Comparative Studies on Homopolymers of Adenylic Acid Possessing Different C-2′ Substituents of the Furanose. Poly(deoxyriboadenylic acid), Poly(riboadenylic acid), Poly(2′-*O*-methyladenylic acid), and Poly(2′-*O*-ethyladenylic acid)†

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ABSTRACT: A comparative study of the single-stranded $d(A)_n$, $r(A)_n$, $r(2'\text{-MeA})_n$, and $r(2'\text{-EtA})_n$ by ultraviolet absorbance, circular dichroism, and proton magnetic resonance reveals the following order of decreasing base stacking: $d(A)_n > r(A)_n > r(2'\text{-MeA})_n > r(2'\text{-EtA})_n$. This order is related to the size of the C-2′ substituents which, in turn, is related to their steric hindrance to the base–base overlap and their perturbation of the furanose conformation. The thermal stabilities of the double-stranded homocomplexes ($A^+ \cdot A^+$) and heterocomplexes ($A \cdot U$) of this series of adenine polynucleotides were also compared. There is a noticeable stabilization of the homoduplex in acidic solution with an increase in size of the C-2′substituents, resulting in the following order of homocomplex thermal stabilities (T_m): $[r(2'\text{-EtA})_n^+]_2 > [r(2'\text{-MeA})_n^+]_2 > [r(A)_n^+]_2 > [d(A)_n^+]_2$. The heterocomplexes give a more complicated series of thermal stabilities: $r(A)_n \cdot r(U)_n \geq r(2'\text{-MeA})_n \cdot r(U)_n > r(2'\text{-EtA})_n \cdot r(U)_n > d(A)_n \cdot r(U)_n$. An explanation of the order of thermal stabilities among these adenine homoduplexes can be made on the basis of the single-stranded polynucleotides. The difference between the homoduplexes and the heteroduplexes with respect to their orders of thermal stabilities is discussed with respect to the potential steric effect of the C-2′-alkyl substituent.

In continuing our effort (Ts'o *et al.*, 1966; Fang *et al.*, 1971; Kondo *et al.*, 1972) to understand the physicochemical differences between RNA and DNA, a comparative study was made of the physicochemical properties of a series of adenine homopolynucleotides possessing different substituents at the C-2′ position with either H, OH, OCH_3, or OCH_2CH_3. Some of these polymers have been the subject of research in many laboratories (Alderfer and Smith, 1971; Adler *et al.*, 1969; Bobst *et al.*, 1969a–c; Pilet *et al.*, 1973). In this paper, the ultraviolet absorbance, the circular dichroism, and the proton magnetic resonance of this series of adenine homopolynucleotides in the single-stranded state were compared. In addition, the properties of the double-stranded complexes of the 2′-*O*-ethyladenine polynucleotides, *i.e.* $[r(2'\text{-EtA})_n^+]_2$ and $r(2'\text{-EtA})_n \cdot r(U)_n$,[1] measured in this investigation were compared with those of other adenine polynucleotide duplexes reported in the literature. Preliminary reports on these compounds have been made (Alderfer *et al.*, 1971, 1973; Tazawa *et al.*, 1972).

Experimental Section

Materials and Methods. The sources of the compounds used were: 5′-rAMP, Sigma Chemical Co., St. Louis, Mo.; $r(A)_n$ and $r(U)_n$, P-L Biochemicals, Inc., Milwaukee, Wis.; $d(A)_n$ was prepared according to published procedures of Bollum (1966); 2′-*O*-alkylated nucleotides and polynucleotides were prepared according to the published procedures of Rottman and Heinlein (1968) and Tazawa *et al.* (1972a); D_2O (99.8 and 100.0 mol %), Bio-Rad, Richmond, Calif. The phosphorus analysis procedure of Chen *et al.* (1956) was used for determination of the polymer extinction coefficients.

Instrumentation. The ultraviolet (uv) spectra were obtained on either a Cary 14 or 15 spectrophotometer. The temperature studies were accomplished with a Haake Brinkmann Model KT-62 constant-temperature circulator and were measured with a YSI Model 42SC Tele-Thermometer connected to a type 421 thermistor probe in contact with the cell. The circular dichroism (CD) spectra were obtained using a Cary 60 spectropolarimeter equipped with the 6001 CD unit. A water-jacketed fused quartz window cell of 1-cm path length (Optical Cell Co., Beltsville, Md.) was used with the constant-temperature circulator described above. The average temperature at the cell entrance and exit (1–2° difference) was measured. The proton magnetic resonance (pmr) spectra were obtained on either (or both) a Varian HR-220[2] with the 620i 8K data system in the Fourier transform mode yielding an inherent resolution of 0.5 Hz or a Varian HA-100. The experiments

† From the Division of Biophysics, Department of Biochemical and Biophysical Sciences, The Johns Hopkins University, Baltimore, Maryland 21205. *Received October 26, 1973.* This work was supported in part by a grant from the National Institutes of Health (GM-16066-05), a grant from the National Science Foundation (GB-30725X), and a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research (J. L. A., 1970–1972). This is paper No. 2 in a series entitled Studies on 2′-*O*-Alkyl Polynucleotides. This paper was presented in part at the 15th Annual Meeting of the Biophysical Society, New Orleans, La., 1971, and at the 17th Annual Meeting of the Biophysical Society, Columbus, Ohio, 1973.

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[1] Abbreviations used are: $d(A)_n$, poly(deoxyriboadenylic acid); $r(A)_n$, poly(riboadenylic acid); $r(2'\text{-MeA})_n$, poly(2′-*O*-methyladenylic acid); $r(2'\text{-EtA})_n$, poly(2′-*O*-ethyladenylic acid); $r(U)_n$, poly(uridylic acid).

[2] Experiments with the 220-MHz instrument were performed at the NMR Regional Facilities Center at the University of Pennsylvania established by National Institutes of Health Research Grant No. 1 P07 RR-00542-03 from the Division of Research Facilities and Resources.

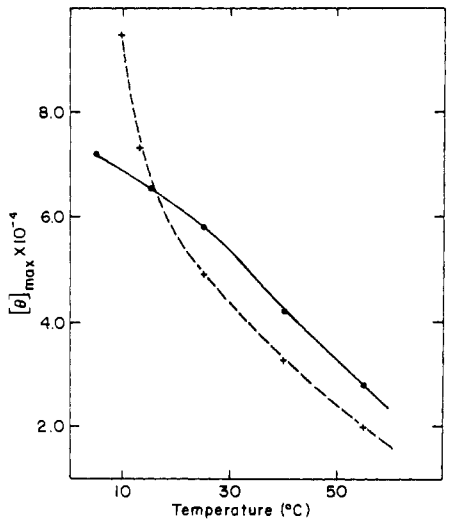

FIGURE 1: Circular dichroic $[\theta]_{max}$ dependence on temperature of (●) $r(A)_n$ and (+) $r(2'\text{-EtA})_n$ in 0.1 M Na^+ (pH 8.0).

performed at 100 MHz were obtained in two different ways. (1) Data reported with respect to an external tetramethylsilane capillary were obtained in the conventional frequency-sweep continuous wave mode, using the Varian C-1024 computer of average transients to increase the signal-to-noise ratio. (2) Data *expressed* with respect to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate were obtained in the Fourier transform mode using the Digilab (Cambridge, Mass.) FTS/NMR-3 data system containing 4K words of core and 128K words of disk. Spectral system parameters used in data acquisition were: a spectrum band width of 1000 Hz; an 8K or 16K transform size (with a pulse repetition rate of 4 or 8 sec, respectively) yielding an inherent resolution of 0.24 or 0.12 Hz, respectively; a pulse width of 37 μsec and 100–400 pulses. Sample tubes contained a 0.9 mm o.d. capillary filled with C_6F_6 which was used as the ^{19}F lock signal. In both the HR-220 and HA-100 spectrometer systems the temperature was regulated by a Varian V-6507 (or similar type model) variable-temperature accessory and measured by either methanol and ethylene glycol spectra or by using a Digetec Model 220C (United Systems, Inc., Dayton, Ohio) digital thermometer equipped with a YSI 731 thermistor probe which was inserted in the pmr sample probe.

Sample Preparation. Optical density (OD) studies were made at concentrations of 1–2 OD units at λ_{max} (~0.1 mM in base unit) in aqueous (H_2O) buffer–salt solutions. Buffer–salt solutions consisted of phosphate buffer (0.005 M) and sufficient NaCl to yield a total sodium ion concentration of 0.10 M. Samples for proton magnetic resonance (pmr) were 10–20 mM in base unit. These samples were repeatedly lyophilized and dissolved in D_2O to remove exchangeable protons; pD adjustments of the unbuffered samples were made with DCl or NaOD and were maintained at pD 7.5–8.5. Those samples run on the HR-220 or HA-100 FTS/NMR-3 spectrometers contained as an internal chemical-shift reference 0.5–1.5 mM *tert*-butyl alcohol or *p*-dioxane. In an independent experiment the chemical shifts of *tert*-butyl alcohol and *p*-dioxane were determined with respect to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate as a function of temperature. This permitted the chemical shifts of the samples run with *tert*-butyl alcohol or *p*-dioxane to be *expressed* with respect to the sulfonate. It should be noted that samples run in this way or using the external tetramethylsilane reference capillary described previously both yield the same values for the polymerization shifts since the corresponding nucleotide 5′-phosphate experiments were always performed under the same conditions as polymers.

TABLE I: Extinction Coefficient and Per Cent Hypochromicity of Adenylic Acid Homopolymers.

Polymer	$\epsilon_{max} \times 10^{-3}$	% h^a
$d(A)_n$	9.1 (9.45)[b]	41
$r(A)_n$	9.8 (10.1)[c]	37
$r(2'\text{-MeA})_n$	9.9	36
$r(2'\text{-EtA})_n$	10.1	34

[a] ϵ monomer was taken to be 15.4×10^3 for the adenylic acid monophosphates. [b] Cassani and Bollum (1969). [c] Holcomb and Tinoco (1965).

Results and Discussion

Single-Stranded Polynucleotides

Ultraviolet Absorbance Studies. The uv absorbance spectra of $r(A)_n$, $r(2'\text{-MeA})_n$, and $d(A)_n$ are similar in shape and in the spectral position of the maximum (Bobst *et al.*, 1969c; Bollum *et al.*, 1964). The uv characteristics of $r(2'\text{-EtA})_n$ are reported here and compared with $r(A)_n$ (in brackets): λ_{max} (pH 8.0) 255.7 [256.7]; λ_{max} (pH 5.5) 251.8 [252.1]; A_{max}/A_{min} = 3.8 [3.8]. The maximum molar extinction coefficients (ϵ) of these polynucleotides are listed in Table I together with those previously reported for $d(A)_n$ and $r(A)_n$ by others. It is clear that the ϵ of $d(A)_n$ is much lower than those of the $r(A)_n$, $r(2'\text{-MeA})_n$, and $r(2'\text{-EtA})_n$. The ϵ value of $r(2'\text{-EtA})_n$ is most likely to be higher than that of $r(A)_n$, since the error range of the determination is estimated to be about 1–2%. Thus, the ϵ values of these polynucleotides can be put in the following order: $d(A)_n < r(A)_n$, $r(2'\text{-MeA})_n < r(2'\text{-EtA})_n$. Since the ϵ values of the monomers of these polynucleotides are identical, the hypochromicities of these adenine polynucleotides in single-stranded conformation are ordered in a reversed manner (Table I). Therefore, the extent of the base–base interaction of these polymers as measured by hypochromicity appears to be reduced by the increasing size of the C-2′ substituents (H *vs.* OH *vs. O*-methyl *vs. O*-ethyl).

Circular Dichroism Studies. While CD data were used frequently as an indication of base–base interaction, it has been repeatedly noted that the CD spectra are sensitive to both the extent as well as the mode of base–base interaction (Bush and Tinoco, 1967; Johnson and Tinoco, 1969; Kondo *et al.*, 1970, 1972). This problem becomes immediately apparent when the ORD/CD spectra of $r(A)_n$ are compared with those from $d(A)_n$ (Ts'o *et al.*, 1966; Bush and Scheraga, 1969; Adler *et al.*, 1969). The vast dissimilarity in the CD spectra between these two adenine polynucleotides reveals that not only the extent of base overlap but also the mode of the base overlap are different between the ribosyl- and deoxyribosyladenine polynucleotides. For this reason, a direct comparison of $[\theta]_{max}$ as a measure of extent of base–base overlap is not meaningful. While the circular dichroism spectra of $r(A)_n$ and $d(A)_n$ cannot be compared in this respect, $r(A)_n$, $r(2'\text{-MeA})_n$, and $r(2'\text{-EtA})_n$ have similar CD spectra (Bobst *et al.*, 1969a, and Figures 1 and 2). A comparison of values of $[\theta]_{max}$ among these three polynucleotides is more meaningful. Therefore, Figure 1 shows a comparison of the tempera-

TABLE II: Polymerization Shifts ($\Delta\delta_p$)[a] and Chemical Shifts (δ)[b] as a Function of Temperature for Poly(adenylic acids) Possessing Different Furanoses.

Temp (°C)	Shift	r(A)$_n$			r(2′-MeA)$_n$				d(A)$_n$[c]		
		H-8	H-2	H-1′	H-8	H-2	H-1′	CH_3O	H-8	H-2	H-1′
30	$\Delta\delta_p$	0.67	0.55	0.61					0.55	0.89	1.00
35	$\Delta\delta_p$	0.59	0.54	0.59	0.65	0.54	0.47	−0.07	0.53	0.86	0.97
	δ	8.37	8.21	6.01	8.32	8.22	6.21	4.00			
40	$\Delta\delta_p$				0.53	0.48	0.44	*d*	0.53	0.83	0.92
45	$\Delta\delta_p$	0.53	0.47	0.47	0.47	0.44	0.42	−0.04	0.49	0.78	0.88
50	$\Delta\delta_p$	0.49	0.44	0.43					0.48	0.74	0.84
55	$\Delta\delta_p$	0.43	0.42	0.41	0.39	0.40	0.38	0.00	0.46	0.69	0.80
	δ	8.55	8.34	6.20	8.57	8.36	6.31	3.93			
60	$\Delta\delta_p$	0.38	0.39	0.38	0.34	0.37	0.34	0.03	*e*	0.64	0.76
65	$\Delta\delta_p$	0.34	0.36	0.36	0.30	0.35	0.31	*d*	*e*	0.61	0.72
70	$\Delta\delta_p$	0.32	0.34	0.33	0.30	0.33	0.30	0.05	*e*	0.56	0.66
	δ	8.61	8.41	6.27	8.64	8.43	6.37	3.87			
75	$\Delta\delta_p$	0.28	0.32	0.32	0.25	0.32	0.28	0.07			

[a] In parts per million; $\Delta\delta_p = \delta_{polymer} - \delta_{monomer}$. Chemical shifts downfield from tetramethylsilane are negative; therefore, a positive $\Delta\delta_p$ indicates the polymer is upfield relative to the monomer. [b] All values are negative in parts per million from a tetramethylsilane capillary. [c] From Alderfer and Smith (1971). [d] Value not recorded. [e] Not observed due to exchange with solvent deuterons.

ture dependence of r(A)$_n$ and r(2′-EtA)$_n$. At low temperatures the larger rotation of r(2′-EtA)$_n$ is the result of helix formation and will be discussed more fully in a later section. At 25° and above, both polymers show a similar temperature dependence with the CD at the long-wavelength maximum for r(A)$_n$ being consistently greater than that of r(2′-EtA)$_n$. The similarity in spectral shape of the two polymers at 25° is shown in Figure 2. The diminished ellipticity of r(2′-EtA)$_n$ suggests a reduction of base–base overlap relative to that of r(A)$_n$. A similar effect was reported by Bobst *et al.* (1969a) in noting that the circular dichroism for r(2′-MeA)$_n$ is slightly less than r(A)$_n$. This effect of 2′-*O*-methylation appears more pronounced at the dimer level. Bobst *et al.* (1969b) observed approximately a 50% reduction in the magnitude of the positive CD band of 2′-MeAp-2′-MeA relative to ApA. By optical rotatory dispersion (ORD) measurements, Singh and Hillier (1971) reported a 46% reduction in peak-to-trough amplitude of 2′-MeApA compared to ApA. Reduction in CD ellipicity of the homodimer and homopolymer can be caused by either a reduction of the extent of the base–base overlap or a change of the mode of the base–base overlap (see discussion in Kondo *et al.*, 1970, 1972). Leaving the discussion on the details of these changes to later sections, these data indicate that 2′-*O*-alkylation of an adenine dimer and polymer decreases the base–base interaction of the single-stranded form. Comparing the $[\theta]_{max}$ of this series of polynucleotides, one concludes the order of the base–base interactions to be r(A)$_n$ > r(2′-MeA)$_n$ > r(2′EtA)$_n$.

Proton Magnetic Resonance Studies. The pmr spectrum of a homopolynucleotide usually contains the same number of proton resonances as that of the corresponding monomer since all the nucleotidyl units in the polymer are essentially magnetically equivalent. Significant differences do exist between the two spectra, particularly the exact chemical shifts and coupling constant (J) values. These differences between monomer and polymer spectra originate from the intramolecular base–base interactions in the polymer and the conformational changes in the backbone as a result of these interactions. The polymerization shift, $\Delta\delta_p$ ($\Delta\delta_p = \delta_{polymer} - \delta_{monomer}$), reflects the degree of base–base interaction. The polymerization shifts and certain chemical shifts of these adenine polynucleotides are plotted in Figures 3a–d and are selectively shown in Tables II and III for a more quantitative comparison. The data for the base protons and H-1′ protons depicted in Figures 3a–c are separable into two groups: (1) r(A)$_n$, r(2′-MeA)$_n$, and r(2′-EtA)$_n$; and (2) d(A)$_n$. The magnitude and the temperature dependence of the $\Delta\delta_n$ values of the r(A)$_n$, r(2′-MeA)$_n$, and r(2′-EtA)$_n$ are quite similar, both smaller than those of the d(A)$_n$ except for H-8. As reported by Alderfer and Smith (1971), d(A)$_n$ is more extensively stacked than r(A)$_n$ and probably with a different mode. At the dimer level, our laboratory (Kondo *et al.*, 1972) reported schematic representations which indicated that the vertically stacked dApdA has a nearly parallel base–base overlap, while the overlap in rAprA has a more oblique arrangement. These

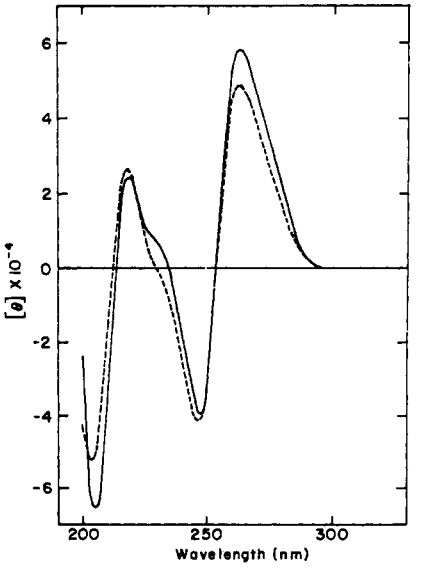

FIGURE 2: Circular dichroism spectra of (–) r(A)$_n$ and (- - -) r(2′-EtA)$_n$ at 25°, 0.1 M Na^+ (pH 8.0).

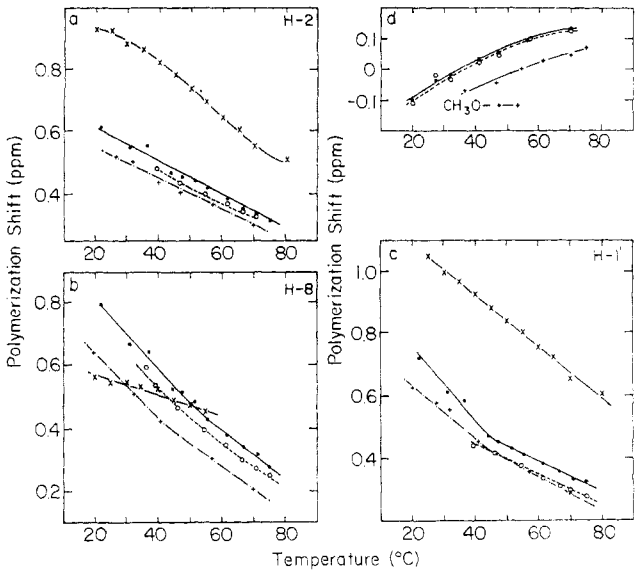

FIGURE 3: The temperature dependence of polymerization shift ($\Delta\delta_p$) of various protons in the adenine polynucleotides [(X) dA_n; (●) $r(A)_n$; (○) $r(2'\text{-MeA})_n$; (+) $r(2'\text{-EtA})_n$]: (a) H-2; (b) H-8; (c) H-1'; (d) C-2' O-alkyl [(●) CH_3 and (○) CH_2O of $r(2'\text{-EtA})_n$; (+) CH_3O of $r(2'\text{-MeA})_n$].

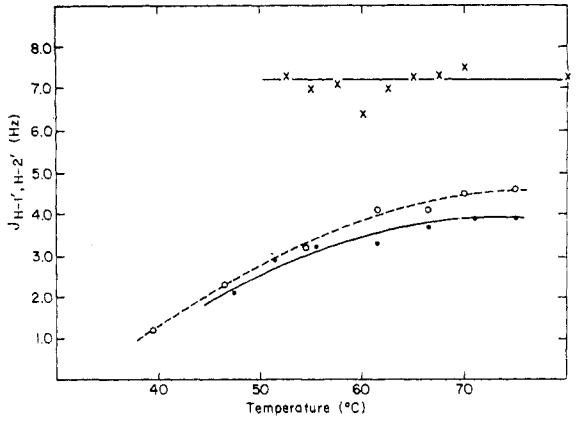

FIGURE 4: The temperature dependence of the coupling constants ($J_{H-1',H-2'(2'')}$) of adenine polynucleotides: (X) $d(A)_n$; (○) $r(2'\text{-MeA})_n$; (●)$r(A)_n$.

schemes were constructed from the dimerization shifts ($\Delta\delta_D$) of dApdA which are all larger than the corresponding $\Delta\delta_D$ of rAprA except for the H-8 of the 5' portion (pdA). The explanation for this variation comes from the reduction of ring current experienced by the H-8 (pdA) in the nearly parallel base–base overlap in dApdA as compared to the more oblique overlap in rAprA where that H-8 is more shielded. Similar types of base–base overlap are envisioned in the single-stranded polynucleotides; consequently one anticipates the $\Delta\delta_p$ of H-8 in $d(A)_n$ to be less than the $\Delta\delta_p$ of H-8 of $r(A)_n$ as observed (Figure 3b and Alderfer and Smith, 1971).

The furanose conformation as related to the stacking of these two groups of adenine polynucleotides can be compared by their H-1' coupling constants, $J_{H-1',H-2'(H-2'')}$, at various temperatures (Figure 4). The magnitude of $J_{H-1',H-2'}$ for both $r(A)_n$ and $r(2'\text{-MeA})_n$ is dependent on temperature. These coupling constants increase with elevation in temperature and approach their monomer values at high temperatures (the coupling constants of these to monomers are identical; Tazawa *et al.*, 1972). In contrast, the coupling constant of $d(A)_n$, $^1/_2|J_{H-1',H-2'} + J_{H-1',H-2''}|$, is not temperature dependent and consistently has a value nearly equal to 5'-dAMP (Alderfer and Smith, 1971). These results at the polymer level are supported by those analyzed in detail for dApdA and rAprA by our laboratory (Fang *et al.*, 1971; Kondo *et al.*, 1972). These results suggest that the C-2' OH and C-2' OCH_3 moieties provide steric interference to the base–base stacking in the dimer or polymer, a situation which is not present for dimer and polymer with C-2' H.

TABLE III: Chemical Shifts (δ)[a] and Polymerization Shifts ($\Delta\delta_p$)[b] as a Function of Temperature for Poly(2'-*O*-ethyladenylic acid).

Temp (°C)	Shift	H-8	H-2	H-1'	OCH_2	CH_3
20	δ	7.834	7.693	5.568	3.795	1.221
	$\Delta\delta_p$	0.65	0.55	0.625	−0.11	−0.10
32	δ	7.956	7.754	5.644	3.715	1.135
	$\Delta\delta_p$	0.53	0.50	0.55	−0.04	−0.02
41	δ	8.044	7.827	5.728	3.650	1.073
	$\Delta\delta_p$	0.425	0.44	0.46	0.02	0.04
57	δ	8.151	7.911	5.824	3.573	1.010
	$\Delta\delta_p$	0.30	0.36	0.35	0.095	0.095
70	δ	8.244	7.980	5.893	3.541	0.968
	$\Delta\delta_p$	0.20	0.30	0.28	0.12	0.13

[a] Chemical shifts are *expressed* in parts per million with respect to internal sodium 2,2-dimethyl-2-silapentane-5'-sulfonate (see Experimental Section for a detailed explanation). [b] Same as in Table II.

The pmr data on the ribosyladenine polynucleotides indicate that no dramatic conformational changes occur as a result of alkylation at the C-2' hydroxyl moiety. The changes which do occur are the result of polymer formation since Hruska *et al.* (1973) have shown that 2'-O-methylation has little or no effect on nucleoside conformation at the monomer level. The effect of the introduction of these two alkyl groups on the polymer conformation mimics the effect of thermal denaturation. For example, the $\Delta\delta_p$ values of $r(2'\text{-EtA})_n$ and $r(2'\text{-MeA})_n$ at a given temperature are comparable to that of $r(A)_n$ at a temperature 10–15° higher (Figure 3, Tables II and III). The same effect is seen in Figure 4 for the $J_{H-1',H-2'}$ of $r(2'\text{-MeA})_n$ and $r(A)_n$. Unfortunately a similar comparison of the $J_{H-1',H-2'}$ of $r(2'\text{-EtA})_n$ can not be made because of the peak broadening of H-1' of $r(2'\text{-EtA})_n$; the origin of this broadening effect is currently under investigation.

The order of the $\Delta\delta_p$ values of the four adenine polynucleotides is: $d(A)_n > r(A)_n > r(2'\text{-MeA})_n > r(2'\text{-EtA})_n$. This order reflects the comparative pmr measurements of the base–base interaction of this series of polynucleotides. This was found to be related to the conformation of the sugar and the C-2' substituents. The lack of variance of the coupling constant ($^1/_2|J_{H-1',H-2'} + J_{H-1',H-2''}| = 7.1 \pm 0.3$ Hz) of $d(A)_n$ with temperature strongly suggests a consistent conformation is maintained at that region of the sugar, which is essentially identical with the value of the monomer, 5'-dAMP ($J = 6.8$ Hz). This conformation is most likely C-2' endo (or C-2' endo–C-3' exo) since the averaged value (7.1 Hz) is essentially the same as the averaged value of the dAp portion of dApdA (7.0 Hz) reported by us earlier (Fang *et al.*, 1971). Inspection of Corey–Pauling–Koltun (CPK) molecular models indicates that base–base overlap in the dimer/polymer can easily take place with the furanose arranged in the C-2' endo conforma-

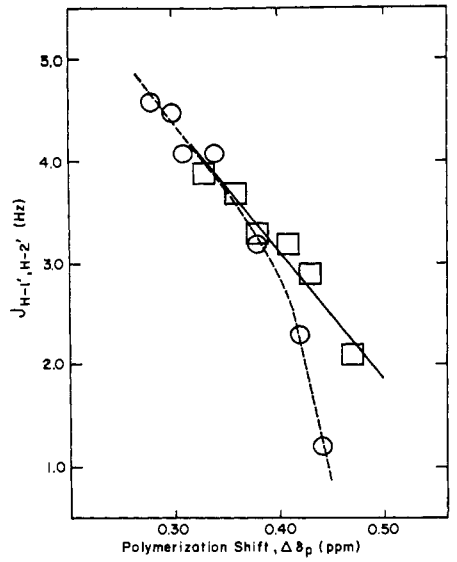

FIGURE 5: Dependence of $J_{H-1',-2'}$ on $\Delta\delta_p$ (H-1') in (□) r(A)$_n$ and (○) r(2'-MeA)$_n$.

tion; with this conformation the C-2' H provides virtually no steric interference to stacking.

The temperature dependence of $J_{H-1',H-2'}$ for r(A)$_n$ and r(2'-MeA)$_n$ is quite different from that for d(A)$_n$ (Figure 4). A similar, thermally induced change for $J_{H-1',H-2'}$ was observed in rAprA (Hruska and Danyluk, 1968; Kondo *et al.*, 1972). This thermal effect on the dimer (~ 5°) is displaced toward lower temperatures relative to the polymer (~ 50°), indicating a more stabilized conformation of the polymer relative to the dimer. Considerations of the coupling constant values and molecular models reveal that base–base stacking can more easily occur in the ribosyl and C-2' *O*-methylribosyl compounds with a furanose conformation of (1) C-3' endo, (2) C-2' exo, and in the extreme case (3) C-2' exo–C-3' endo. These conformations have a reduced dihedral angle ($\phi_{1'-2'}$) between H-1' and H-2', and as predicted by the Karplus equation (Karplus, 1959) the coupling constant, $J_{H-1',H-2'}$, of these conformers would be smaller. Hruska and Danyluk (1968) suggested that the averaged ribose conformation of rAprA changes from C-3' endo at low temperature to C-2' endo at high temperatures. The preferred ribose conformation of the monomeric adenylic acid is C-2' endo (or C-3' exo, or C-2' endo–C-3' exo), where $\phi_{1',2'}$ is large (140–160°) (Hruska and Danyluk, 1968). Thus, in order for a large extent of base–base overlap to occur in the dimer or polymer, the conformation of the ribose has to change from C-2' endo to C-3' endo. This change decreases the angle $\phi_{1'-2'}$ to about 100° and $J_{H-1',H-2'}$ to about 2 Hz.

The above conclusion on the steric hindrance of the C-2' OH group to base–base stacking receives support from the data in Figure 5 where the coupling constant ($J_{H-1',H-2'}$) is plotted against the H-1' polymerization shift ($\Delta\delta_p$) for both r(A)$_n$ and r(2'-MeA)$_n$. In general, the $\Delta\delta_p$ values are large and the $J_{H-1',H-2'}$ values are small at low temperatures where extensive base–base overlap occurs, and the $\Delta\delta_p$ values are small and the $J_{H-1',H-2'}$ values are large at high temperatures where the overlap is greatly decreased. This plot for the adenine polynucleotides is essentially in agreement with a similar plot for rAprA as reported by Hruska and Danyluk (1968). In addition, the slopes in Figure 5 for both r(A)$_n$ and r(2'-MeA)$_n$ are equivalent at high temperatures, but the slope for r(2'-MeA)$_n$ is larger than that for r(A)$_n$ at low temperatures where extensive base–base overlap occurs. Since a similar $\Delta\delta_p$ value reflects a similar extent of base–base overlap, the more rapid decrease of $J_{H-1',H-2'}$ (and, thus, $\phi_{1',2'}$) indicates a more drastic change of the furanose conformation is needed for the r(2'-MeA)$_n$ than for r(A)$_n$ to accommodate the same extent of base–base overlap. In other words, for a given extent of base–base overlap, the dihedral angle $\phi_{1',2'}$ of the furanose of r(2'-MeA)$_n$ is smaller than that of r(A)$_n$.

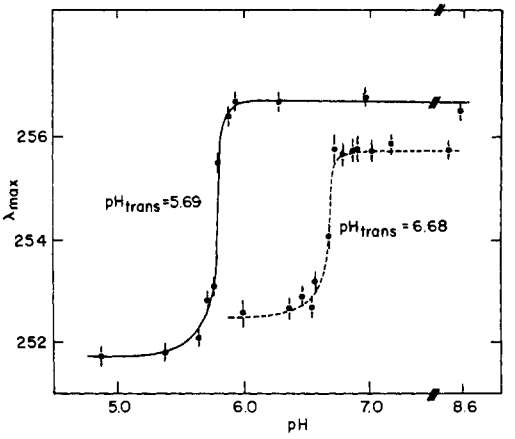

FIGURE 6: Ultraviolet λ_{max} dependence on pH of (–) r(A)$_n$ and (- - -) r(2'-EtA)$_n$ at 24°, 0.1 M Na^+.

Double-Stranded Complexes

Homocomplex. Extensive studies have been made on all of the adenine-containing helical complexes reported here (for a compilation see Janik, 1971) with the exception of r(2'-EtA)$_n$. Recently some preliminary data have been published by ourselves and others on the preparation and properties of r(2'-EtA)$_n$ (Khurshid *et al.*, 1972; Tazawa *et al.*, 1972a; Alderfer *et al.*, 1973).

As reported in the section on single-stranded complexes, the uv spectra of r(2'-EtA)$_n$ exhibits a blue shift at lower pH, similar to that observed for r(A)$_n$. This acid-induced transition of λ_{max} is shown in Figure 6. In this figure, the pH of this helix–coil transition for r(2'-EtA)$_n$ is 6.7 *vs.* 5.7 for r(A)$_n$.

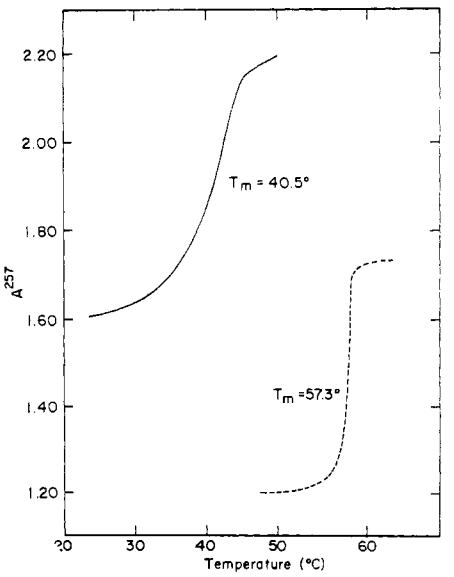

FIGURE 7: Helix–coil transition of (–) r(A)$_n$ and (- - -) r(2'-EtA)$_n$ in 0.1 M Na^+ (pH 5.5), measured by uv absorbance at 257 nm.

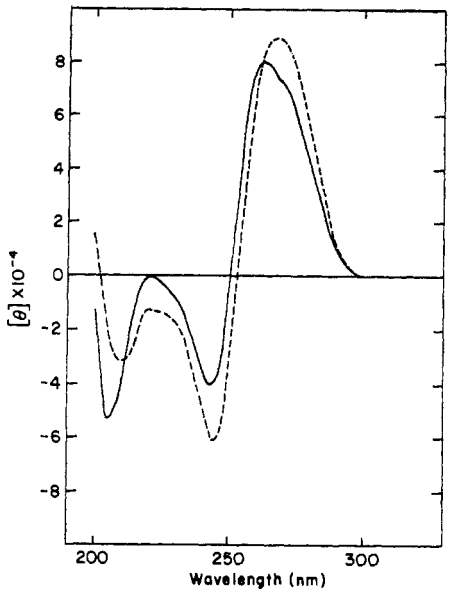

FIGURE 8: Circular dichroism spectra of (–) $r(A)_n$ and (- - -) $r(2'\text{-EtA})_n$ at 25°, 0.1 M Na^+ (pH 5.5).

This difference (~1.0 pH unit) in $pH_{transition}$ between $r(2'\text{-EtA})_n$ and $r(A)_n$ is 2.5-fold larger than the difference of 0.4 pH unit between $r(2'\text{-MeA})_n$ and $r(A)_n$ reported by Bobst *et al.* (1969a). A pH titration of adenosine 3′,5′-cyclic phosphate (p<A) and 2′-*O*-ethyladenosine 3′,5′-cyclic phosphate (p<2′-EtA) in D_2O by pmr technique yielded a pK_a of 3.7 for p<A and 3.8 for p<2′-EtA. Thus, clearly the difference in $pH_{transition}$ of ~1.0 pH unit between $r(A)_n$ and $r(2'\text{-EtA})_n$ is related to the properties of the polymers and is not due to the small differences in the pK_a between the monomeric residues.

The difference of 1 pH unit change in $pH_{transition}$ corresponds to a dramatic increase in thermal stability, when the meltings of $r(2'\text{-EtA})_n$ and $r(A)_n$ are compared at identical conditions. As shown in Figure 7, the helical polynucleotide with ethoxyl groups is 17° more stable than the helix with hydroxyl groups at the same pH. This strong tendency of $r(2'\text{-EtA})_n$ toward helix formation is also indicated in Figure 1 where a large increase in ellipticity occurs below 25° even

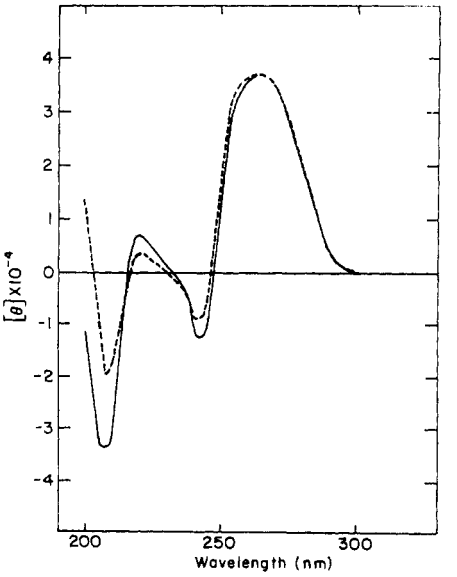

FIGURE 9: Circular dichroism spectra of (–) $r(A)_n \cdot r(U)_n$ and (- - -) $r(2'\text{-EtA})_n \cdot r(U)_n$ at 25°, 0.1 M Na^+ (pH 7.0).

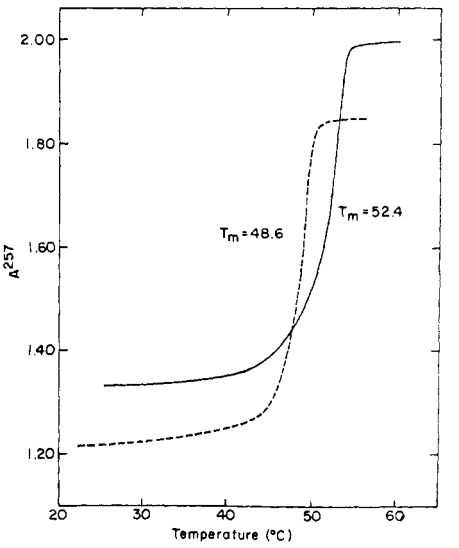

FIGURE 10: Helix–coil transition of (–) $r(A)_n \cdot r(U)_n$ and (- - -) $r(2'\text{-EtA})_n \cdot r(U)_n$ in 0.1 M Na^+ (pH 7.0), measured by uv absorbance at 257 nm.

at pH 8.0. A somewhat broadened cooperative transition was observed by uv absorption for $r(2'\text{-EtA})_n$ at 0.1 M Na^+ (pH 8.0) with a T_m of 11°.

From a comparison of the $pH_{transition}$ at a given temperature or the comparison of the T_m at a given pH (Barszcz and Shugar, 1968; Bobst *et al.*, 1969a), the stabilities of the acidic self-complexes of these adenine polynucleotides can be ordered as follows: $[r(2'\text{-EtA})_n^+]_2 > [r(2'\text{-MeA})_n^+]_2 > [r(A)_n^+]_2 > [d(A)_n^+]_2$. This order is exactly *opposite* to the order describing the magnitude of the base–base interaction of this series of polynucleotides in the single-stranded state.

The circular dichroism spectra of these self-complexes in acidic solution are shown in Figure 8. The relative differences between $[r(A)_n^+]_2$ and $[r(2'\text{-EtA})_n^+]_2$ are similar to those between $[r(A)_n^+]_2$ and $[r(2'\text{-MeA})_n^+]_2$, as reported by Bobst *et al.* (1969c). The spectral differences are: (1) $[r(A)_n^+]_2$ has a shoulder at 270 nm while $[r(2'\text{-EtA})_n^+]_2$ does not, (2) the crossover point or the $[r(2'\text{-EtA})_n^+]_2$ spectrum is shifted toward longer wavelength as compared to that of $[r(A)_n^+]_2$, and (3) the spectrum of $[r(2'\text{-EtA})_n^+]_2$ has a slightly larger ellipticity (particularly the negative band), resulting in a more conservative spectrum than $[r(A)_n^+]_2$.

Heterocomplex. The CD spectra of the double-stranded helix of $r(2'\text{-EtA})_n \cdot r(U)_n$ is essentially identical with that of $r(A)_n \cdot r(U)_n$ as shown in Figure 9. A similar result was obtained by Bobst *et al.* (1969c) in their comparison of the CD spectra of $r(2'\text{-MeA})_n \cdot r(U)_n$ and $r(A)_n \cdot r(U)_n$.

The influence of the C-2′ *O*-ethyl group on the stability of a double-stranded $r(2'\text{-EtA})_n \cdot r(U)_n$ helix is seen in Figure 10. The $r(2'\text{-EtA})_n \cdot r(U)_n$ complex is *destabilized* relative to the $r(A)_n \cdot r(U)_n$ complex by approximately 4°. This observation is in sharp contrast to the *stabilizing* effect of a C-2′ *O*-ethyl group in the $[r(2'\text{-EtA})_n^+]_2$ complex. A possible explanation of the destablization of the $r(2'\text{-EtA})_n \cdot r(U)_n$ complex relative to the $r(A)_n \cdot r(U)_n$ complex could come from the increased stability of $[r(2'\text{-EtA})_n^+]_2$ relative to $[r(A)_n^-]_2$, assuming under these experimental conditions some $[r(2'\text{-EtA})_n^+]_2$ is formed upon melting the heterocomplex. If this is the case then the T_m of the heterocomplex would be pH dependent, and increasing the pH (thus reducing the portion of $[r(2'\text{-EtA})_n^+]_2$) should increase the T_m of $r(2'\text{-EtA})_n \cdot r(U)_n$. However, the T_m values at pH 8 of both $r(2'\text{-EtA})_n \cdot r(U)_n$ (49.0°) and $r(A)_n \cdot r(U)_n$

(52.5°) are essentially identical with their value at pH 7 (Figure 10), indicating the formation of $[r(2'\text{-EtA})_n^+]_2$ is not responsible for the destabilization of $r(2'\text{-EtA})_n \cdot r(U)_n$. In comparison, $r(2'\text{-MeA})_n \cdot r(U)_n$ and $r(A)_n \cdot r(U)_n$ have identical T_m values (51°) in 0.04 M NaCl–0.01 M cacodylate (pH 7) (Bobst *et al.*, 1969c). At higher salt conditions (0.2 M) Bobst *et al.* (1969c) report a slight destabilization of $r(2'\text{-MeA})_n \cdot r(U)_n$ compared to $r(A)_n \cdot r(U)_n$ (65° *vs.* 64°, respectively). On the other hand, the T_m of $d(A)_n \cdot r(U)_n$ and the T_m of $r(A)_n \cdot r(U)_n$ were found to be 45 and 57°, respectively, under identical conditions (Riley *et al.*, 1966). Thus the stabilities of these A·U heterocomplexes can be ordered as follows: $(rA_n) \cdot r(U)_n \geq r(2'\text{-MeA})_n \cdot r(U)_n > r(2'\text{-EtA})_n \cdot r(U)_n > d(A)_n \cdot r(U)_n$.

Comparison of the Various Physicochemical Studies of the Adenine Polynucleotides with Different C-2′ Substituents

Single-Stranded Polynucleotides. The extent of the base–base interaction through vertical stacking in a single-stranded polynucleotide is measured by hypochromicity (per cent) in the uv absorbance study, by ellipticity (θ) in the CD study, and by polymerization shift ($\Delta\delta_p$) in the pmr study. The uv hypochromicity (Table I) gives the following order for the base–base interaction of this series of adenine polynucleotides: $d(A)_n > r(A)_n \geq r(2'\text{-MeA})_n > r(2'\text{-EtA})_n$. The CD spectral data indicate that the mode of base–base overlap in $d(A)_n$ is different from that in the ribosyl and 2′-*O*-alkyl polynucleotides; therefore, the CD data of these two groups cannot be compared directly. The comparison among the ellipticity data of $r(A)_n$, $r(2'\text{-MeA})_n$, and $r(2'\text{-EtA})_n$ can still be done meaningfully, since their spectral shapes are similar. The ellipticity data indicate the following order of base–base interaction: $r(A)_n > r(2'\text{-MeA})_n > r(2'\text{-EtA})_n$. The data on polymerization shifts from pmr studies clearly support the conclusions from the optical investigation: (1) the mode of base–base overlap in $d(A)_n$ (more parallel) is different from that of $r(A)_n$ (more oblique); (2) the order of the extent of the base–base overlap is $d(A)_n > r(A)_n > r(2'\text{-MeA})_n > r(2'\text{-EtA})_n$. It is noteworthy that this order of base–base stacking interaction is identical with the order determined from the dimers previously reported from our laboratories and others (Kondo *et al.*, 1972, and references therein).

The pmr study on both the $\Delta\delta_p$ values and $J_{H\text{-}1',H\text{-}2'}$ values reveals why such an order of base–base overlap is related to the size of the C-2′ substituents at the furanose ring. In $d(A)_n$ there is no significant conformational change of the furanose on stacking, whereas in $r(A)_n$ and especially $r(2'\text{-MeA})_n$, a pronounced alteration of furanose conformation from C-2′ endo to C-3′ endo occurs with increasing base–base stacking. This strongly implies that in order to facilitate base–base overlap the furanose conformation must be changed to accommodate a bulkier C-2′ substituent.

Double-Stranded Helices. Alkylation at C-2′ OH affects the thermal stability of a helical complex in a manner depending on the nature of the complex. A thermodynamic model has been constructed to assist our understanding of the above phenomena. In this model, the free energy (F_D) of the double-stranded duplex, the free energy of the base-stacked single strands (F_S), and the free energy of the *un*stacked single strands (F_U) are considered. It is further assumed that the free energy of the unstacked, single-stranded state (F_U) is virtually identical for all polynucleotides, independent of the C-2′ substituent. Thus, the free-energy difference ($F_S - F_U$) between the stacked, single-stranded state and the unstacked, single-stranded state varies among these polynucleotides, depending on the C-2′ substituents. The order of ($F_S - F_U$) among these adenine polynucleotides should be the same as the order of the extent of base stacking described in the preceding section, *i.e.*, $d(A)_n > r(A)_n > r(2'\text{-MeA})_n > r(2'\text{-EtA})_n$.

Consequently, the free-energy difference ($F_D - F_S$) between the helical duplex state and the stacked single-stranded state should also vary among those adenine polynucleotides depending on their C-2′ substituents. This quantity, $F_D - F_S$, at the melting temperature, is directly related to T_m; the larger the $(F_D - F_S)_{T_m}$ is, the higher the T_m. Obviously, this quantity ($F_D - F_S$) can be affected either by changing the value of F_D or by changing the value of F_S or by changing both.

The order of the T_m values of the homoduplexes of this series of adenine polynucleotides in acidic solution can be readily understood from the above discussion. The order of the T_m values of the homoduplexes, $[r(2'\text{-EtA})_n^+]_2 > [r(2'\text{-MeA})_n^+]_2 > [r(A)_n^+]_2 > [d(A)_n^+]_2$, is exactly opposite to the order of the values of ($F_S - F_U$) described in the preceding paragraphs. This relationship would be expected if the increase in size of the C-2′ substituents from H to OCH_2CH_3 would have either no significant effect or an enhancement effect on the value of F_D, the free energy of the helical homoduplexes. Under such a situation, the order of ($F_D - F_S$) for this series of adenine polynucleotides, which reflects the observed order of the T_m values, would be exactly *opposite* to the order of ($F_S - F_U$) which has been shown above to be related to the steric hindrance of the 2′ substituents to base–base stacking. It is conceivable that alkylation at C-2′ could decrease the F_D values of the homoduplexes by changing the microscopic dielectric constant at the surface of the helix; however, postulation of such an effect is not needed to explain the order of the T_m values of the homoduplexes of this series of adenine polynucleotides.

For the heteroduplexes (A·U), it is assumed that the contribution of the $r(U)_n$ component to these complexes is the same and therefore needs no special consideration in the comparative study. The order of the T_m values of these heteroduplexes is: $(rA)_n \cdot r(U)_n \geq r(2'\text{-MeA})_n \cdot r(U)_n > r(2'\text{-EtA})_n \cdot r(U)_n > d(A)_n \cdot r(U)_n$. The experimental results suggest that this order of T_m values should be separated into two parts for its consideration: $(rA)_n \cdot r(U)_n > d(A)_n \cdot r(U)$; and $r(A)_n \cdot r(U)_n \geq r(2'\text{-MeA})_n \cdot r(U)_n > r(2'\text{-EtA})_n \cdot r(U)_n$. The first part of this order could be explained again on the basis that the C-2′ substitution of H by OH does not change the F_D values of these duplexes. Under such a condition, the $F_D - F_S$ value for $r(A)_n \cdot r(U)_n$ is larger than the $F_D - F_S$ value for $d(A)_n \cdot r(U)_n$, since conversely $F_S - F_U$ of $d(A)_n$ is larger than the $F_S - F_U$ of $r(A)_n$. Consequently, the T_m of $r(A)_n \cdot r(U)_n$ is higher than the T_m of $d(A)_n \cdot r(U)_n$ as observed. For the second part of this order such an explanation is inadequate, since the $F_S - F_U$ of $r(2'\text{-EtA})_n$ is smaller than that of $r(A)_n$. Therefore, the T_m of $r(2'\text{-EtA})_n \cdot r(U)_n$ should be higher than the T_m of $r(A)_n \cdot r(U)_n$ based on this consideration alone, while the opposite was observed. The logical explanation, therefore, is that the alkylation (especially the ethylation) at the C-2′ OH increases the F_D values of the heteroduplex. Such an effect has to be sufficiently large to overcome the difference in contribution from the $F_S - F_U$ values. A destabilizing effect of the C-2′ *O*-alkyl group on the heteroduplex can be conceived easily, though the CD study (Figure 9) indicates the general geometry of the base planes in the 2′-*O*-alkylated A·U duplex must be very similar to that of the ribosyl duplex.

In conclusion, this study reveals the important effects of the C-2′ substituent on the base–base stacking of the single-

stranded polynucleotides and on the furanose conformation at the backbone. This knowledge provides the basis for the establishment of a relationship between the comparative thermal stabilities of the helical duplexes to the base-stacking interaction of the single-stranded components. To further our understanding of this problem concerning the C-2′ substituents, a similar comparative study has been made on the hypoxanthine and cytosine polynucleotides (Alderfer *et al.*, 1972). The results of this study will be the subject of the next paper in this series.

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