanishes in both cases. In other words, at variance with the isotopic shift of $T_{m4}$ which depends only upon

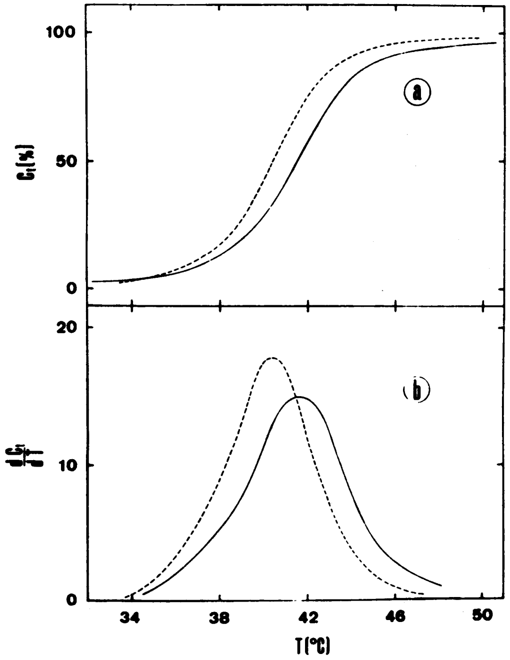

Figure 4: a) interconversion transition profiles (reaction (4) in the text). Dashed line: sample formed and diluted in $H_2O$. Solid line: sample formed in $H_2O$ and diluted in $D_2O$. Other conditions as in Fig.3. b) First derivatives of curves shown in a). At variance with the case of Fig.3, there is no observable difference between the $H_2O$ and the $D_2O$ cases.

TABLE V. Dependence of critical temperature $T_{m4}$ and of transition width $\Delta T_4$ upon sequence of treatments. Final polymer concentration $10^{-1}$M (in base pairs); $|K^+| = 8.4 \cdot 10^{-}$ M.

| SAMPLE | | | |
|---|---|---|---|
| Formed in | Diluted in | $T_{m4}$ °C | $\Delta T_4$ °C |
| $H_2O$ | $H_2O$ | 40.2±.2 | 3.2±.1 |
| $D_2O$ | $H_2O$ | 40.2±.2 | 3.2±.1 |
| $H_2O$ | $D_2O$ | 41.2±.2 | 3.3±.1 |
| $D_2O$ | $D_2O$ | 41.3±.2 | 3.7±.1 |

the H/D atomic proportion and not upon the previous history of the sample, the width of the transition profile is affected by deuteration, only if double helices are formed and kept or further diluted in $D_2O$. If, instead, they are formed in $H_2O$ and transferred (by dilution) in $D_2O$, or viceversa, the effect is no longer observed. It was not possible to measure the time of disappearance of the effect upon dilution with $H_2O$, in view of the long temperature scanning times required in this experiment. It can only be stated that this time is shorter than that (about 2 hrs) needed for a complete temperature scan.

In partially deuterated samples obtained upon dilution with $D_2O$ of samples originally formed and kept in $H_2O$, $T_{m4}$ is progressively shifted upwards by progressive deuteration, while $\Delta T_4$ and the transition profile as a whole are not affected in this case. If partially deuterated samples were obtained by diluting with normal water samples originally formed and kept in $D_2O$, the effect on $T_{m4}$ in this and in the previous case were found to be identical. Nevertheless, in agreement with the data in Fig.3 and Tab.V, and at variance with the case of Fig.5, $\Delta T_4$ was now found to be progressively affected.

## DISCUSSION

### I) Isotopic shifts of transition temperatures

As seen from Tab.III, dependence upon polymer concentration is, if any, negligible. We thus disregard interactions such as

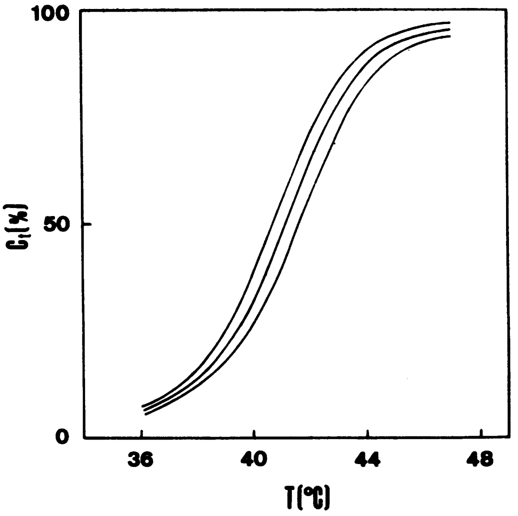

Figure 5: profiles of interconversion transition (reaction (4) in the text) From left to right: 100% $H_2O$; 50% $D_2O$ in $H_2O$; 96% $D_2O$ in $H_2O$. All samples were formed in $H_2O$ and then diluted to the desired H/D proportion. Other conditions as in Fig.4. Note the identical shapes of the curve.

those among helices. We write:

$$T_{mi} = \Delta H_i / \Delta S_i \quad \text{and} \quad \Delta G_i = \Delta H_i - T\Delta S_i \qquad (i = 1,2,3,4)$$

for the critical temperatures of our transitions and for the Gibbs free energy change in the i-th passage from one configuration to another. Since the bulk isotope effect on the thermodynamic parameters is expected to be small, we introduce the much more significant difference effects. Analogous to

$$\delta T_{mi} = T^d_{mi} - T^h_{mi} = \frac{\Delta H^d_i}{\Delta S^d_i} - \frac{\Delta H^h_i}{\Delta S^h_i} \qquad (5)$$

we have

$$\delta \Delta H_i = \Delta H^d_i - \Delta H^h_i \quad \text{and} \quad \delta \Delta S = \Delta S^d_i - \Delta S^h_i \qquad (6)$$

If we substitute (6) into (5) we obtain

$$\delta T_m = \frac{\Delta H^d \Delta S^h - \Delta H^h \Delta S^d}{\Delta S^d \cdot \Delta S^h} = \frac{\Delta H^d \Delta S^h - \Delta H^h\ (\delta\Delta S + \Delta S^h)}{\Delta S^h\ (\delta\Delta S + \Delta S^h)}$$

Since $(\Delta S^h)^2 >> \Delta S^h \cdot \delta\Delta S$ we find

$$\delta T_m \simeq \frac{\delta \Delta H}{\Delta S^h} - T_m \frac{\delta \Delta S}{\Delta S^h} \qquad (7)$$

which specifies how the observed isotope effect may be due to an altered ΔH or ΔS value or to both. The possibility of partly compensating ΔH and ΔS values should not be disregarded: in this con nection we recall that at the melting of the classic hydrogen-bond crystal, ice I, the deuterium isotope effect is a 4.5% increase in $\Delta H$ (65 cal/mole) and a 3% increase in $\Delta S$ (0.16 e.u.), originating a partial entropy-enthalpy compensation and a net 1.4% increase of melting temperature[29].

According to equation (7) the relative role of $\delta\Delta H$ and $\delta\Delta S$ contribution to $\delta T_m$ does not result explicitely from our experimental data. To the purpose of sorting them out, we have followed Massoulié's approach[15]. Accordingly, we write e.g., for transition (2) (and similarly for the remaining ones): $\Delta G_2 = 0$ that is:

$$\Delta H_2 - T_{m2}\Delta S'_2 + RT_{m2}(C_{12} - C_{22}\mathrm{Log}[K^+]) - RT_{m2}\log(1+\exp(\Delta G_A/RT_{m2})) = 0 \qquad (8)$$

where $\Delta S'_2$ is the salt-independent (and also, expectedly, isotope-independent) part of the polymer configurational entropy, and

$$\Delta S''_2 = R(C_{12} - C_{22}\mathrm{Log}[K^+]) \qquad (9)$$

accounts for the part of entropy which depends upon electrostatic and salt-dependent interactions (and, in our case, also upon H-D isotopic substitution). It follows:

$$\Delta S_i^x = \Delta S_i'^x + \Delta S_i''^x \qquad (x = h,d) \qquad (10)$$

Further, $\Delta G_A = \Delta H_A - T\Delta S_A$ is the standard Gibbs free energy (per base) for the passage of one poly(A) strand from the unstacked to the single strand stacked configuration[15,16]. Parameters in equation (8) and similar ones obey to the following relationships[15,16,30]:

$$C_{1i} = \frac{1}{2} C_{2i} \qquad (i = 1,2,3,4)$$

$$C_{21} = C_{23} - C_{22}; \qquad \Delta H_1 = \Delta H_3 - \Delta H_2; \qquad \Delta S'_1 = \Delta S'_3 - \Delta S'_2$$

$$C_{24} = 2C_{22} - C_{23}; \qquad \Delta H_4 = 2\Delta H_2 - \Delta H_3; \qquad \Delta S'_4 = 2\Delta S'_2 - \Delta S'_3$$

so that there are left six independent parameters only, that is:

$$\Delta H_2,\ \Delta H_3,\ \Delta S'_2,\ \Delta S'_3\ ,\ C_{22},\ C_{23}$$

The method consists in the computerized optimization of these independent thermodynamic parameters, providing the best fit of the entire set of experimental data[15,16]. The present case, however, differs from Massoulié's since the information seeked does not concern the bulk values of thermodynamics parameters but, rather, the much smaller values of their alterations caused by H-D substitution. The suitability of the method to this new task had to be established.We attempted four different fittings,as specified below(in all cases $\Delta H_A$=7,900 cal/mol and $\Delta S_A$=25 e.u.)[15,31]:

a) all six independent parameters, that is $\Delta H_2$, $\Delta H_3$, $\Delta S'_2$, $\Delta S'_3$, $C_{22}$ and $C_{23}$ were adjustable in the course of optimization

b) conditions $\Delta S'^d - \Delta S'^h = \delta\Delta S' = 0$ and $C_2^d - C_2^h = \delta C_2 = 0$ were imposed.Enthalpy parameters only($\Delta H_2$, $\Delta H_3$)remained adjustable.

c): condition $\Delta H^d - \Delta H^h = \delta\Delta H = 0$ was imposed. Entropy parameters only (that is, $\Delta S'_2$, $\Delta S'_3$, $C_{22}$ and $C_{23}$) remained adjustable.

d): conditions $\Delta H^d - \Delta H^h = \delta\Delta H = 0$ and $\Delta S'^d - \Delta S'^h = \delta\Delta S' = 0$ were imposed. Parameters $C_{22}^d$ and $C_{23}^h$ only remained adjustable.

Results of these fittings are shown pictorially, together with experimental points, in Fig.1 a), b), c) and d) and quantitatively in Tab.VI a, b, c, and d. Fittings where obtained by the refinement procedure discussed in the Materials and Methods section.

We now discuss results obtained with the four different types of fittings listed above. Case a) will be more conveniently considered at the end of this discussion, while we discard case b) on the basis of the quality of the fitting in Fig.1,b). This fitting predicts on branch No.3 a sizeable isotope effect, and on branch No.1 a much smaller isotope effect than on branch No.2. This is grossly contradicted by our experimental findings and our discarding of case b) will be further substantiated in the discussion which follows.

In case c),our optimization procedure ended up to $\frac{\delta\Delta S'}{\Delta S'}=4\cdot10^{-4}$ i.e. $\delta\Delta S'\simeq0$. We checked the unicity of this minimum by leaving $\delta\Delta H$ free to vary (together with $\delta C_2$) and by imposing instead the $\delta\Delta S'=4\cdot10^{-4}$ condition. Very satisfactorily, the reciprocal condition $\frac{\delta\Delta H}{\Delta H}<10^{-5}\sim0$ resulted from this new optimization. In short, whichever of the two conditions $\delta\Delta S'\sim0$ or $\delta\Delta H\sim0$ was imposed, the other was obtained by the optimization procedure.

This led us to case d),where the two conditions $\delta\Delta H=0$ and $\delta\Delta S'=0$ were simultaneously imposed. (Fig.1,d) and Tab.VI,d). The quality of fitting of branches 1, 2 and 3 is again excellent,while that of branch No.4 is still good,although of a somewhat lower quality. (On this last reaction nevertheless we expect other effects due to high salt concentration). This, together with results relative to case c) above,already focuses our attention on the $C_2$ parameters, that is on $\delta\Delta S$".

The quality of fitting in case c),the smaller number of parameters involved in case c) and particularly in case d) and the fact that in case d) the fitting of at least branches 1, 2 and 3 is excellent, make us favour case d) among the four, either in itself or as derived from case c). This choice is further supported by the following remarks:

i) parameters $C_2$ in expression (9) accounts by definition for the salt dependent part of entropy. Thus it must also depend at least upon dielectric constant. Since dielectric constants are different for $D_2O$ and $H_2O$[29], condition $\delta C_2 = 0$ appears unphysical in terms of our definitions (case b).

ii) If we now consider case a) (see Tab.VI,a), $\delta\Delta H$ values of the order of 1 kcal/mole,are far too large for being acceptable,since they amount to some 25÷50% of hydrogen bond energies[29,32]. Isotope effects on $\Delta H$ concern the breaking of intrahelical hydrogen bonds and the making of an equal number of similar bonds with solvent water. $\delta\Delta H$ values are therefore expected not to exceed a fraction of the entire isotope effect on the hydrogen bond strength, which is itself expected to be of the order of only a few percent of the total bond energy. On these grounds, we can discard case a).

iii) A vanishingly small $\delta\Delta H$ term is also fully consistent with the invariance of transition profiles in Fig.2, taking into account the fact that hydrogen exchange is not rapid[21]. A sizeable $\delta\Delta H$ term should, in fact, cause a broadening of these melting profiles in the mixed $H_2O/D_2O$ cases, as it is for co-polymers compared to homopolymers.

We see therefore that appropriate use of Massoulié's treatment allows an evaluation of the effects of deuteration on thermodynamic parameters governing reactions (1) to (4).Since results of this analysis concur in favouring case d), they suggest that our effects cannot merely be traced to the greater strength of deuterium (as compared to hydrogen) bond. The salt- and isotope dependent $C_2$ terms of polymer-solvent interaction entropy seem to have a prevailing role in these isotope effects.

A negative $\delta C_2$ term may be due either to the coil configura-

TABLE VI. Values of thermodynamic parameters for reactions (1) to (4) in the text, obtained following Massoulié's method. Cases a), b) and c) are described in the text. $\Delta_1, \Delta_2, \Delta_3$ and $\Delta_4$ are the r.m.s. differences between experimental and calculated $T_m$ values for reactions 1,2,3 and 4 respectively. $\bar{\Delta}$ is the r.m.s. difference between experimental and calculated $T_m$ values for the entire set of experimental points (39).

Case a)

| Reaction | $H_2O$ |  |  | $D_2O$ |  |  | Isotopic effect |  |  |
|---|---|---|---|---|---|---|---|---|---|
|  | ΔH kcal/mole | ΔS' e.u. | $C_2$ | ΔH kcal/mole | ΔS' e.u. | $C_2$ | δΔH kcal/mole | δΔS' e.u. | $\delta C_2$ |
| 1 | 3.17 | 9.19 | .533 | 3.74 | 10.85 | .590 | + .57 | +1.66 | + .057 |
| 2 | 8.32 | 24.01 | .442 | 8.92 | 25.78 | .486 | + .60 | +1.77 | + .044 |
| 3 | 11.49 | 33.20 | .975 | 12.66 | 36.63 | 1.075 | +1.17 | +3.43 | + .100 |
| 4 | 5.15 | 14.82 | -.091 | 5.18 | 14.93 | - .103 | + .03 | + .11 | -0.012 |
|  | $\Delta_1 = .3$ $\Delta_3 = .3$ |  |  | $\Delta_1 = .5$ $\Delta_3 = .2$ |  |  |  |  |  |
|  | $\Delta_2 = .6$ $\Delta_4 = .7$ |  |  | $\Delta_2 = .5$ $\Delta_4 = .7$ |  |  |  |  |  |
|  | $\bar{\Delta} = .5$ |  |  | $\bar{\Delta} = .5$ |  |  |  |  |  |

Case b)

| Reaction | $H_2O$ |  |  | $D_2O$ |  |  | Isotopic effect |  |  |
|---|---|---|---|---|---|---|---|---|---|
|  | ΔH kcal/mole | ΔS' e.u. | $C_2$ | ΔH kcal/mole | ΔS' e.u. | $C_2$ | δΔH kcal/mole | δΔS' e.u. | $\delta C_2$ |
| 1 | 3.73 | 10.85 | .590 | 3.74 | 10.85 | .590 | + .01 | .00 | .000 |
| 2 | 8.90 | 25.78 | .486 | 8.92 | 25.78 | .486 | + .02 | .00 | .000 |
| 3 | 12.63 | 36.63 | 1.075 | 12.66 | 36.63 | 1.075 | + .03 | .00 | .000 |
| 4 | 5.17 | 14.93 | - .103 | 5.18 | 14.93 | - .103 | + .01 | .00 | .000 |
|  | $\Delta_1 = 1.1$ $\Delta_3 = .7$ |  |  | $\Delta_1 = .5$ $\Delta_3 = .2$ |  |  |  |  |  |
|  | $\Delta_2 = .6$ $\Delta_4 = 1.0$ |  |  | $\Delta_2 = .5$ $\Delta_4 = .7$ |  |  |  |  |  |
|  | $\bar{\Delta} = .9$ |  |  | $\Delta = .5$ |  |  |  |  |  |

(continued)

Case c)

| Reaction | $H_2O$ |  |  | $D_2O$ |  |  | Isotopic effect |  |  |
|---|---|---|---|---|---|---|---|---|---|
|  | $\Delta H$ kcal/mole | $\Delta S'$ e.u. | $C_2$ | $\Delta H$ kcal/mole | $\Delta S'$ e.u. | $C_2$ | $\delta\Delta H$ kcal/mole | $\delta\Delta S'$ e.u. | $\delta C_2$ |
| 1 | 3.74 | 10.85 | .611 | 3.74 | 10.85 | .590 | .00 | .00 | - .021 |
| 2 | 8.92 | 25.79 | .507 | 8.92 | 25.78 | .486 | .00 | - .01 | - .021 |
| 3 | 12.66 | 36.64 | 1.118 | 12.66 | 36.63 | 1.075 | .00 | - .01 | - .043 |
| 4 | 5.18 | 14.94 | - .104 | 5.18 | 14.93 | - .103 | .00 | - .01 | + .001 |
|  | $\Delta_1 = .3$ $\Delta_3 = .4$<br>$\Delta_2 = .5$ $\Delta_4 = .8$<br>$\bar{\Delta} = .5$ |  |  | $\Delta_1 = .5$ $\Delta_3 = .2$<br>$\Delta_2 = .5$ $\Delta_4 = .7$<br>$\bar{\Delta} = .5$ |  |  |  |  |  |

Case d)

| Reaction | $H_2O$ |  |  | $D_2O$ |  |  | Isotopic effect |  |  |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3.74 | 10.85 | .612 | 3.74 | 10.85 | .590 | .00 | .00 | - .022 |
| 2 | 8.92 | 25.78 | .511 | 8.92 | 25.78 | .486 | .00 | .00 | -0.025 |
| 3 | 12.66 | 36.63 | 1.123 | 12.66 | 36.63 | 1.075 | .00 | .00 | -0.048 |
| 4 | 5.18 | 14.93 | - .101 | 5.18 | 14.93 | - .103 | .00 | .00 | +0.002 |
|  | $\Delta_1 = .3$ $\Delta_3 = .3$<br>$\Delta_2 = .5$ $\Delta_4 = 1.0$<br>$\bar{\Delta} = .5$ |  |  | $\Delta_1 = .5$ $\Delta_3 = .2$<br>$\Delta_2 = .5$ $\Delta_4 = .7$<br>$\bar{\Delta} = .5$ |  |  |  |  |  |

tion entropy, as lowered by the smaller dielectric constant of $D_2O$[29], ot to the stronger hydrophobic interactions in $D_2O$[33,38]. It is impossible at the present stage to make definite statements on the relative weight of these two contributions. It is of interest, however, to compare the present case with that of a pure polysaccharide (Agarose). This has no charged groups, it forms ordered double helical structures and undergoes sharp helix-coil transitions which are shifted by $D_2O$-$H_2O$ substitution much in the same way as in the present case[39-42]. In this case, the coil configurational entropy will not be affected by alterations of the solvent dielectric constant. This notwithstanding,thermodynamic analysis of experimental data supports also in that case a prevailing rolo of a negative entropic term[42].

An additional entropic contribution might be due to the freeing, at the helix-to-coil transition, of solvent water molecules immobilized along with small ions in the helical structure[43-45]. Comparison of ice I fusion entropies[29] of pure $H_2O$ and $D_2O$ shows nevertheless that the sign of $\delta\Delta S$ term due to this type of effect in opposite to that found here, which corresponds with the onset rather than with a release of constraints of some water molecules at the helix-to-coil transition.

### II) Isotopic effects on width of transition profiles

Transition (4) only is affected in its profile by deuteration.The larger width observed in the $D_2O$ case could be attributable to a less marked cooperative character of the transition itself or alternatively,to a wider distribution of situations (such as branched structures)of double helices taking part in the reaction.The first possibility appears unlikely in view of data in Figs.4 and 5 and Tab.V.Branched helical structures on the other hand are expected,owing to the homopolymer character of our samples and to the dispersity of chain lenghts[14,46,48]. Alterations in their distribution could affect the width of the interconversion reaction (4)more dramatically than that of"melting"reactions. This possibility is also favoured by the observed dependence of $\Delta T_4$ upon conditions in which double helices are formed and kept. If differences in branched helix structures obtained when mixing sufficiently high concentrations of poly(A)and poly(U)chains in $D_2O$ are responsible for the observed effects,our results suggest that:i)polymer-solvent-polymer interaction is involved in the formation and not only in the stability of ordered structures; ii) this interaction is effective for polymer concentrations larger than $10^{-4}$M (per base pairs), that is when no much more than $10^4$ water molecules per base are present,and iii)breathing and re-adjustment kinetics of helical structures are considerably slower in $D_2O$ than in $H_2O$(samples formed and diluted in $D_2O$ still show, after more than 100 hrs of incubation, the broader transition,while samples formed in $D_2O$ and diluted in $H_2O$ go immediately back to the narrower transition).

Since isotope effects depend on hydrogen mass,and concern a (critical)temperature,their study provides,despite their small size,an approach for bringing the role of the dynamics of hydrogen ions into the picture of the thermodynamic stability and of formation of functional structures of nucleic acids.

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