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Influence of the Phosphodiester Linkage (3′–5′, 2′–5′, and 5′–5′) on the Conformation of Dinucleoside Monophosphate*

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ABSTRACT: The interaction of two adenines in dinucleoside monophosphates joined at different positions by a phosphodiester linkage (2′–5′, 3′–5′, and 5′–5′) have been studied by proton magnetic resonance, circular dichroism, and ultraviolet absorbance over the temperature range of 4–60°. The proton magnetic resonance spectra have been measured in dimethyl sulfoxide-d_6 and in D_2O. Similarly, the interactions of adenine and cytosine in $A_{2'}p_{5'}C$ and $A_{3'}p_{5'}C$ were also investigated. While these five dimers possess certain basic features in common in their conformations, the geometrical relationship between the two constitutive units as well as the temperature effect on this geometrical relationship is quite different for each dimer when examined in sufficient detail. These results indicate that the conformation of the dimers is strongly influenced by the position of the phosphodiester linkage. Studies on the interaction of adenine–adenine (or adenine–cytosine) having three types of geometrical relationships by three physicochemical methods offer a unique comparison among different types of dependence of these three methods (proton magnetic resonance, circular dichroism, and ultraviolet absorbance) on the geometrical relationships between two bases. From the analysis of the proton magnetic resonance data at 4° based on a most recent calculation on diamagnetic effects of ring-current magnetic anisotropy (Giessner-Prettre, C., and Pullman, B. (1970), *J. Theor. Biol. 27*, 87, 341), conformational models for these five dimers have been proposed. From these models, both the magnetic resonance data and the optical data can be correlated and unified qualitatively in accordance with our current understanding of the effect of base–base interaction on the optical properties in a homodimer (Glaubiger *et al.* (1968), *Biopolymers 6*, 409). The base planes in these five dimer models are parallel to each other with a vertical distance of about 3–4 Å. In this model the nucleosidyl units all have the *anti* conformation with respect to the sugar–base torsion angle, and the turn of the screw axis of the stack is right handed. The models for $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, $A_{3'}p_{5'}C$, and $A_{2'}p_{5'}C$ are asymmetric, but the model of $A_{5'}p_{5'}A$ is constructed with a twofold axis of symmetry (C_{2v}) located at the phosphate group.

In our previous paper of this series, we reported the studies by proton magnetic resonance on 15 nucleosides and nucleotides and on 25 dinucleoside mono- and diphosphates (Ts'o *et al.*, 1969a). From these studies, a general conformational model for all the dimers is constructed. In this model the nucleosidyl units all have the *anti* conformation with respect to the sugar–base torsion angle, and the turn of the 3′–5′ screw axis of the stack is right handed. In this paper, we wish to study the influence of the phosphodiester linkage on the conformation of the dinucleoside monophosphates. For this reason, a comparative study was made on $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, and $A_{5'}p_{5'}A$ as well as on $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$. The base–base interactions in these five dimers have been investigated concurrently by proton magnetic resonance, circular dichroism, and ultraviolet absorbance over the temperature range of 4–60°. The proton magnetic resonance spectra have been measured in dimethyl sulfoxide-d_6 and in D_2O. The results indicate that the geometric relationships between the two bases in these isomeric dimers are strongly influenced by the position of the phosphodiester linkage they possess. In applying three physicochemical methods to examine the adenine–adenine (or adenine–cytosine) interaction in dimers having different geometrical relationships, we are able to compare different types of depen-

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dence of these three methods on the geometrical relationship between two bases. Conformational models for these five dimers have been constructed. From these models, both the magnetic resonance data and the optical data can be correlated and unified in light of the existing theories on the physicochemical basis of these measurements.

Experimental Section

Instrumentation. The circular dichroism curves were measured on a Cary 60 spectropolarimeter with the 6001 circular dichroism attachment. The cell temperature was controlled by the use of a Haake Brinkman Model KT-62 constant-temperature circulator. A water-jacketed fused quartz window cell of 1-cm path length obtained from the Optical Cell Co., Beltsville, Md., was used. A thermometer was placed at the flow-inlet chamber and the other thermometer at the flow-outlet chamber; both chambers are located next to the jacketed cell. An average was taken from the readings of these two thermometers which provided an accurate measurement of the temperature of the fluid circulating through the jacketed cell. The concentration of dinucleoside monophosphates employed was at the level of 1.2 optical density units at 260 mμ, dissolved in 0.05 M $NaClO_4$ (pH 7.3).

The proton magnetic resonance spectra were recorded on a Varian HA-100 spectrometer operating in the frequency sweep mode. Probe temperatures were regulated by a Varian V-6507 variable temperature accessory and monitored by observing the splitting in methanol and ethylene glycol. Chemical shifts were measured from an external tetramethylsilane capillary and are reliable to better than ±0.005 ppm. Infinite dilution values were obtained by extrapolation and are measured with an accuracy of about ±0.01 ppm. No bulk susceptibility corrections have been made. A Varian C-1024 computer of average transients was used to enhance the signal to noise ratio of dilute solutions.

Ultraviolet spectra were recorded on a Cary 15 spectrophotometer.

Assay Methods. The hypochromicity values for the 2′–5′, 3′–5′, and 5′–5′-dinucleoside monophosphates are calculated from the absorption maximum of the ultraviolet spectrum before and after hydrolysis in accordance with

$$H\,(\%) = \left(1 - \frac{\epsilon(\text{dimer})}{\epsilon(\text{monomer})} \times 100\right)$$

Alkaline hydrolysis was employed to determine the hypochromicity of the 2′–5′ and 3′–5′ dimers. Samples of known absorbance in phosphate buffer (pH 7.0, 0.01 M) were weighed and the exact volume calculated using the known density (0.01 M K-PO_4 solution, $p = 1.0015$) for this solution. The solutions were then made 0.3 N with respect to KOH by the addition of 5 N KOH. After incubation at 37° for 18–20 hr, the solutions were neutralized with 1 M H_3PO_4 and weighed, and the exact volume was calculated using $p = 1.021$. The spectra of these weighed solutions were then measured against an appropriate blank. In the case of $A_{5'}p_{5'}A$, hydrolysis was accomplished using snake venom phosphodiesterase. A 10-μl aliquot of an $A_{5'}p_{5'}A$ stock solution (1.75 mg in 1 ml of H_2O) was diluted to 1 ml with phosphate buffer (pH 7.0, 0.05 M) and the ultraviolet spectrum determined against the appropriate blank. Another 10-μl aliquot of the $A_{5'}p_{5'}A$ stock solution was digested by treatment with 5 μl of enzyme solution (2 mg in 1 ml of H_2O) and 10 μl of ammonium bicarbonate buffer (pH 9.0, 0.2 M) at 37° for 4 hr. After incubation, the sample was diluted to 1 ml with phosphate buffer (pH 7.0, 0.05 M). The spectrum of the hydrolysate was then measured against an appropriate blank.

Phosphorus was determined by the method of Chen *et al.* (1956) following digestion by the method of Baginski *et al.* (1968). Periodate consumption was determined by the method of Dixon and Lipkin (1954).

Paper chromatography was carried out on Whatman 1 paper by the descending technique with 2-propanol–concentrated ammonium hydroxide–H_2O (7:1:2, v/v) as solvent. Paper electrophoresis was carried out on Whatman No. 3MM paper, with potassium phosphate buffer (pH 8.1, 0.03 M) using a Savant power supply and a unit built to the specifications of Savant HV 5000-3.

Materials. Commercially available compounds of the highest degree of purity were used without further purification. All dinucleoside monophosphates except 5′–5′-adenylyladenosine were obtained from Zellstoffabrik, Waldhof, Mannheim, Germany. The following compounds were purchased from Sigma Chemical Co., St. Louis, Mo.: 2′,3′-*O*-isopropylideneadenosine, 5′-AMP, 3′-AMP, and 5′-CMP. Deuterium oxide of 99.5% purity was purchased from Matheson Scientific, Beltsville, Md. Snake venom phosphodiesterase and spleen phosphodiesterase were supplied by Worthington Biochemical Corp.

Synthesis and Characterization of Adenylyl-(5′–5′)-adenosine ($A_{5'}p_{5'}A$). This compound has been previously reported by Sulston *et al.* (1968). It was obtained as one of the products in the condensation of 5′-AMP with adenosine on a poly U template, and was only partially characterized. In the present work, the general phosphorylation method of Yoshikawa *et al.* (1967) was followed. 2′,3′-*O*-Isopropylideneadenosine (307 mg, 1 mmole) was added to a solution of freshly distilled phosphoryl chloride (0.18 ml, 2 mmoles) in redistilled triethyl phosphate (2 ml). After standing 2 hr in an ice bath, the colorless solution was slowly added to 10 ml of ether for the removal of triethyl phosphate and excess phosphoryl chloride. The mixture was centrifuged and the ether removed by decantation. The solid was washed with ether, isolated by centrifugation, and dissolved in triethyl phosphate (5 ml) containing isopropylideneadenosine (307 mg, 1 mmole). The mixture, after standing overnight in a refrigerator, was added to ether. The precipitate, isolated as described above, was slowly added to ice-water. After adjusting the pH to 1.5 with 1 N HCl the solution was heated at 70° for about 1 hr and then neutralized with concentrated ammonium hydroxide. The solution was found to contain large amounts of adenosine, 5′-AMP, and $A_{5'}pp_{5'}A$ in addition to the desired $A_{5'}p_{5'}A$ by paper chromatography and electrophoresis.[1] This solution was applied to a 1.25 × 30 cm column of Dowex 1-X2 (Cl^-). After a water wash, the column was eluted with 0.0025 N HCl. The first peak

[1] In a subsequent preparation, a yield of *ca.* 70% of 2′,3′-*O*-isopropylideneadenyl-(5′–5′)-2′,3′-*O*-isopropylideneadenosine was obtained (based on chromatographic analysis) when stoichiometric amounts of nucleoside and phosphoryl chloride (2:1) were used in a one-step reaction; this is the recommended procedure.

TABLE I: Chemical Shifts of Base and H-1′ Protons of Adenine and Cytosine Dinucleoside Monophosphates at 4, 30, and 60° (D_2O, pD 7.4)[a] (Parts per Million from Tetramethylsilane Capillary).

Dinucleoside	Temp (°C)	A_{H-8}	A_{H-2}	C_{H-6}	C_{H-5}	H-1′ (1)	H-1′ (2)
$A_{3'}p_{5'}A$	4	8.570 (5′)	8.465 (5′)			6.31 (5′)	6.20 (3′)
		8.575 (3′)	8.26 (3′)				
	30	8.69 (5′)	8.61 (5′)			6.41 (5′)	6.29 (3′)
		8.66 (3′)	8.47 (3′)				
	60	8.835 (5′)	8.715 (5′)			6.555 (5′)	6.43 (3′)
		8.745 (3′)	8.65 (3′)				
$A_{2'}p_{5'}A$	4	8.55 (2′)	8.52 (5′)			6.525 (2′)	6.17 (5′)
		8.36 (5′)	8.08 (2′)				
	30	8.615 (2′)	8.615 (5′)			6.58 (2′)	6.255 (5′)
		8.44 (5′)	8.20 (2′)				
	60	8.70 (2′)	8.755 (5′)			6.65 (2′)	6.36 (5′)
		8.58 (5′)	8.41 (2′)				
$A_{5'}p_{5'}A$	4	8.445 (5′)	8.41 (5′)			6.295 (5′)	
	30	8.525 (5′)	8.50 (5′)			6.38 (5′)	
	60	8.66 (5′)	8.63 (5′)			6.48 (5′)	
$A_{3'}p_{5'}C$	4	8.74	8.48	8.03	5.98	6.39	6.01
	30	8.79	8.61	8.15	6.12	6.49 (A)	6.18 (C)
	60	8.83	8.715	8.275	6.37	6.57	6.36
$A_{2'}p_{5'}C$	4	8.705	8.33	7.71	6.055	6.55	5.89
	30	8.76	8.455	7.80	6.15	6.615 (A)	5.995 (C)
	60	8.81	8.59	7.92	6.27	6.675 (A)	6.135 (C)

[a] All data have been extrapolated to infinite dilution except those of $A_{5'}p_{5'}A$, $A_{3'}p_{5'}C$, and $A_{2'}p_{5'}C$ at 60° which were measured at 0.005 M. As indicated in Table II, the $\Delta\delta_C$ values at 60° and at the concentration of 0.005 M are expected to be within 0.01–0.02 ppm to those at infinite dilution.

in the acidic eluate was neutralized with triethylamine and concentrated to dryness *in vacuo*. The residue, after extraction with chloroform to remove triethylammonium chloride, was dissolved in water. Lyophilization furnished 82 mg of the anhydrous triethylammonium salt of $A_{5'}p_{5'}A$; $\lambda_{max}^{0.1\ N\ HCl}$ 257 mμ (ε 13,600); $\lambda_{max}^{pH\ 7}$ 259 mμ (ε 12,000). The product $A_{5'}p_{5'}A$ was chromatographically and electrophoretically homogeneous with R_F 0.18 (1.72 relative to 5′-AMP; Sulston *et al.* (1968) report 1.67) and +0.40 mobility relative to 5-AMP (Sulston *et al.* (1968) report +0.40).

Anal. Calcd for $C_{26}H_{25}N_{11}O_{16}P$: P, 4.44; periodate consumption: P, 2.00. Found: P, 4.49, periodate consumption: P, 2.00.

The compound was further characterized in the following manner. Digestion with snake venom phosphodiesterase (the conditions were essentially the same as those described above for the hypochromicity determination) yielded adenosine (48%) and 5′-AMP (52%). On the other hand, treatment of about 0.1 μmole of compound with spleen phosphodiesterase (20 μl of a 16 units/ml of H_2O–enzyme stock and 20 μl of 0.2 M ammonium acetate, pH 6.5) at 37° for 4 hr or 0.3 N KOH at 40° for 24 hr did not hydrolyze this compound to adenosine and 5′-AMP. In order to eliminate the possibility of enzyme inhibition in the case of spleen phosphodiesterase a mixed incubation of the compound and $A_{3'}p_{5'}A$ was examined. It was found that while the compound was not hydrolyzed, complete digestion of $A_{3'}p_{5'}A$ occurred in its presence.

Results

Proton Magnetic Resonance Studies. SPECTRAL ASSIGNMENTS. The chemical shifts at infinite dilution of the base protons and the H-1′ protons of the three adenine dimers joined by 3′–5′, 2′–5′, and 5′–5′ phosphodiester linkages and of the two adenine–cytosine dimers joined by 3′–5′ and 2′–5′ linkages are shown in Table I.

The assignments of the $A_{3'}p_{5'}A$, $A_{3'}p_{5'}C$, and $A_{2'}p_{5'}C$ spectra at 30 and 60° have been reported previously in the first paper of this series (Ts'o *et al.*, 1969a). The resonances in the spectra at 4° of these three dimers have the same relative order of field positions with respect to each other as those in spectra at 30 and 60°. Therefore, the resonances in the 4° spectra can be assigned readily (Table I).

The assignment for the $A_{5'}p_{5'}A$ is greatly facilitated by the fact that the spectrum consisted of only two base protons and one H-1′ resonance. At 30° the $A_{5'}p_{5'}A$ resonances are located at 6.38, 8.50, and 8.525 ppm (Table I). The doublet centered at 6.38 ppm is assigned to the H-1′ proton because of the position and the multiplicity. For the assign-

ment of the base protons, the procedure of deuterium exchange was used. This procedure is based on the observation that the exchange of the H-8 proton of adenine takes place much more readily than the exchange of the H-2 proton (Schweizer *et al.*, 1964; Bullock and Jardetzky, 1964). An $A_{5'}p_{5'}A$ solution (5×10^{-3} M) in D_2O was heated for 2 hr at 85°. The exchanged proton magnetic resonance spectrum revealed that the resonance at the lowest field position (8.525 ppm) should be assigned to the H-8 proton and the resonance at 8.50 ppm to the H-2 proton. The relative order of field positions of the resonances in the 4° spectrum and 60° spectrum is the same as that in the 30° spectrum (Table I).

The assignment of the $A_{2'}p_{5'}A$ spectrum was a more formidable task. At 30°, the resonances of the two H-1′ protons are centered at 6.58 and 6.255 ppm (Table I). The H-1′ proton of the 2′-AMP is located at a lower field (about 0.05 ppm) than the H-1′ proton of the 3′- or 5′-AMP due to the deshielding effect of the vicinal phosphate group (Schweizer *et al.*, 1968; Ts'o *et al.*, 1969a). Also, the H-1′ proton of $A_{2'}p$- in $A_{2'}p_{5'}C$ is located at 6.615 ppm (Table I) which is very close to the chemical shift of the resonance (6.58 ppm) at a lower field. Thus, the resonance at 6.58 ppm is assigned to the H-1′ of the $A_{2'}p$- portion and the resonance at 6.255 ppm to the H-1′ of the $-p_{5'}A$ portion.

At 30°, the $A_{2'}p_{5'}A$ spectrum at the base proton region consists of the three resonances at 8.615, 8.44, and 8.20 with the intensity ratio of 2:1:1 (Table I). At 4 and 60°, the resonances of the four base protons are separate from each other (Table I). Therefore, the spectral line at 8.615 observed at 30° consists of resonances from two base protons. Heating the $A_{2'}p_{5'}A$ in D_2O resulted in a loss of approximately 50% intensity of the spectral line at 8.615 ppm and nearly all of the intensity of the line at 8.44 ppm. Thus, the resonance at 8.615 ppm consists of a H-8 proton and a H-2 proton; the resonance at 8.44 consists of a H-8 proton; and the resonance at 8.20 ppm consists of a H-2 proton. The procedure of Mn^{2+} binding was used to assist the assignment of the two H-8 protons, a procedure employed by Chan and Nelson (1969) in their assignment of the H-8 protons of $A_{3'}p_{5'}A$. They reported that this paramagnetic ion is bound to the phosphate and thus broadens the proton in its proximity by dipole-dipole interaction. Since this interaction is extremely distance dependent, the resonance of the H-8 protons close to the phosphate–Mn^{2+} group is much more affected than that of the H-8 proton farther away. Thus, the broadening effect of Mn^{2+} on the H-8 proton of the $-p_{5'}A$ portion should be larger than that on the H-8 protons of the $-p_{3'}A$ or $-p_{2'}A$ portion. The half-line widths of the resonances of $A_{2'}p_{5'}A$ (0.01 M) were measured in Mn^{2+} solutions. At the range of Mn^{2+} employed, the line width of the H-8 resonance located at higher field broadened from 2.5 cps at 3×10^{-5} M Mn^{2+} to 6 cps at 3×10^{-4} M Mn^{2+} concentration. The increase of the line widths of all other proton resonances over this concentration range of Mn^{2+} was less than 0.6 cps. Therefore, the H-8 proton located at higher field (8.44 ppm) is assigned to the $-p_{5'}A$ portion and the H-8 proton located at lower field (8.615 ppm) is assigned to the $A_{2'}p$- portion. This assignment is in agreement with the molecular model of the dimer and the temperature effect discussed in later sections.

The assignment of the H-2 protons in the $A_{2'}p_{5'}A$ spectrum has to rely on considerations of the conformation of the dimer, especially that of the nucleosidyl units. Extensive studies on mononucleotides and dinucleotides indicate that the adenosinyl unit in AMP and in ApA is predominantly in the *anti* conformation with respect to the rotation of the C–N bond between the base and the ribose (Schweizer *et al.*, 1968; Ts'o *et al.*, 1969a; Chan and Nelson, 1969). The Mn^{2+} experiment cited above has shown that the adenosinyl unit in the $-p_{5'}A$ portion of $A_{2'}p_{5'}A$ also is in the *anti* conformation. Thus, the adenosinyl unit in the $A_{2'}p$- portion of the dimer most likely is in the *anti* conformation as well. When both adenosinyl units in the dimer are in the *anti* conformation ($\phi_{cn} = 0$ to $-20°$; Davis, 1967), then the H-2 proton of the $A_{2'}p$- portion is shielded much more than the H-2 proton of the $-p_{5'}A$ portion regardless of whether the 2′–5′ screw axis of the stack of the dimer is right or left handed. In other words, regardless of the handedness of the screw axis of the dimer, the H-2 proton of the $A_{2'}p$- portion is always located *inside* the stack, shielded especially by the five-membered ring of the adenine of the $-p_{5'}A$ portion, and the H-2 proton of the $-p_{5'}A$ portion is always located *outside* the stack and thus is not shielded (Figure 11). Hence, the H-2 proton of the $A_{2'}p$- portion should be located at the high field and the H-2 proton of the $-p_{5'}A$ portion located at the low field (Table I). In addition, the resonance of the high-field proton should be much more temperature sensitive than the resonance of the low-field proton, since the chemical shifts of the former depend on the extent of stacking of the dimer, while those of the latter do not. This is indeed what was observed (Table I). Based on the above arguments, the high-field resonance (8.20 ppm) is assigned to the H-2 proton of the $A_{2'}p$- portion and the low-field resonance (8.615 ppm) is assigned to the H-2 proton of the $-p_{5'}A$ portion.

All the resonances in the $A_{2'}p_{5'}A$ spectrum at 60° move to lower field positions by about 0.07–0.21 ppm as compared with the spectrum at 30° (Table I). This downfield shift is due to the effect of temperature on the conformation of the dimer, the proton–solvent interaction (Ts'o *et al.*, 1969b), and the change of bulk magnetic susceptibility due to the use of an external standard as reference. The relative spectral positions of these resonances with respect to each other, however, are the same as those in the 30° spectrum. There is one important exception. The singlet at 8.615 ppm in the 30° spectrum with the intensity of two protons has separated into two peaks located at 8.755 and 8.70 ppm in the 60° spectrum. The deuterium-exchange experiments showed that the resonances at 8.70 and 8.58 ppm are due to the H-8 protons. Therefore, the assignment of the 60° spectrum is as follows (in ppm): H-2 (5′), 8.755; H-8 (2′), 8.70; H-8 (5′), 8.58; H-2 (2′), 8.41; H-1′ (2′), 6.65; and H-1′ (5′), 6.36.

At 4°, the resonances of the $A_{2'}p_{5'}A$ move upfield by 0.06–0.12 ppm as compared with those in the 30° spectrum (Table I). Again, the relative spectral positions of these resonances with respect to each other are the same as those in the 30° spectrum with one exception. The singlet at 8.615 ppm in the 30° spectrum with the intensity of two protons has now also separated into two peaks located at 8.55 and 8.52 ppm. The deuterium-exchange experiment demonstrated that the resonances at 8.55 and 8.36 ppm are due to the H-8 protons. Therefore, the assignment of the 4° spectrum is as follows (in parts per million): H-8 (2′), 8.55; H-2 (2′), 8.52; H-8 (5′), 8.36; H-2 (2′), 8.08; H-1′ (2′), 6.525; and

TABLE II: Concentration Dependence of the Chemical Shifts, $\Delta\delta_C$, of the Base and H-1′ Protons of $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, $A_{5'}p_{5'}A$, $A_{3'}p_{5'}C$, and $A_{2'}p_{5'}C$ at 4, 30, and 60° ($\Delta\delta_c = \delta_0 - \delta_{0.02\ M}$).

	Temp (°C)	H-8 (5′)	H-8 (3′ or 2′)	H-2 (5′)	H-2 (3′ or 2′)	H-1 (5′)	H-1 (3′ or 2′)
$A_{3'}p_{5'}A$	4	0.06	0.09	0.14	0.13	0.05	0.06
	30	0.05	0.06	0.11	0.11	0.03	0.04
	60	0.025	0.025	0.025	0.045	0.015	0.03
$A_{2'}p_{5'}A$	4	0.045	0.04	0.08	0.06	0.04	0.05
	30	0.04	0.04	0.04	0.05	0.04	0.03
	60	0.03	0.025	0.035	0.035		
$A_{5'}p_{5'}A$	4	0.06		0.10		0.06	
	30	0.03		0.06		0.04	
		H-8	H-2	H-5	H-6	H-1′ (A)	H-1′ (C)
$A_{3'}p_{5'}C$	4	0.05	0.065	0.01	0	0.04	0.01
	30	0.035	0.05	0.01	0.02	0.04	0.01
$A_{2'}p_{5'}C$	4	0.03	0.055	0	0.03	0.02	0.02
	30	0.02	0.04	0.01	0.015	0.02	0.01

H-1′ (5′), 6.17. It is interesting to note the resonance positions of the H-8 (2′) proton and the H-2 (5′) proton at 4, 30, and 60°. At 30°, both protons coincidentally have the same chemical shift, *i.e.*, 8.615 ppm. At 60°, the H-8 (2′) proton (8.70 ppm) is upfield from the H-2 (5′) proton (8.755 ppm), while at 4°, the H-8 (2′) proton (8.55 ppm) is downfield from the H-2 (5′) proton (8.52 ppm). Therefore, the relative spectral positions of these two protons change in the temperature range of 4–60° and cross each other at 30°. Hence, the deuterium-exchange data were essential in the proper assignment of these protons.

CONCENTRATION DEPENDENCE. The concentration dependence of the chemical shifts, $\Delta\delta_c$, from 0 to 0.02 M of the three ApA isomers and the two ApC isomers at 4, 30, and 60° is shown in Table II. The concentration dependence of $A_{3'}p_{5'}A$ (Ts'o *et al.*, 1969a; Chan and Nelson, 1969) and of $A_{3'}p_{5'}C$ (Bangerter and Chan, 1969) at 30° has been studied before over a much wider concentration range. In agreement with these previous studies, the $\Delta\delta_c$'s of the adenine base protons are larger than those of the cytosine base protons, the $\Delta\delta_c$'s of the base protons are generally larger than those of the H-1′ protons, and the $\Delta\delta_c$ of the H-2 proton of the adenine base is larger than that of the H-8 proton. These observations can be anticipated from the known properties of the monomers (Schweizer *et al.*, 1965; Broom *et al.*, 1967) and the conformation of the dimers (Ts'o *et al.*, 1969a). As expected the $\Delta\delta_c$ values are largest at 4°, moderate at 30°, and smallest at 60° (Table II). For the $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$, there is virtually no concentration dependence at 60°.

A comparison of $\Delta\delta_c$ values among these isomers indicates that the concentration dependence of the 3′–5′ dimers is generally larger than that of the 2′–5′ dimers. At 30°, among the adenine homodimers this difference is especially significant between the base protons of $A_{3'}p_{5'}A$ and those of $A_{2'}p_{5'}A$, while those of $A_{5'}p_{5'}A$ are situated between the two. These differences are presumably due to the dissimilarities in conformation and in intramolecular interaction of these three types of dimers. It is interesting to note the correlation of this observation to the differences in the concentration dependence among the three 2′-, 3′-, and 5′-adenine mononucleotides (Schweizer *et al.*, 1968). The overall concentration dependence, $\Delta\delta_c$, is largest for 3′-AMP, next for 5′-AMP, and smallest for 2′-AMP (Schweizer *et al.*, 1968). This difference in $\Delta\delta_c$ among the three isomeric mononucleotides is due to the variation of the distance of the repulsive phosphate group to the adenine base where the stacking takes place. The closer the phosphate group to the base, the lesser the tendency of stacking for the monomer (Schweizer *et al.*, 1968). The distance between the phosphate group and the base is shorter for 2′-AMP than for 5′-AMP and 3′-AMP. It appears that this argument may also be applicable to the stacking of these isomeric dimers.

CONFORMATION MODELS for $A_{3'}p_{5'}A$, $A_{3'}p_{5'}C$ (Ts'o *et al.*, 1969a; Chan and Nelson, 1969; Bangerter and Chan, 1969), and $A_{2'}p_{5'}C$ (Ts'o *et al.*, 1969a) have previously been constructed mainly on the basis of the proton magnetic resonance results. The construction of these models was based on two conformational requirements. The first requirement is that all the nucleosidyl units in the dinucleoside monophosphates have the *anti* conformation and the second requirement is that the 3′–5′ or 2′–5′ screw axis of the dinucleoside monophosphates has a right-handed turn in its stacked form. The model constructed from these two requirements can account for the proton magnetic resonance results semiquantitatively and is also supported by the optical rotatory dispersion–circular dichroism data (Ts'o *et al.*, 1969a). One procedure successfully adopted for the analysis of the chemical shift data of the dimer is to compare the δ values of the 5′-nucleotidyl unit or the 3′-nucleotidyl unit in the dimer with the δ values of the corresponding 5′-mononucleotides or 3′-mononucleotides. This difference is termed dimerization shift ($\Delta\delta_D$, usually negative) which reflects the shielding of this proton by the neighboring nucleotidyl unit in the dimer. The values of the dimerization shifts ($\Delta\delta_D$) at 4, 30, and 60°

TABLE III: Dimerization Shifts, $\Delta\delta_D$, of the Base and H-1′ Protons of $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, and $A_{5'}p_{5'}A$ at Various Temperatures.[a,b]

		3′ or 2′			5′		
	Temp (°C)	H-8	H-2	H-1′	H-8	H-2	H-1′
$A_{3'}p_{5'}A$	4	0.17	0.345	0.285	0.30	0.125	0.19
	30	0.11	0.215	0.26	0.235	0.075	0.15
	60	0.12	0.145	0.19	0.175	0.105	0.11
	% change[c]	*d*	58	33	42	*d*	42
$A_{2'}p_{5'}A$	4	0.195	0.53	+0.01	0.53	0.07	0.33
	30	0.155	0.46	0.02	0.485	0.07	0.315
	60	0.155	0.385	0.03	0.445	0.04	0.29
	% change[c]	*d*	28	*d*	16	*d*	12
$A_{5'}p_{5'}A$	4				0.43	0.185	0.21
	30				0.40	0.185	0.18
	60				0.35	0.16	0.18
	% change[c]				18	*d*	*d*

[a] pD 5.9; in parts per million, all negative unless otherwise indicated. For 5′-nucleotidyl unit $\Delta\delta_D$ = δpA − δApA, and for 3′- or 2′-nucleotidyl unit $\Delta\delta_D$ = δAp − δApA. [b] The chemical shift data on 2′-AMP, 3′-AMP, and 5′-AMP at 30 and 60° needed for the calculation of the dimerization shifts are obtained from a previous publication (Ts'o *et al.*, 1969a). The chemical shift data at 4° and infinite dilution are listed as follows (parts per million from TMSi capillary): 5′-AMP: H-8 (8.87); H-2 (8.59); H-1′ (6.50). 3′-AMP: H-8 (8.745); H-2 (8.605); H-1′ (6.485). 2′-AMP: H-8 (8.745); H-2 (8.605); H-1′ (6.52). [c] Percentage change = $(1 - (\Delta\delta_D{}^{60^\circ}/\Delta\delta_D{}^{4^\circ})) \times 100$. [d] The magnitude of change is too small to be accurate or significant.

TABLE IV: Dimerization Shifts of the Base and H-1′ Protons of $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ at Various Temperatures.[a,b]

	Temp (°C)	H-8	H-2	H-1′	H-6	H-5	H-1′
$A_{3'}p_{5'}C$	4	0.01	0.13	0.10	0.39	0.51	0.35
	30	−0.015	0.08	0.06	0.28	0.41	0.24
	60	0.03	0.07	0.05	0.20	0.25	0.14
	% change[c]	*d*	46	50	49	50	60
$A_{2'}p_{5'}C$	4	0.04	0.28	0	0.70	0.43	0.46
	30	0.01	0.20	+0.015	0.63	0.38	0.42
	60	0.04	0.21	0.02	0.55	0.35	0.36
	% change[c]	*d*	25	*d*	21	19	22

[a] pD 5.9; in parts per million, all negative unless otherwise indicated. For 5′-nucleotidyl unit $\Delta\delta_D$ = δpC − δApC and for 3′- or 2′-nucleotidyl unit $\Delta\delta_D$ = δAp − δApC. [b] The chemical shift data on the 2′-AMP, 3′-AMP, and 5′-CMP at 30 and 60° needed for the calculation of the dimerization shifts are obtained from a previous publication (Ts'o *et al.*, 1969). The data on 3′-AMP at 4° is from the footnote in Table III. The chemical shift data of 5′-CMP at 4° and 0.04 M concentration are listed as follows (parts per million from TMSi capillary): H-6 (8.41), H-5 (6.49), H-1′ (6.35). [c] Percentage change = $(1 - (\Delta\delta_D{}^{60^\circ}/\Delta\delta_D{}^{4^\circ})) \times 100$. [d] The magnitude of change is too small to be accurate or significant.

for the ApA dimers and for the ApC dimers are shown in Tables III and IV, respectively. At present, the discussion on the construction for the model is based mainly on the data at 30°.

As shown in Table III, both $A_{2'}P_{5'}A$ and $A_{3'}p_{5'}A$ have similar patterns of $\Delta\delta_D{}^{30^\circ}$ for the base protons. The $\Delta\delta_D{}^{30^\circ}$ values for three base protons of $A_{2'}p_{5'}A$, *i.e.*, H-8 in ($A_{2'}p$-) unit, H-2 in ($A_{2'}p$-) unit, and H-8 in (-$p_{5'}A$) unit, are 50–100% larger than the $\Delta\delta_D{}^{30^\circ}$ values for the corresponding protons of $A_{3'}p_{5'}A$. The $\Delta\delta_D{}^{30^\circ}$ value for the H-2 proton in (-$p_{5'}A$) unit of $A_{2'}p_{5'}A$ is about the same as that for the corresponding unit of $A_{3'}p_{5'}A$. It should be noted that part of the $\Delta\delta_D{}^{30^\circ}$ (about 0.1 ppm) of the H-8 in (-$p_{5'}A$) of both $A_{3'}p_{5'}A$ and $A_{2'}p_{5'}A$ comes from the reduction of the deshielding effect of the phosphate when the Δδ values are compared between the monomer and dimer. The transformation of a monoester phosphate group in a monomer to a diester phosphate group in a dimer reduces the deshielding effect of the phosphate group. The $A_{2'}p_{5'}A$ and $A_{3'}p_{5'}A$ have different patterns of $\Delta\delta_D{}^{30^\circ}$ for the H-1′ protons (Table

III). For $A_{2'}p_{5'}A$, the $\Delta\delta_D^{30°}$ of H-1′ of the ($-p_{5'}A$) is much higher than the $\Delta\delta_D^{30°}$ of H-1′ of the ($A_{2'}p-$), while for $A_{3'}p_{5'}A$, the $\Delta\delta_D^{30°}$ of H-1′ of ($-p_{5'}A$) is smaller than the $\Delta\delta_D^{30°}$ of H-1′ of the ($A_{5'}p-$).

These data suggest that the $A_{2'}p_{5'}A$ also has an *anti,anti* right-handed stack. The *anti* conformation of the adensoyl moiety in the ($-p_{5'}A$) unit of $A_{2'}p_{5'}A$ has been confirmed independently based on the Mn^{2+} relaxation experiment described in the section on "spectral assignment." The fact that the $\Delta\delta_D^{30°}$ of H-1′ in ($-p_{5'}A$) is much larger than the $\Delta\delta_D^{30°}$ of H-1′ in the ($A_{2'}p-$) also indicates that the average conformation for the stack of the $A_{2'}p_{5'}A$ is right handed according to the examination of the CPK model. This conclusion is also supported by the circular dichroism results. As shown in a later section, the circular dichroism spectrum of $A_{2'}p_{5'}A$ is similar to that of the $A_{3'}p_{5'}A$ which has been shown to have an *anti,anti* right-handed stack (Ts'o *et al.*, 1969a; Chan and Nelson, 1969). In the examination of the Corey–Pauling–Koltun (CPK) models, the conformation of $A_{2'}p_{5'}A$ has the following interesting differences from that of the $A_{3'}p_{5'}A$: (a) the direct lateral distance between the two C-1′ atoms in $A_{2'}p_{5'}A$ is longer than that in $A_{3'}p_{5'}A$ when the bases are laid down alongside each other on the same plane; (b) thus, the arc in the turn of the $A_{2'}p_{5'}A$ stack is larger than that in the $A_{3'}p_{5'}A$. (c) The base of the ($-p_{5'}A$) unit tends to overlap the 6-NH_2, C-6, N-1, and C-2 region of the ($A_{2'}p-$) unit in the 2′–5′ dimer, while in the 3′–5′ dimer, the base of the ($-p_{5'}A$) unit tends to overlap the C-2, N-3, and C-2′ portion of the ($A_{3'}p-$) unit. In other words, the overlap of the base of the ($-p_{5'}A$) tends to be away from C-1′ of the ($A_{2'}p-$) unit in the $A_{2'}p_{5'}A$, while the overlap of the base of the ($-p_{5'}A$) is comparatively closer to the C-1′ of the ($A_{3'}p-$) unit in $A_{3'}p_{5'}A$. We believe that this is the reason why the $A_{2'}p_{5'}A$ and $A_{3'}p_{5'}A$ have different patterns for the $\Delta\delta_D^{30°}$ of the H-1′ protons even though both are assigned with a right-handed stack. In the $A_{3'}p_{5'}A$ situation, the left-handed model is excluded because of the substantial shielding (0.15 ppm) of the H-1′ of ($-p_{5'}A$) and the difference between the $\Delta\delta_D^{30°}$ of the two H-1′ protons is only about 0.11 ppm. Therefore, the right-handed model of $A_{3'}p_{5'}A$ with the overlapping of the center of the adenine of the ($-p_{5'}A$) unit over the H-1′ of the ($A_{3'}p-$) unit is more compatible with the observed pattern of $\Delta\delta_D^{30°}$ for the two H-1′ protons. As for the base protons, the large value of the $\Delta\delta_D^{30°}$ of the protons of $A_{2'}p_{5'}A$ (three values out of four are 50–100% larger than those of $A_{3'}p_{5'}A$) indicates that the degree of stacking and base–base overlapping is very extensive. The situation of the $A_{5'}p_{5'}A$ is quite different from those of the 2′–5′ and the 3′–5′ isomers. As shown in Table I, the same protons in both residues have the identical resonance, *i.e.*, both H-8 protons are located at 8.525 ppm, both H-2 protons at8. 50 ppm, and both H-1′ protons at 6.38 ppm. (Preliminary data indicate that the H-5′ and H-5′′ protons of both residues are also located at the same position, 4.64 ppm. However, owing to the multiplicity of these proton peaks, more work is needed before a firm conclusion can be reached.) This magnetic equivalence of the same protons in both residues requires a symmetry consideration in building the conformation model. Another point to consider is that the $\Delta\delta_D^{30°}$ value for the H-8 proton is considerably larger than that for the H-2 proton. The model depicted in Figure 11 is one of the two models that can satisfy these two requirements. The model is constructed with a twofold axis of symmetry, C_{2v}, parallel to the two base planes and bisecting the phosphorus atom of the 5′–5′ dimer. This twofold axis is generated by the intersection of the two perpendicular planes formed by the four oxygen atoms (one plane contains the two ester oxygens and the other plane contains the two ionized oxygen atoms) in a tetrahedron of the phosphate group. As shown, this model of $A_{5'}p_{5'}A$ has an *anti,anti* right-handed symmetrical conformation. The other model which also satisfies these two requirements is an *anti,anti* left-handed symmetrical conformation. However, the H-1′ protons of both residues are located between the two base planes in a fully stacked conformation of a right-handed model, while the H-1 protons are located outside the two base planes in a left-handed model. The $\Delta\delta_D^{30°}$ value of the H-1′ proton is rather large and is the same as that of the H-2 proton (Table III). These data favor the right-handed model in which the H-1′ proton and the H-2 proton are nearly in the same plane with the H-1′ protons closely shielded by the neighboring five-membered ring and the H-2 protons more distantly shielded by the neighboring six-membered ring (Figure 11). As shown in the later sections, the circular dichroism spectrum of $A_{5'}p_{5'}A$ (Figure 3) is very similar to that of the $A_{3'}p_{5'}A$ (Figure 1). The observed similarity supports this model that the screw axis of the stack of the $A_{5'}p_{5'}A$ is right handed, the same as that of the $A_{3'}p_{5'}A$ (Ts'o *et al.*, 1969a). The model of *syn,syn* right-handed symmetrical conformation is excluded since the $\Delta\delta_D^{30°}$ of the H-8 protons is much larger than that of the H-2 protons, while H-1′ and H-2 have the same $\Delta\delta_D^{30°}$ (Table III), an observation contrary to the expectation of the model of *syn* conformation. There is an interesting asymmetrical model in which the two 5′-AMP residues are in *anti* conformation and parallel to each other. In this model, a plane of symmetry exists between the two adenine bases but not between the ribosyl units. This model is excluded since $A_{5'}p_{5'}A$ in this conformation will not have optical activity arising from the base–base interaction, and also the H-2 protons in this model will have a greater $\Delta\delta_D^{30°}$ value than the H-8 protons. Both circular dichroism and proton magnetic resonance data are contrary to the predicted properties of this conformational model. In summary, the nuclear magnetic resonance data require the model for the stack of $A_{5'}p_{5'}A$ to have *anti,anti* and symmetrical conformation (Figure 11). The data also favor the screw axis to be right handed, and this preference is supported by the circular dichroism data.

The conformation model for $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ has been proposed in our previous publication (Ts'o *et al.*, 1969a). The additional data acquired (Tables I and IV) in the present work continue to indicate the correctness of this model of *anti,anti* right-handed conformation. The chemical shifts of $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ shown in Table I are at a slightly lower field position (about 0.03–0.05 ppm) than those published previously (Ts'o *et al.*, 1969a). It is because the previous values were obtained at 0.02 M concentration while the present values are obtained at infinite dilution (Table II). Correspondingly, the $\Delta\delta_D^{30°}$ values of the $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ are slightly less than those published previously.

TEMPERATURE AND SOLVENT EFFECTS. The effects of temperature in the range of 4–60° on the chemical shifts and the coupling constant $J_{H\text{-}1'\text{-}H\text{-}2}$ have been studied. In addi-

TABLE V: Chemical Shifts of the Base and H-1′ Protons of $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, and $A_{5'}p_{5'}A$ in Dimethyl-d_6 Sulfoxide at 30°.

	H-8	H-2	NH_2	H-1′
$A_{3'}p_{5'}A$[a]	8.78 (5′)	8.50 (5′)	7.59 (5′)	6.29 (5′)
	8.70 (3′)	8.48 (3′)	7.67 (3′)	6.23 (3′)
$A_{2'}p_{5'}A$	8.75 (5′)[b]	8.47 (5′)[c]	7.61 (5′)[c]	6.22 (5′)[b]
	8.68 (3′)	8.45 (2′)	7.57 (2′)	6.35 (2′)
$A_{5'}p_{5'}A$	8.83	8.50	7.60	6.28

[a] The δ values are cited from our previous paper (Ts'o *et al.*, 1969a). [b] The assignment of the protons to the ($A_{2'}p$-) unit and the (-$p_{5'}A$) unit is based on the consideration of the phosphate deshielding effect on the H-1′ (2′ unit) and on the H-8 (5′ unit) in an *anti* conformation. [c] The assignment of the protons to the ($A_{2'}p$-) unit and the (-$p_{5'}A$) unit is based on the residual stacking of an *anti,anti*, right-handed model. Such an assignment, especially based on a small difference in δ value, is considered to be tentative.

tion, the effect of dimethyl sulfoxide-d_6 as a solvent on the resonance of the dimers has also been measured. As shown in previous investigations (Helmkamp and Ts'o, 1961; Chan *et al.*, 1964; Ts'o *et al.*, 1969a; Schweizer, 1969), dimethyl sulfoxide is a very effective denaturing solvent. The hydrophobic stacking interactions of the bases in nucleic acids and dinucleotides are reduced to a minimum when dissolved in dimethyl sulfoxide. Therefore, the procedure of dissolving the dimers in dimethyl sulfoxide has been adopted as a substitute for high-temperature perturbation. This method of denaturation or destacking by organic solvent reduces the problem of degradation and the problem of exchange of H-8 protons with D_2O, which occur at high temperature.

The temperature dependence of the dimerization shifts, $\Delta\delta_D$, of the three adenine homodimers are shown in Table III. The results clearly indicate that the effect of temperature on the $\Delta\delta_D$ values of $A_{3'}p_{5'}A$ is much more profound than on those of $A_{2'}p_{5'}A$, even though the $\Delta\delta_D$ values of the 2′–5′ dimer are larger than those of the 3′–5′ dimer. For instance, the $\Delta\delta_D$ value of the H-2 proton in the ($A_{3'}p$-) unit of $A_{3'}p_{5'}A$ is 0.345 ppm at 4° and 0.145 ppm at 60°, for a change of 63%; the $\Delta\delta_D$ value of the H-2 proton in the ($A_{2'}p$-) unit of $A_{2'}P_{5'}A$ is 0.53 ppm at 4° and 0.385 ppm at 60°, for a change of only 28%. The same situation occurs for the H-8 proton and the H-1′ proton in the (-$p_{5'}A$) unit, *i.e.*, while the $\Delta\delta_D$ values of the $A_{2'}p_{5'}A$ are larger, they are much less affected by temperature than the corresponding $\Delta\delta_D$ values of $A_{3'}p_{5'}A$. The situation in $A_{5'}p_{5'}A$ is not directly comparable. Nevertheless, the effect of temperature on the $\Delta\delta_D$ value of the H-8, H-2, and H-1′ protons of $A_{5'}p_{5'}A$ is rather small although these protons are substantially shielded, especially the H-8 protons, by their neighboring units. The chemical shifts and dimerization shifts of the three adenine homodimers dissolved in dimethyl sulfoxide-d_6 are shown in Tables V and VI, respectively. These results indicate that the chemical shifts of these three dinucleoside monophosphates dissolved in dimethyl sulfoxide-d_6 are very similar to the chemical shifts of the constitutive monomers in dimethyl sulfoxide-d_6. As shown in Table VI, the $\Delta\delta_D$ values in dimethyl sulfoxide-d_6 have been reduced to a negligible level, confirming the conclusion that in dimethyl sulfoxide, the dimers are mostly destacked and free of the influence of the neighboring units in the dimer. Even under this condition, the $\Delta\delta_D$ values of $A_{2'}p_{5'}A$ in dimethyl sulfoxide-d_6 appear to be slightly higher than those of $A_{3'}p_{5'}A$.

The temperature dependence of the dimerization shifts of the two isomeric adenine–cytosine dimers is shown in Table IV. Again, the effect of temperature on the $\Delta\delta_D$ values of $A_{3'}p_{5'}C$ is much more profound than on those of the $A_{2'}p_{5'}C$, even though the $\Delta\delta_D$ values of the $A_{2'}p_{5'}C$ (except the H-5 proton) are larger than those of the $A_{3'}p_{5'}C$. This situation is especially noticeable for the H-6 protons and H-1′ protons in the same (-$p_{5'}C$) unit of both dimers, which are shielded by neighboring ($A_{3'}p$-) or ($A_{2'}p$-) units (Table IV). For instance, the $\Delta\delta_D$ value of the H-6 proton in $A_{3'}p_{5'}C$ is 0.39 ppm at 4° and 0.20 ppm at 60°, for a change of 49%; the $\Delta\delta_D$ value of the H-6 proton in $A_{2'}p_{5'}C$ is 0.70 ppm at 4° and 0.55 ppm at 60° for a change of 21%. The comparison of the temperature effect on the two H-5 protons of these two isomers is rather interesting. At 4°, the H-5 proton of $A_{3'}p_{5'}C$ ($\Delta\delta_D^{4°}$ = 0.51 ppm) is more shielded than the H-5 proton of $A_{2'}p_{5'}C$ ($\Delta\delta_D^{4°}$ = 0.43 ppm). At 60°, owing to the smaller temperature effect, the H-5 proton of $A_{2'}p_{5'}C$ ($\Delta\delta_D^{60°}$ = 0.35 ppm) is now more shielded than the H-5 proton of $A_{3'}p_{5'}C$ ($\Delta\delta_D^{60°}$ = 0.25 ppm). This situation actually can be predicted from the conformation models of these two isomers as discussed in a later section (Figure 11).

When the temperature dependence of the $\Delta\delta_D$ values is normalized in terms of per cent change or presented in Table III and Table IV, the magnitude of the temperature effect varies with the location of the proton within the dimer, especially for the ApA. For instance, in $A_{3'}p_{5'}A$, per cent change (4–60°) is different for H-2 (58%) and H-1′ (33%) in the ($A_{3'}p$-) unit, and for H-8 or H-1′ (42%) in the (-$p_{5'}A$) unit. It is difficult to explain this differential temperature effect on dimerization shifts by a two-state model, since this model would predict an equal temperature dependence (normalized as per cent change) for all protons within a given dimer.

In Table VII, the chemical shifts of the base and H-1′ protons of $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ in dimethyl sulfoxide-d_6 at 30° are shown. These δ values are very close to the δ values of the constitutive monomers (3′-AMP, 2′-AMP, and 5′-CMP) in dimethyl sulfoxide-d_6 at 30°. Most of the $\Delta\delta_D$ values in dimethyl sulfoxide are less than 0.02 ppm, indicating the interaction between the bases is reduced to a minimal level.

The effect of temperature and the change of solvent (from D_2O to dimethyl sulfoxide-d_6) on the coupling constants ($J_{H\text{-}1'\text{-}H\text{-}2'}$) between the H-1′ proton and the H-2′ proton of five dinucleoside monophosphates is shown in Table VIII. The change of $J_{H\text{-}1'\text{-}H\text{-}2'}$ of $A_{3'}p_{5'}A$ was first reported by Hruska and Danyluk (1968) and was interpreted as an indication of the change of the conformation of the ribose ring in accordance with the Karplus relationship. Subsequently, the effect of temperature on the $J_{H\text{-}1'\text{-}H\text{-}2'}$ of $A_{3'}p_{5'}A$, $A_{3'}p_{5'}C$, and $C_{3'}p_{5'}A$ has also been described by Chan and Nelson (1969) and by Bangerter and Chan (1969) in terms that "the ribose conformations of both the adenosine

TABLE VI: Dimerization Shifts, $\Delta\delta_D$, of the Base and H-1′ Protons of $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, and $A_{5'}p_{5'}A$, in Dimethyl Sulfoxide-d_6 at 30°[a] (in Parts Per Million, All Negative Unless Indicated Otherwise).

	H-8	H-2	NH_2	H-1′
$A_{3'}p_{5'}A$	0.07 (5′)	+0.01 (5′)	0.07 (5′)	+0.01 (5′)
	0.02 (3′)	0.0 (3′)	+0.01 (3′)	+0.05 (3′)
$A_{2'}p_{5'}A$[b]	0.10 (5′)	0.02 (5′)	0.05 (5′)	0.06 (5′)
	0.04 (2′)	0.03 (2′)	0.09 (2′)	0.01 (2′)
$A_{5'}p_{5'}A$	0.02	0.01	0.06	0.0

[a] The δ values of 5′-AMP and 3′-AMP in dimethyl sulfoxide-d_6 needed for the calculation of $\Delta\delta_D$ in this table are obtained from a previous publication (Ts'o *et al.*, 1969a). [b] The δ values of 2′-AMP in dimethyl sulfoxide-d_6 are found to be the same as those of 3′-AMP except for the H-1′ proton (6.344 ppm from TMSi capillary).

TABLE VII: Chemical Shifts of the Base and H-1′ Protons of $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ in Dimethyl Sulfoxide-d_6 at 30° (Parts per Million from Tetramethylsilane Capillary).

	($A_{3'}p$-) or ($A_{2'}p$-)				(-$p_{5'}C$)			
	H-8	H-2	NH_2	H-1′	H-6	H-5	NH_2	H-1′
$A_{3'}p_{5'}C$	8.70	8.48	~7.67[b]	6.23[a]	8.20	6.08	~7.44[b]	6.19[a]
$A_{2'}p_{5'}C$	8.70	8.47	~7.64[b]	6.34	8.12	6.04	~7.43[b]	6.11

[a] The doublets of the two H-1′ protons from ($A_{3'}p$-) and (-$p_{5'}C$) overlapped each other. The δ values are accurate only to ±0.02 ppm. [b] The peaks of the NH_2 protons are relatively broad.

and cytidine nucleosides become more 3′-*endo* when the dinucleotide is intramolecularly stacked." In our previous paper (Ts'o *et al.*, 1969a), similar effects of temperature on the $J_{H\text{-}1'\text{-}H\text{-}2'}$ of various dimers including ApA were also reported. Most recently, Hruska *et al.* (1970) reported the coupling constants of all the protons in pseudouridine and most of the protons in uridine. They concluded that there is a rapid interconversion between the $C_{2'\text{-}endo}$ and $C_{3'\text{-}endo}$ (or $C_{2'\text{-}exo}$ and $C_{3'\text{-}exo}$) form, and that the distribution between these forms is not affected by temperatures between 30 and 70°. Davies and Danyluk (1970) have also analyzed the conformation of eight nucleosides dissolved in dry dimethyl sulfoxide–benzene solution and other solvents. They came to the conclusion (D. B. Davies and S. S. Danyluk, private communication) that the ribose ring conformation is in rapid equilibrium between various forms and the ΔH between these forms is very small. It should be noted that the change of the dihedral angle between H-1′ and H-2′ can be brought about either by the change of the H-1′ proton position relative to the ribose ring (C-1′ in *endo* or *exo*) or by the change of relative position of the H-2′ (C-2′ in *endo* or *exo*). Most of the attention in the past has been focused on the latter situation, *i.e.*, the change of C-2′ or C-3′ relative position (Hruska and Danyluk, 1968; Chan and Nelson, 1969; Bangerter and Chan, 1969; Prestegard and Chan, 1969).

From the standpoint of the Karplus relationship (Jardetzky, 1960; Abraham *et al.*, 1962), the large change of the $J_{H\text{-}1'\text{-}H\text{-}2'}$ observed (Table VIII) may indicate a change of the dihedral angle between H-1′ and H-2′ by as much as over 30°. While a proposal has been made that the change of C-2′ *endo* conformation to C-3′ *endo* conformation of the 3′-nucleosidyl unit ($N_{3'}p$-) may relieve certain steric repulsion in the stacked dimer (Bangerter and Chan, 1969), it is very difficult to understand how the C-2′ and C-3′ atoms in the ribosyl ring of the 5′-nucleosidyl unit (-$p_{5'}N$) can be involved in a sufficiently large nonbonding interaction with the adjacent nucleosidyl unit in the dimer in order to account for the large changes of $J_{H\text{-}1'\text{-}H\text{-}2'}$ values observed for all five dimers listed (Table VIII). It is because the C-2′ and C-3′ atoms of the 5′-nucleosidyl unit (including those of $A_{5'}p_{5'}A$) are located *outside* of the stack of these dimers. In comparison with the $J_{H\text{-}1'\text{-}H\text{-}2'}$ value of the appropriate monomers (Ts'o *et al.*, 1969a) or of the noninteracting monomeric units in a totally "unstacked" dimer (such as those dissolved in dimethyl sulfoxide), the $J_{H\text{-}1'\text{-}H\text{-}2'}$ values of the interacting monomeric units in the "stacked" dimers are visibly smaller, indicating a reduction of the dihedral angle between the H-1′ and H-2′ protons. There is one interesting exception in the case of the $A_{2'}p_{5'}C$. The $J_{H\text{-}1'\text{-}H\text{-}2'}$ value (6.6–6.7 cps) of the ($A_{2'}p$-) unit is larger than that of 2′-AMP (5.9 cps; Ts'o *et al.*, 1969a), even though the $J_{H\text{-}1'\text{-}H\text{-}2'}$ value (4.5 cps, at 30°) of the ($A_{2'}p$-) unit in $A_{2'}p_{5'}A$ is smaller than that of 2′-AMP. These are rather puzzling observations, especially if they are to be explained by the change of the relative position (*endo vs. exo*) of the C-2′ atom of the ribose

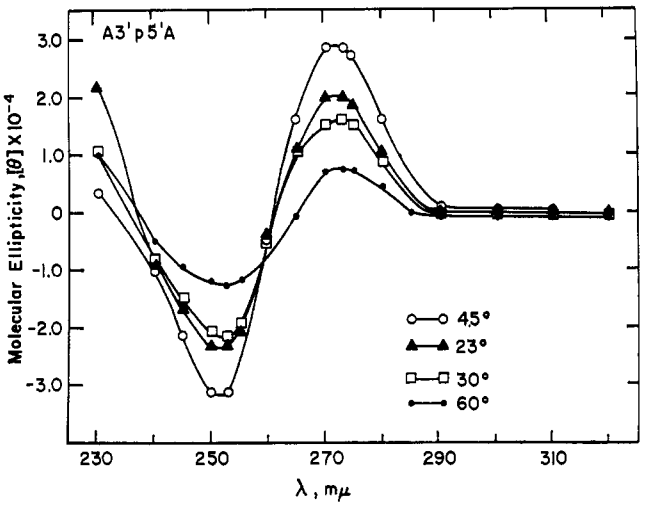

FIGURE 1: Circular dichroism of $A_{3'}p_{5'}A$ in 0.05 M $NaClO_4$ (pH 7.3) at the following four temperatures: 4.5 (○-○), 23 (▲-▲), 30 (□-□), and 60° (●-●).

ring. Some of these data may be more understandable if the change of the relative position of the C-1′ atom is evoked instead. It is possible to envisage a nonbonding interaction of the C-1′ atom and H-1′ proton of the 5′-nucleotidyl unit with the neighboring unit in the stack of dimer. Certainly, much more work is needed for a more thorough understanding of these data on $J_{H\text{-}1'\text{-}H\text{-}2'}$ values. Recently, a complete conformational analysis of the ribose conformations of various nucleoside 3′–5′-cyclic phosphates based on coupling constants has been done by Smith and Jardetzky (1968). The influence of electrolytes on the $J_{H\text{-}1'\text{-}H\text{-}2'}$ values of uracil nucleosides and nucleotides have been studied by Prestegard and Chan (1969). In ribosyl polynucleotides, X-ray diffraction studies indicated that the ribosyl ring has a 3′-*endo* conformation (Arnott, 1970).

Circular Dichroism Studies. The circular dichroism spectra of $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, and $A_{5'}p_{5'}A$ in 0.05 M $NaClO_4$ (pH 7.3) at 4.5, 23, 30, and 60° are shown in Figures 1, 2, and 3, respectively. The spectral range is from 220 or 230 mμ to 300 mμ, thus covering the optical activity of the major transition band near 260 mμ. The spectra of $A_{3'}p_{5'}A$ in Figure 1 are very similar to those previously reported by Van Holde *et al.* (1965). The circular dichroism spectral data of $A_{2'}p_{5'}A$ have also been measured by Brahms as cited by Bush and Scheraga (1969). The [θ] values at both peak and trough positions of $A_{2'}p_{5'}A$ measured in 4.7 M KF, pH 7, 5°, by Brahms are slightly smaller than those of $A_{2'}p_{5'}A$ shown in Figure 2 at 4.5°, 0.05 M $NaClO_4$. The circular dichroism spectra of all these three adenine dinuceloside monophosphates have similar peak position (273 ± 2 mμ) and trough position (~253 ± 2 mμ). The absolute θ values of the peak and the trough are approximately equal in the $A_{3'}p_{5'}A$ and $A_{5'}p_{5'}A$ spectra, indicating the "conservative" nature of the spectra (Bush and Scheraga, 1969). The $A_{2'}p_{5'}A$ spectra are less symmetrical; the absolute peak θ values are slightly larger than the absolute trough θ values. Also, the $[\theta]_{peak}$ and $[\theta]_{trough}$ (or the magnitude between the peak and the trough) of $A_{3'}p_{5'}A$ and of $A_{5'}p_{5'}A$ are close to each other and are significantly larger than those of the $A_{2'}p_{5'}A$. Within the temperature range of 5–60°, the spectral positions of

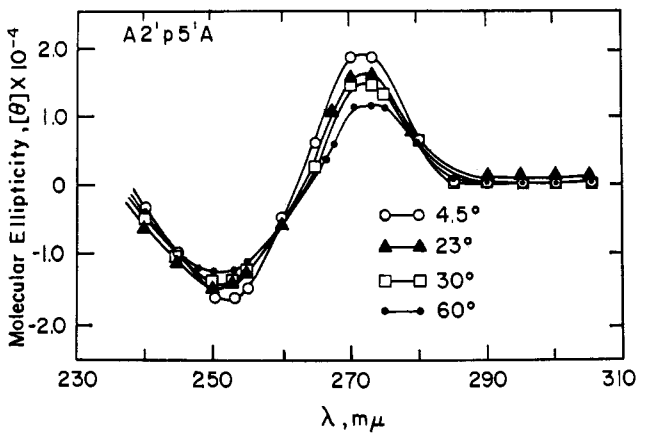

FIGURE 2: Circular dichroism of $A_{2'}p_{5'}A$ in 0.05 M $NaClO_4$ (pH 7.3) at the following four temperatures: 4.5 (○-○), 23 (▲-▲), 30 (□-□), and 60° (●-●).

TABLE VIII: Effect of Temperature and Solvent on the Coupling Constant, $J_{H\text{-}1'\text{-}H\text{-}2'}$, of Five Dinucleoside Monophosphates.[a]

Compounds	Temp (°C) and Solvent	$J_{H\text{-}1'\text{-}H\text{-}2'}$ (3′ or 2′ Residue) (cps)	$J_{H\text{-}1'\text{-}H\text{-}2'}$ (5′ Residue) (cps)
$A_{3'}p_{5'}A$	D_2O, 4	2.5	2.0
	D_2O, 30	3.2	3.5
	D_2O, 60	4.5	4.1
	Me_2SO, 30	6.6[b]	5.8[b]
$A_{2'}p_{5'}A$	D_2O, 4	4.5	3.0
	D_2O, 30	4.5	2.7
	D_2O, 60	5.2	4.0
	Me_2SO, 30	5.9	5.8
$A_{5'}p_{5'}A$	D_2O, 4		4.3
	D_2O, 30		4.6
	D_2O, 60		4.7
	Me_2SO, 30		5.7
$A_{3'}p_{5'}C$	D_2O, 4	2.4	*c*
	D_2O, 30	3.7	2.0
	D_2O, 60	4.5	3[c]
	Me_2SO, 30	>5.0[d]	*d*
$A_{2'}p_{5'}C$	D_2O, 4	6.7	<1
	D_2O, 30	6.6	<1
	D_2O, 60	6.6	<1
	Me_2SO, 30	5.4	*c*

[a] The accuracy of the *J* value is about ±0.2–0.3 cps. [b] The values cannot be measured accurately due to the overlapping of the two H-1′ protons. [c] The values cannot be measured accurately due to the overlapping with H-5′ of C. [d] The values cannot be measured accurately due to the overlapping between H-1′ of A and H-1′ of C.

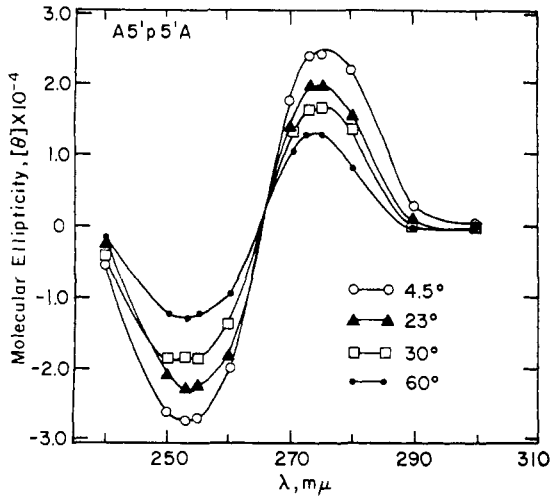

FIGURE 3: Circular dichroism of $A_{5'}p_{5'}A$ in 0.05 M $NaClO_4$ (pH 7.3) at the following four temperatures: 4.5 (O-O), 23 (▲-▲), 30 (□-□), and 60° (●-●).

the peak and trough of these three dimers remain about the same and the changes of the $[\theta]$ values for both the peak and the trough are all linearly dependent on the temperature. The linear temperature dependence of the $[\theta]_{peak}$ and $[\theta]_{trough}$ of $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, and $A_{5'}p_{5'}A$ is shown in Figures 4, 5, and 6, respectively. The temperature dependence of the $[\theta]$ values (both peak and trough) is the largest for $A_{3'}p_{5'}A$, less for $A_{5'}p_{5'}A$, and significantly less for $A_{2'}p_{5'}A$ (Figures 1, 2, and 3). Thus, at 60°, the $[\theta]_{peak}$ and the $[\theta]_{trough}$ values of the three dimers are similar, but at 4° the $[\theta]$ values of the $A_{3'}p_{5'}A$ and $A_{5'}p_{5'}A$ are definitely larger than those of the $A_{2'}p_{5'}A$. The temperature effect on the circular dichroism of $A_{3'}p_{5'}A$ shown in Figure 1 is comparable with those reported previously by Van Holde *et al.* (1965) over the same temperature range, while the temperature effect on the circular dichrosim of $A_{2'}p_{5'}A$ shown in Figure 2 is about twice as large as that observed by Brahms *et al.* (1967) in 4.7 M KF. Nevertheless, the present data do support the conclusion of Brahms *et al.* (1967) that the temperature

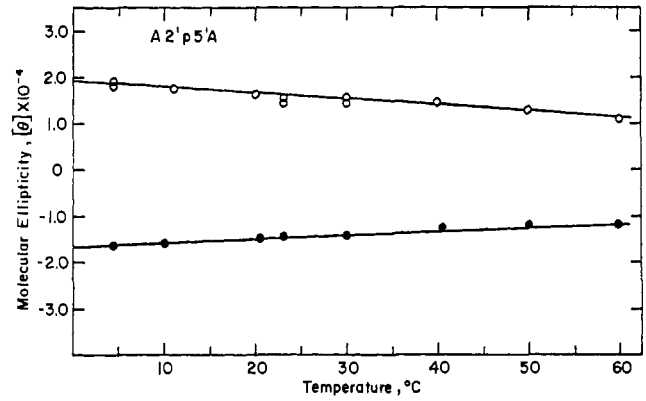

FIGURE 5: The temperature dependence of the circular dichroism ($[\theta]_{peak}$ (O-O) and $[\theta]_{trough}$ (●-●)) of $A_{2'}p_{5'}A$ in 0.05 M $NaClO_4$ at pH 7.3.

effect on the circular dichroism spectra of the 2′–5′ dimer is less than that of the 3′–5′ dimer.

The circular dichroism spectra of $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ in 0.05 M $NaClO_4$ (pH 7.3) at 4.5, 23, 30, and 60° are shown in Figures 7 and 8. The absolute $[\theta]$ values of the peak and the trough of these spectra are not equal: the absolute $[\theta]_{peak}$ value is 80–100% larger than the absolute $[\theta]_{trough}$ value. Within the 5–60° temperature range, the spectral positions of the peak and the trough remained the same, and the $[\theta]$ values changed linearly with respect to the temperature (Figures 9 and 10). The spectral positions of the peak (274 mμ) and the trough (238 mμ) of $A_{3'}p_{5'}C$ are significantly different from the peak position (278 mμ) and the trough position (254 mμ) of $A_{2'}p_{5'}C$. At 60°, the absolute $[\theta]$ values at the peak and the trough of $A_{2'}p_{5'}C$ are slightly larger than those of the $A_{3'}p_{5'}C$, while the reverse is true at 4.5°. The values of $[\theta]_{peak}$ and $[\theta]_{trough}$ of $A_{2'}p_{5'}C$ in 0.05 M salt at 4.5° (Figure 7) are approximately equivalent to those reported by Brahms *et al.* (1967) in 4.7 M KF at −20°. The temperature effects on the circular dichroism of both $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ in 0.05 M $NaClO_4$ in the present experiments appear to be about twice as large as those found by Brahms *et al.* (1967) in 4.7 M KF. Again the data indicate that the temperature

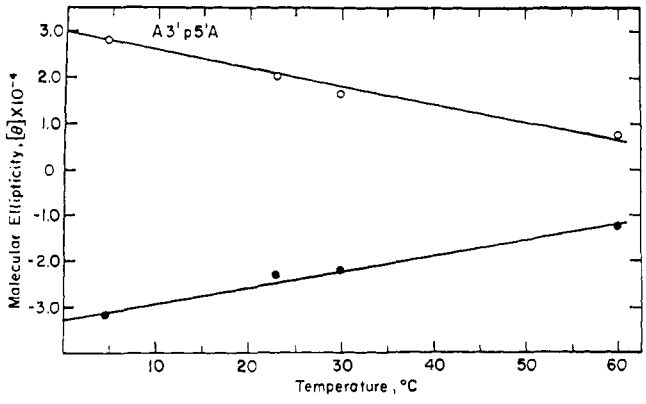

FIGURE 4: The temperature dependence of the circular dichroism ($[\theta]_{peak}$ (O-O) and $[\theta]_{trough}$ (●-●)) of $A_{3'}p_{5'}A$ in 0.05 M $NaClO_4$ at pH 7.3.

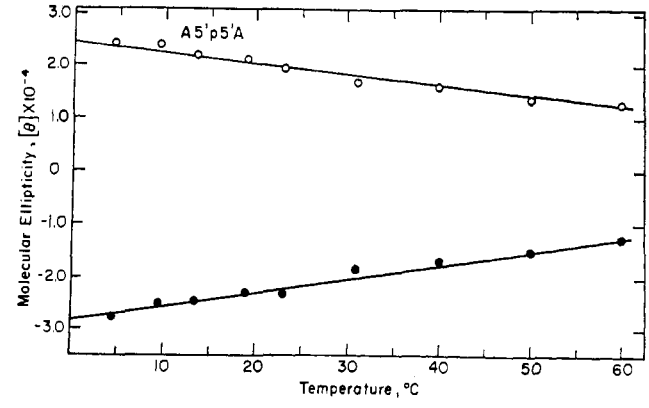

FIGURE 6: The temperature dependence of the circular dichroism ($[\theta]_{peak}$ (O-O) and $[\theta]_{trough}$ (●-●)) of $A_{5'}p_{5'}A$ in 0.05 M $NaClO_4$ at pH 7.3.

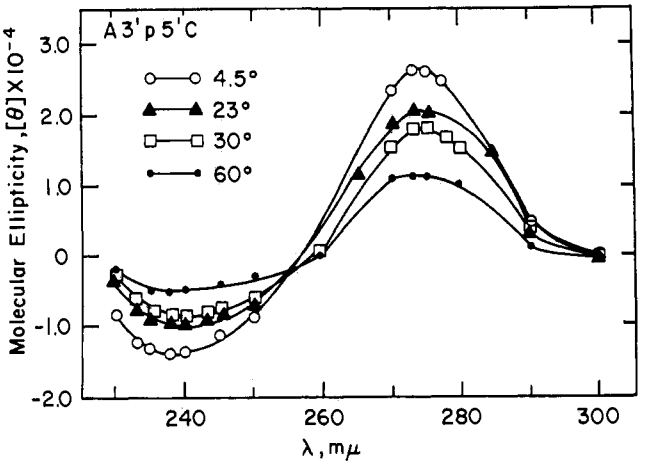

FIGURE 7: Circular dichroism of $A_{3'}p_{5'}C$ in 0.05 M $NaClO_4$ (pH 7.3) at the following four temperatures: 4.5 (O-O), 23 (▲-▲), 30 (□-□), and 60° (●-●).

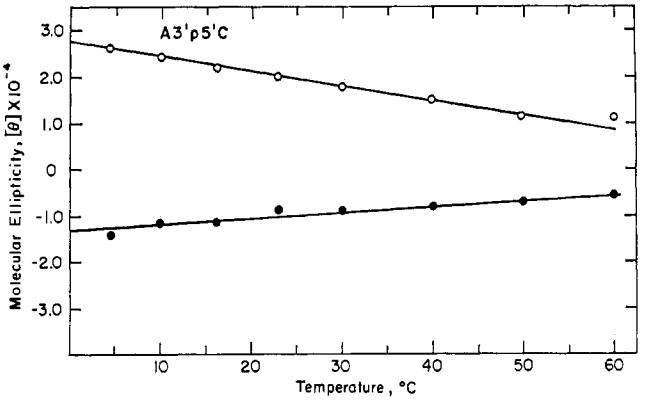

FIGURE 9: The temperature dependence of the circular dichroism ($[\theta]_{peak}$ (O-O) and $[\theta]_{trough}$ (●-●)) of $A_{3'}p_{5'}C$ in 0.05 M $NaClO_4$ at pH 7.3.

effect on the circular dichroism spectra of the 2′–5′ dimer is less than that of the 3′–5′ dimer (Brahms *et al.*, 1967).

The comparative studies discussed above suggest that the absolute $[\theta]$ values at low temperature (5–20°) as well as the temperature dependence of $[\theta]$ values from 5 to 60° for dimers dissolved in 4.7 M KF are less than those for dimers dissolved in dilute salt solutions (0.05 M, etc.). Apparently the high concentration of KF affects the conformation and the conformational changes of the dimers. Davis and Tinoco (1968) reported a 35% reduction in optical rotatory dispersion magnitude when the dimers are dissolved in 25% LiCl instead of dilute buffer.

These measurements on the optical activity provide valuable information about the conformation of these dimers, especially the *handedness* of the screw axis of the stack. As discussed in the above section on proton magnetic resonance data and in the first paper of this series (Ts'o *et al.*, 1969a), the screw axis of the stacks of the 3′–5′ and the 2′–5′ dimer are all predominantly right handed. One of the major supports for this conclusion is the agreement between the observed data and the calculated circular dichroism spectra based on a right-handed model (Warshaw *et al.*, 1965; Warshaw and Tinoco, 1965; Bush and Tinoco, 1967). Since the circular dichroism spectrum of the $A_{5'}p_{5'}A$ is very similar to those of $A_{3'}p_{5'}A$ and $A_{2'}p_{5'}A$, the screw axis of the stack of $A_{5'}p_{5'}A$ is likely to be right handed also (Figure 11). The same conclusion about the right-handed screw axis of $A_{5'}p_{5'}A$ has also been reached by the proton magnetic resonance studies presented above.

Ultraviolet Absorbance Studies. The molar extinction coefficients and the hypochromicity values with respect to the absorbance of the monomeric unit of the five dimers at room temperature (23–25°) have been determined carefully in this work and are reported in Table IX. The accuracy of the extinction coefficient values is definitely within ±2%. These values are in agreement with those previously reported in the literature by several laboratories (Table IX).

Comparison among the ultraviolet absorbance data for the three adenine homodimers clearly indicates that $A_{5'}p_{5'}A$ is the most hypochromic with respect to the absorbance of the monomeric 5′-AMP; $A_{2'}p_{5'}A$ is next and $A_{3'}p_{5'}A$ is the least hypochromic. If the degree of hypochromicity is employed as a measurement of the base–base interaction, then the results suggest that such an adenine–

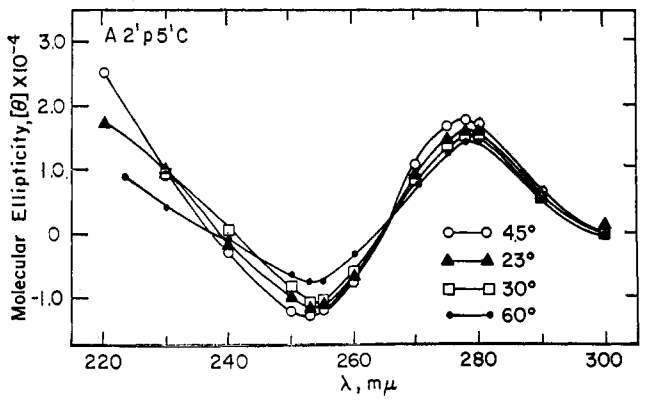

FIGURE 8: Circular dichroism of $A_{2'}p_{5'}C$ in 0.05 M $NaClO_4$ (pH 7.3) at the following four temperatures: 4.5 (O-O), 23 (△-△), 30 (□-□), and 60° (●-●).

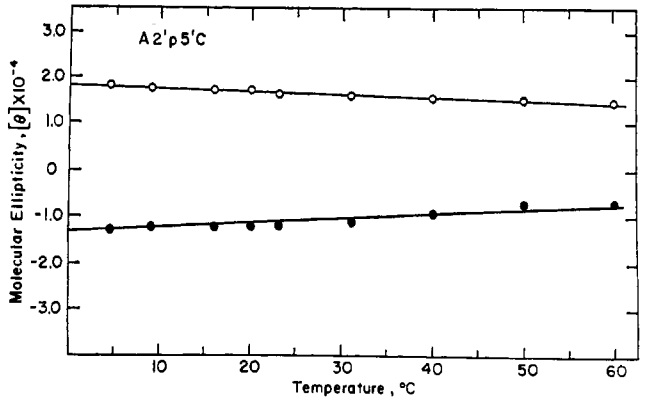

FIGURE 10: The temperature dependence of the circular dichroism ($[\theta]_{peak}$ (O-O) and $[\theta]_{trough}$ (●-●)) of $A_{2'}p_{5'}C$ in 0.05 M $NaClO_4$ at pH 7.3.

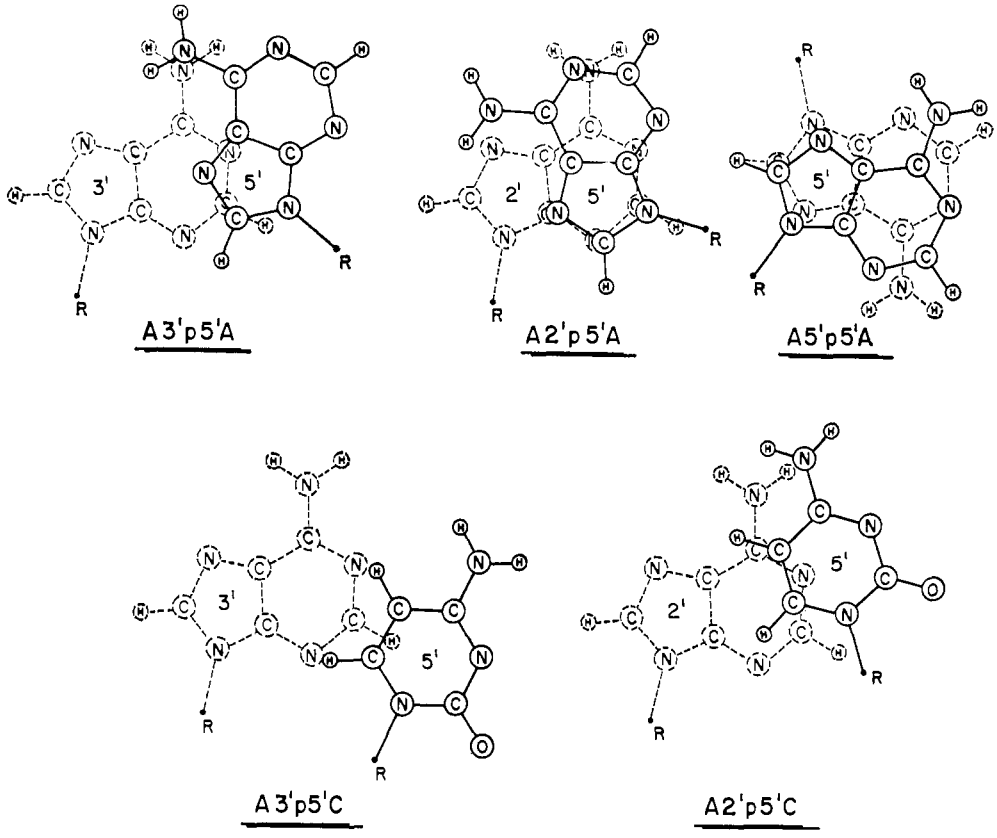

FIGURE 11: Schematic presentation of the front view of the conformational models for $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, $A_{5'}p_{5'}A$, $A_{3'}p_{5'}C$, and $A_{2'}p_{5'}C$. The dimensions of the bases are from X-ray studies and the base planes are parallel to each other (bases drawn by the dotted line are located at the bottom) and to the plane of the paper. The relative orientation between the two bases is positioned from the consideration of the proton magnetic resonance data at 4° and the constraint of the backbone as indicated by the CPK model. As shown, the nucleotidyl units have an *anti* conformation and the screw axis is right handed, *i.e.*, the axis is advancing upward from the plane of the paper and is rotating counterclockwise simultaneously.

adenine interaction is the most extensive in the 5′–5′ dimer, next in the 2′–5′ dimer, and the least in the 3′–5′ dimer. Similarly, the $A_{2'}p_{5'}C$ has a larger hypochromicity value than that of the $A_{3'}p_{5'}C$. Therefore, these data again suggest that the adenine–cytosine interaction is more extensive in the 2′–5′ dimer than in the 3′–5′ dimer.

The temperature dependence of the ultraviolet absorbance at λ_{max} over the 0–70° range has been studied for the five dimers. Within this temperature range of 70° the absorbance is linearly proportional to the temperature except for $A_{2'}p_{5'}C$. This constant relationship between the variation in absorbance *vs.* temperature change can be expressed by $\epsilon_{max(t)} = \epsilon_{max\ 25°}[1 + \theta_{t-25°}]$, where $\epsilon_{max\ 25°}$ is the maximal molar extinction coefficient of the individual dimer at 25° (Table IX), t is the temperature (°C) within the range of 0–70°, and the θ $(1/c^0)$ is the proportionality constant between the temperature change and the absorbance variation after normalization to the ϵ_{max} at 25°. The temperature effect on the density of water in this temperature range has been properly compensated for in these experiments. The value of θ is an indication of the extent of temperature effect on the absorbance. The θ values $(1/c^0)$ for the five dimers were found to be as follows: 8×10^{-4} for $A_{3'}p_{5'}A$; 10×10^{-4} for $A_{2'}p_{5'}A$; 11.7×10^{-4} for $A_{5'}p_{5'}A$; 10×10^{-4} for $A_{3'}p_{5'}C$; and about -1×10^{-4} to -2×10^{-4} for $A_{2'}p_{5'}C$. In other words, among the three adenine homodimers, the absorbance of the 2′–5′ dimer is slightly more sensitive to temperature change than that of the 3′–5′ dimer, but is slightly less sensitive than the 5′–5′ dimer. On the average, the absorbance increases by about 5% over an increase of temperature of 50°. For the adenine–cytosine dimer, the $A_{3'}p_{5'}C$ has a normal θ value of 10×10^{-4}, while the $A_{2'}p_{5'}C$ has an unusual θ value of -1×10^{-4} to -2×10^{-4}. After the proper correction of absorbance change due to the effect of temperature on the water density, the absorbance of $A_{2'}p_{5'}C$ appears to be slightly lower at 60° as compared with 0°, a change of about 1%. This change is very small and at present its experimental significance is not certain, even though essentially the same result was obtained in three trials. Nevertheless, it can be safely concluded that the temperature effect on the absorbance of $A_{2'}p_{5'}C$ is much less than that of $A_{3'}p_{5'}C$ or those of the three adenine homodimers.

Discussion

The main program of this research is to study by three physicochemical techniques (proton magnetic resonance, circular dichroism, and ultraviolet absorbance) the interaction of two identical bases (adenine) in dinucleoside

TABLE IX: Hypochromicity and Molar Extinction Coefficients of Adenine–Adenine and Adenine–Cytosine Dinucleoside Monophosphates at Neutral pH and Room Temperature.

Dimer	λ (mμ)	Hypochromicity (%)	ε (10^3)	Ref
$A_{3'}p_{5'}A$	*a*	11.7[b]	*a*	Michelson (1959)
	260	*a*	13.9	Warshaw and Tinoco (1965)
	257	*a*	13.8	Van Holde *et al.* (1965)
	257	*a*	13.6	Brahms *et al.* (1966)
	258	11.9	13.6[c]	This work[d]
$A_{2'}p_{5'}A$	*a*	15.5[b]	*a*	Michelson (1959)
	258.5	*a*	12.9	Brahms *et al.* (1967)
	258	15.9	12.9[c]	This work[d]
$A_{5'}p_{5'}A$	259	22.1	12.0	This work[d]
$A_{3'}p_{5'}C$	*a*	7.1[b]	*a*	Michelson (1959)
	261	*a*	10.5	Warshaw and Tinoco (1965)
	261	*a*	10.0	Brahms *et al.* (1967)
	260	6.8	10.6	This work[d]
$A_{2'}p_{5'}C$	262	*a*	10.0	Brahms *et al.* (1967)
	260	11	10.1	This work[d]

[a] Not given in the reference. [b] Given as hyperchromicity (hyper) and is converted into hypochromicity (hypo) by the equation: hypo (%) = hyper (%)/(100 + hyper (%)). [c] Calculated from the hypochromicity and from the extinction coefficient of AMP to be 15.4×10^{-3} M at 258 mμ. [d] See Experimental Section for the method of determination.

monophosphates joined at different positions by a phosphodiester linkage (2′–5′, 3′–5′, and 5′–5′). Similarly, the interactions of adenine and cytosine in $A_{2'}p_{5'}C$ and $A_{3'}p_{5'}C$ were also investigated by these three physicochemical methods. The main purpose of this study is (1) to examine the influence of the phosphodiester linkage on the base–base interaction in the dimer; (2) to compare the results obtained by these three different physicochemical techniques (proton magnetic resonance, circular dichroism, and ultraviolet absorbance) on these different dimers joined at various positions by phosphodiester linkages. The study has been carried out from 5 to 60° so that the temperature-dependent properties of these dimers can be compared, and the proton magnetic resonance spectra have also been measured in dimethyl sulfoxide-d_6 as well as in D_2O.

The parameter for the measurement of base–base interaction in a dimer by the proton magnetic resonance method is the "dimerization shifts ($\Delta\delta_D{}^{t°}$)" (Table III and Table IV). This term is defined as the difference between the chemical shift values of the 5′-nucleotidyl unit or the 2′- or 3′-nucleotidyl unit in the dimer and the chemical shift values of the corresponding 5′-mononucleotides or 2′- or 3′-mononucleotides. ($\Delta\delta_D{}^{t°} = \delta_{Np}{}^{t} - \delta_{NpN'}{}^{t}$; or $= \delta_{pN'}{}^{t} - \delta_{NpN'}{}^{t}$). Most of the $\Delta\delta_D{}^{t°}$ originates from the nonbonded interaction between the two neighboring units in the dimer as indicated by the reduction of the $\Delta\delta_D{}^{30°}$ values to a very low level (Table VI and Table VII) when the dimers are dissolved in dimethyl sulfoxide-d_6. The residual $\Delta\delta_D$ values are reduced further to practically zero when the $A_{3'}p_{5'}A$ dissolved in dimethyl sulfoxide-d_6 was measured above 60° (Ts'o *et al.*, 1969a). From this study and our previous work (Helmkamp and Ts'o, 1961), it was concluded that "under this condition (dimethyl sulfoxide-d_6, above 60°), as far as the method of proton magnetic resonance is concerned, the individual nucleotidyl unit in ApA becomes totally free from the influence of the neighboring unit in the dimer (Ts'o *et al.*, 1969a). There is one special case where a contribution to the dimerization shift is made indirectly by the formation of the phosphodiester linkage. The deshielding of the H-8 proton of the 5′-nucleotidyl unit (or the H-6 proton of the 5′-pyrimidine nucleotidyl unit) by the 5′ phosphate (Schweizer *et al.*, 1968) is reduced by the conversion of the phosphomonoester to the phosphodiester in the formation of the dimer from the 5′-mononucleotides (Ts'o *et al.*, 1969a). Other than this interesting exception, all the contribution to the dimerization shift is apparently from the nonbonded interaction. This is because the phosphate group exerts little direct influence on the chemical shift of the base and H-1′ protons in a nucleotide other than the deshielding effect of the 5′-phosphate group to the H-8 of the purinyl unit or H-6 of the pyrimidinyl unit (Schweizer *et al.*, 1968). Therefore the conversion of a phosphomonoester into a phosphodiester induces no direct effect on the chemical shifts due to the formation of this bond. Most of the dimerization shift apparently originates from the shielding effect of the ring-current magnetic anisotropy of the neighboring base as indicated by a series of extensive research on the effect of concentration of the chemical shifts of the monomers (Chan *et al.*, 1964; Schweizer *et al.*, 1964, 1965; Broom *et al.*, 1967; Helmkamp and Kondo, 1968).

The parameter for the measurement of base–base interaction in a dimer by the circular dichroism method is the

amplitude in mean residue ellipticity, $[\theta]$, between the peak and the trough of the circular dichroism curve related to the optical transition(s) above 230 mμ. Both experimental results (Van Holde *et al.*, 1965; Brahms *et al.*, 1966, 1967; and others) and theoretical consideration (Bush and Tinoco, 1967) have indicated that the contribution from the interaction between the residues in the dimer are much larger than the inherent contribution from the monomeric unit. In the present experiment, the inherent contribution from the monomeric unit can be totally ignored since all the dimers being compared have the same monomeric units. Two points in this approach should be cautioned. First, when the spectral positions of the peak and the trough are not the same for the two dimers under consideration, then the amplitude values between the peak and the trough should not be used in a simple comparison between these two dimers. While these amplitude values are still related to the base–base interaction, different types of interaction may actually be involved. Second, there are other optical transition(s) below 230 mμ which are informative and should be considered. Experimentally, these transitions are more difficult to measure, especially in experiments with variable temperatures. For the circular dichroism measurements, amplitude in ellipticity is a better parameter for measurement of base–base interaction than the rotational strength of a given peak or trough, since the rotational strengths of the circular dichroism bands of the dimer are derived from the splitting of the energy level of the transition and therefore the summation of the rotational strengths of the dimer bands should be equal to zero.

The parameter for the measurement of base–base interaction in a dimer by the ultraviolet absorbance method in the present experiments is the hypochromicity, *i.e.*, the difference in molar (or residue) extinction coefficient of the constitutive monomer(s) and the dimer at a given wavelength (at or very near the maximum). From the theoretical point of view, hypochromism (fractional decrease in integrated intensity) is more appropriate than hypochromicity (or point hypochromism) as a parameter. However, the difference in absorbance between monomer and dimer is relatively small (Table IX). Measurement of hypochromism involves the separation of the overlapping absorption bands, a step which can introduce additional error in calculation, especially for such a complex spectrum as that of ApC. Therefore, values of hypochromicity which are measured directly from experiments are used instead. It has been shown for the purpose of comparison that the results from hypochromism are the same as those from hypochromicity (Cantor and Tinoco, 1965; Davis and Tinoco, 1968).

As discussed in the Results section, the general conformational models for these five dimers have been determined by the proton magnetic resonance and circular dichroism methods. The results indicate that $A_{3'}p_{5'}A$, $A_{2'}p_{5'}A$, $A_{3'}p_{5'}C$, and $A_{2'}p_{5'}C$ all have an *anti,anti*, right-handed, asymmetrical conformation while $A_{5'}P_{5'}A$ has an *anti,anti*, right-handed, symmetrical conformation. As far as the comparison of the base–base interaction among the three ApA(s) and between the two ApC(s) is concerned, a rather complex situation is found. As indicated by the proton magnetic resonance studies on six protons in $A_{3'}p_{5'}A$ and $A_{2'}p_{5'}A$ (Table IV), two protons (H-1′ in $A_{3'}$p- and H-2 in -$p_{5'}$A) have larger $\Delta\delta_D^t$ values in $A_{3'}p_{5'}A$, four protons (H-8 in $A_{2'}$p-, H-2 in $A_{2'}$p-, H-8 in -$p_{5'}$A, and H-1′ in -$p_{5'}$A) have larger δ_D^t values in $A_{2'}p_{5'}A$. Comparing the three protons in the (-p_5′A) unit among the three ApA(s), the following series can be formulated in order of the decreasing values of $\Delta\delta_D^t$: for the H-8 and H-1′ protons, $A_{2'}p_{5'}A > A_{5'}p_{5'}A > A_{3'}p_{5'}A$; for the H-2 proton, $A_{5'}p_{5'}A > A_{3'}p_{5'}A > A_{2'}p_{5'}A$. As for the comparison of the six protons in $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ (Table IV), one proton (H-1′ in $A_{3'}$p-) has a larger $\Delta\delta_D^t$ value in $A_{3'}p_{5'}C$, three protons (H-2 in $A_{2'}$p-, H-6 in -$p_{5'}$C, and H-1′ in -$p_{5'}$C) have larger $\Delta\delta_D^t$ values in $A_{2'}p_{5'}C$, and two protons (H-8 in $A_{3'}$p- or $A_{2'}$p- and H-5 in -$p_{5'}$C) have about the same $\Delta\delta_D^t$ values in these two isomeric dimers. The temperature dependence of the $\Delta\delta_D^t$ values observed is different for each dimer. Generally speaking, the effect of temperature on the $\Delta\delta_D$ value is larger for the $A_{3'}p_{5'}A$ than for the other two ApA(s), and is larger for the $A_{3'}p_{5'}C$ than the $A_{2'}p_{5'}C$. As for the circular dichroism measurements among the three ApA(s), the amplitude between the peak (~273 mμ) and the trough (~253 mμ) at 23 and 5° is largest for the $A_{3'}p_{5'}A$, next for the $A_{5'}p_{5'}A$, and smallest for the $A_{2'}p_{5'}A$ (Figures 1–6). However, the effect of temperature on the amplitude is also the most for the $A_{3'}p_{5'}A$, next for the $A_{5'}p_{5'}A$, and the least for the $A_{2'}p_{5'}A$. The circular dichroism measurements on the $A_{3'}p_{5'}C$ and $A_{2'}p_{5'}C$ show that the amplitude and the temperature dependence are larger for the $A_{3'}p_{5'}C$ than for the $A_{2'}p_{5'}C$ (Figures 7–10). However, the peak and trough positions are quite different in the circular dichroism spectra of these two isomeric dimers. Therefore, these amplitude values should not be compared in a simple manner. As for the hypochromicity obtained from the ultraviolet absorbance measurements, $A_{5'}p_{5'}A$ has the highest value (~22%), $A_{2'}p_{5'}A$ has the next highest value (~16%), and the $A_{3'}p_{5'}A$ has the lowest value (~12%), among the three isomeric ApA(s). The effect of temperature on the maximal extinction coefficient (therefore on the hypochromicity as well) is also the highest for $A_{5'}p_{5'}A$, next for $A_{2'}p_{5'}A$, and lowest for $A_{3'}p_{5'}A$. $A_{2'}p_{5'}C$ was found to have a higher hypochromicity than $A_{3'}p_{5'}C$; however, the effect of temperature on the maximal extinction coefficient is higher for $A_{3'}p_{5'}C$ than for $A_{2'}p_{5'}C$.

Three general conclusions emerge from this complex picture about the base–base interactions in these five dimers as studied by the three physicochemical methods. First, while these five dimers possess certain basic features in common in their conformations (such as *anti* conformation for the nucleosidyl units and right-handed turn for the screw axis of the stack), the geometrical relationship between the two constitutive units as well as the temperature effect on this geometrical relationship is quite different for each dimer when examined in sufficient detail. Second, each physicochemical method measures a different type of base–base interaction, and the dependence of each measurement on the geometrical relationship between the two constitutive units is different. Third, therefore, a direct comparison of the base–base interaction (or commonly expressed as base stacking) among various dimers based on numerical values derived from a single physicochemical method is not necessarily meaningful and can be quite misleading. For example, from circular dichroism measurement at or below room temperature, the order of base–base interaction can be arranged as follows: $A_3p_{5'}A > A_5p_{'5'}A > A_{2'}p_{5'}A$;

and $A_{3'}p_{5'}C > A_{2'}p_{5'}C$. However, from the ultraviolet absorbance measurement, the order is the following: $A_{5'}p_{5'}A > A_{2'}p_{5'}A > A_{3'}p_{5'}A$; $A_{2'}p_{5'}C > A_{3'}p_{5'}C$. Similarly, from the $\Delta\delta_D^t$ values of the H-8 proton of ($-p_{5'}A$), the order is $A_{2'}p_{5'}A > A_{5'}p_{5'}A > A_{3'}p_{5'}A$, while from the $\Delta\delta_D^t$ values of the H-2 proton of ($-p_{5'}A$), the order is $A_{5'}p_{5'}A > A_{3'}p_{5'}A > A_{2'}p_{5'}A$. Davis and Tinoco (1968) have reported previously that different $\Delta H°$ (unstacking) values were obtained by optical rotation, circular dichroism, and hypochromism (or hypochromicity) measurements. They felt that the two-state model is "too simple" for the study on dimers and proposed an oscillating dimer model. The three general conclusions stated here may appear to be merely common sense; nevertheless, a rigorous establishment of these conclusions based on experimental facts provides us with the necessary caution and perspective. It appears that, in order for us to understand and to harmonize these physicochemical measurements properly, we should correlate these data in the framework of three-dimensional conformational models for these dimers. The initial attempt here is likely to be crude and inadequate, but it will serve as a reference point for future development. For the discussion of the model we shall further assume that the base planes are always parallel to each other in the dimers, and that the vertical distance between the base planes is about the same for all the dimers and is kept to a minimum (probably about 3 Å). In examining the CPK models of these dimers, the base–base vertical distance of the $A_{3'}p_{5'}A$ can be slightly longer than those of $A_{2'}p_{5'}A$ and $A_{5'}p_{5'}A$ because of the possible interference of the H-2′ proton from the ($A_{3'}p$-) unit. (Can this be the reason why the dihedral angle between the H-1′ and H-2′ protons is reduced in stacking so that the H-2′ is out of the way?) At this juncture, we shall temporarily ignore the stereochemistry of various rotatable bonds in the dimers (Sasisekharan *et al.*, 1967; Sundaralingam, 1969; Lakshminarayanan and Sasisekharen, 1969), even though these are the most important factors to be considered in the future for a more precise model.

In the consideration of the relationship of proton magnetic resonance data to the geometry of the dimer conformation, we shall assume that the values of $\Delta\delta_D^t$, the parameter for the measurement of base–base interaction, are originated entirely from the influence of diamagnetic anisotropy of the neighboring bases. Johnson and Bovey (1958), following the approach of Waugh and Fessenden (1957), have applied the ring-current concept to account quantitatively for the additional shielding observed for the aromatic protons (such as those in benzene) over the closely related olefinic protons (such as those in 1,3-cyclohexadiene). In this approach, the electrons are assumed to circulate in two loops of radius a (radius of the benzene ring) separated by $+P$ and $-P$ above and below from the plane of the carbon. Satisfactory agreement between the calculation and the observed data were obtained. Most recently Giessner-Prettre and Pullman (1970) have adopted this approach to the heterocyclic pyrimidines and purines. The ring-current intensities of these bases have been calculated previously (Giessner-Prettre and Pullman, 1965, 1969) and are normalized to that of benzene as unity. The computed effects of ring current on the chemical shifts in the δ scale are normalized by an empirical constant factor to obtain the 1.5-ppm value observed in the benzene–olefin comparison. Their equation (Giessner-Prettre and Pullman, 1970) is

$$\Delta\delta = C\sum_{N}\frac{I_R}{a_R}\left[\frac{2}{[(1+\rho)^2+z_-^2]^{1/2}}\left(K+\frac{1-\rho^2-z_-^2}{(1-\rho)^2+z_-^2}E\right)+\frac{2}{[(1+\rho)^2+z_+^2]^{1/2}}\left(K+\frac{1-\rho^2-z_+^2}{(1-\rho)^2+z_+^2}E\right)\right] \quad (1)$$

where N is the number of the rings of the molecule; I_R is the ring-current intensity of the ring R expressed as a fraction of the benzene ring current; a_R is the radius of the ring; ρ and $z_\pm$ are the cylindrical coordinates of the point under investigation measured from the center of the ring in ring radius unit; K and E are complete elliptic integrals of first and second kind; and $z_\pm$ means $z + \langle\bar{z}\rangle$ and $z - \langle\bar{z}\rangle$, where $2\langle\bar{z}\rangle$ is the distance separating the two loops. From this equation, the shielding zones of purines and pyrimidines (including adenine and cytosine) have been calculated in a plane 3.4 Å distant from the molecular surface (or z = 3.4 Å). The shielding zones for the adenine are elliptical in shape following the general envelope of the base, while the zones for cytosine are circular. Because of the complexity of eq 1, it is difficult to discuss the geometrical dependence of $\Delta\delta_D^t$ on ρ and z (distances in cylindrical coordinates from the base) in an analytical manner as we have done with the equations on the optical properties described below. The conformational models of the five dimers depicted in Figure 11 are constructed in accordance with the data of $\Delta\delta_D$[4°] (Tables III and IV) with the shielding zones from eq 1 (Giessner-Prettre and Pullman, 1970), and with the aid of the CPK models, especially in regard to the constraint of the ribosyl phosphodiester backbone and the position of the H-1′ protons. The base planes are parallel to each other with a vertical distance of 3.4 Å and the dimensions of the bases are from the X-ray results (Steiner and Beers, 1961). The experimental $\Delta\delta_D^t$ values agree rather well with the computed shielding values from this geometry and from the zones to about ±0.05 ppm in most cases. The uncertainty with H-1′ protons is probably larger since they are out of the base planes and have slightly different distances (*i.e.*, z values are different). In terms of the angle between the principal axes of the bases in the stack, a variation of ±15° from the present model is likely to be acceptable from the proton magnetic resonance data. In addition, it should be remembered that these models represent only the statistical average of a dynamic situation. The shielding zones of adenine and cytosine at z = 2-, 3-, 4-, and 5-Å distance from the plane have been recently calculated (B. Pullman and C. Giessner-Prettre, unpublished data). Examination of these calculations indicates that models cannot be built to correlate the observed data with the patterns computed at z = 2 and 5 Å. This study suggests that patterns of shielding zones, computed at a distance of 3–4 Å, are most suitable for model construction. It appears, therefore, that the choice of 3.4 Å as the distance between the two base planes in the dimer is very reasonable. It should be noted that this 3.4-Å distance used for the models of dimers (Figure 11) is the same as the distance between the two base planes in the DNA helix determined from X-ray diffraction (Langridge *et al.*, 1960). At this juncture, only the proton magnetic resonance data obtained at low temperature (4°) are used. While the data at higher temperature can be explained by the move-

ment of the bases away from each other through rotation of bonds in the phosphodiester linkage, our present knowledge does not warrant a description of this movement in detail at this time. It suffices to state that the observed temperature effects on $\Delta\delta_D$ (Tables III and IV) are in qualitative agreement with the proposed models (Figure 11) and with the computed shielding zones (Geissner-Prettre and Pullman, 1970). When the temperature dependence of the dimerization shifts is normalized in terms of per cent change, the magnitude of the temperature effect varies considerably with the location of the proton within the dimer, especially for ApA. These results are not in agreement with the interpretation by a two-state model since this model would predict an equal temperature dependence (normalized as per cent change) for all protons within a given dimer. The differences among these models in Figure 11 also illustrate the large influence of the phosphodiester linkage on the conformation and interaction of the dimers.

For the analyses of the optical properties in relationship to conformational model, we are especially interested in reconciling the circular dichroism data and the hypochromicity data from ultraviolet absorbance. Fortunately, Glaubiger *et al.* (1968) have already made a very helpful contribution in this direction, and we shall adopt their equations here. In their model, the two identical base planes of the homodimers are also assumed to be parallel to each other. As the first approximation, the relative motion between these base planes in their model is restricted to rotation without stretching or lateral displacement. The optical property is analyzed on the basis that each chromophore has an isolated, optically inactive, electronically allowed transition at frequency ν_a. According to their analysis, the circular dichroism (the original derivation was for optical rotatory dispersion, but it is the same for circular dichroism) is proportional to the $V_{12}R_{0a}$, where V_{12} is the interaction energy term arisen from the splitting of the degenerate transition into two energy levels which have the rotational strength, R_{0a}, of equal magnitude but opposite sign. In the dipole approximation, V_{12} and R_{0a} can be written as follows (Glaubiger *et al.*, 1968)

$$V_{12} = \frac{1}{R_{12}^3}\left[\mu_{10a}\cdot\mu_{20a} - 3\frac{(R_{12}\cdot\mu_{10a})(R_{12}\cdot\mu_{20a})}{R_{12}^2}\right] \quad (2)$$

$$R_{0a} = \frac{\pi\nu_{0a}}{2c} R_{12}\cdot(\mu_{10a}\times\mu_{20a}) \quad (3)$$

where R_{12} is the distance from the transition dipole in base 1 to the transition dipole in base 2 of the dimer, and μ_{10a} and μ_{20a} are the transition dipole moment in the planes of base 1 and base 2, respectively. Since these two planes are always parallel, R_{12} is perpendicular to both μ_{10a} and μ_{20a}. Under this condition, the second term in V_{12} vanishes and one obtains

$$[\theta]_{CD} \propto R_{0a}V_{12} \propto \frac{\mu_{10a}^2\mu_{20a}^2}{R_{12}^2}\sin\theta\cos\theta \quad (4)$$

where θ (not $[\theta]_{CD}$) is the angle between μ_{10a} and μ_{20a}. From expressions 3 and 4, we obtain the following results in regard to the dependence of circular dichroism measurement on the geometrical relationship of two bases in the dimer. (1) The circular dichroism value is very sensitive to the angle θ between the two transition dipoles. It becomes zero when θ is at 0, 90, and 180° and reaches a maximum when θ is near 45°. The circular dichroism value is dependent on the sign of θ; therefore it is dependent on the handedness of the screw axis. A detailed calculation on the dependence of optical activity of $A_{3'}p_{5'}A$ on the angle θ has been reported (Bush and Tinoco, 1967). (2) The circular dichroism value is dependent on the R_{12}^{-2}. Although eq 4 was developed for a model without relative lateral displacement, the circular dichroism value is affected by the lateral displacement between the bases when allowed, only to the same extent that this displacement affects R_{12}^{-2}. This is because the lateral component of the interaction terms $(R_{\perp}\cdot(\mu_{10a}\times\mu_{20a}))$ becomes zero as substituted into expression 3.

In the analysis of hypochromism, Glaubiger *et al.* (1968) had made the following additional simplification: the effect of permanent dipoles is negligible, and the symmetry of the polarizability term $(\alpha(\nu_a))$ is that of an elliptical cylinder with its axis perpendicular to the plane of base. From this approximation, the following expression for hypochromism, H, was derived[2]

$$H(\theta) = \frac{2}{R_{12}^3}\left[\alpha_1\cos^2(\theta+\phi) + \alpha_2\sin^2(\theta+\phi)\right] \quad (5)$$

where α_1 and α_2 are the polarizabilities at frequency, ν_a, along the principal axes e_1 and e_2 which lie in the plane of the base, and ϕ is the angle between μ_{20a} and e_1. From eq 5 and other considerations, we obtain the following results in regard to the dependence of hypochromicity on the geometrical relationship of two bases in the dimer. (1) If the base (such as adenine) is isotropic with respect to the polarizabilities (*i.e.*, $\alpha_1 = \alpha_2$), then eq 5 is reduced to

$$H(\theta) = \frac{2}{R_{12}^3}[\alpha] \quad (6)$$

Under this condition, the hypochromism is not sensitive to θ and therefore not sensitive to rotation of the base planes relative to each other. Recently, Takashima (1969) has calculated the polarizability of adenine along two axes (α_x and α_y) by both Hückel and SCF methods. The results indicated that the ratio of $\alpha_x:\alpha_y$ is less than 1.4. Though the calculation of Takashima is for the ground-state polarizabilities and the terms in eq 5 are the polarizabilities at excited state (at V_a), the ratio of $\alpha_y:\alpha_x$ (at V_a) is unlikely to be much larger than the ratio at ground state. This consideration suggests that for certain bases at least hypochromism is rather insensitive to the value of θ. In addition, since the angle term (θ) in eq 5 is a square term, the sign of the angle (θ) will not be important. Hypochromicity will be the same regardless of whether the screw axis of the dimer is right handed or left handed. (2) Hypochromism is dependent on the R_{12}^{-3}. (3) In the dimer model of Glaubiger *et al.* (1969), no lateral movement of the two base planes relative to each other is allowed. The CPK models of these dimers

[2] The bracket in $[\alpha_1\cos^2(\theta+\phi) + \alpha_2\sin^2(\theta+\phi)]$ has been left out inadvertently from eq 10 in the paper of Glaubiger *et al.* (1968).

clearly show that lateral movement must be involved upon rotation of the phosphodiester bonds. Therefore, we shall consider the term $G_{ia,jb}$ used by DeVoe and Tinoco (1962, eq 5) instead of the T_{12} term used by Glaubiger *et al.* (1968, eq 9), where $G_{ia,jb}$ (or T_{12}) represents the spatial relationship for the interaction term $V_{ia,jb}$ (or $V_{ia,2b}$). For our present parallel model, $G_{ia,jb}$ can be simplified to

$$\left(\frac{1 - 3\cos^2 a}{R_{ij}^3}\right)$$

where a is the angle between R_{ij} (the distance between the center of two bases) and transition moment μ_{ia}, and H is directly proportional to this term as

$$H \propto \frac{1}{R_{ij}^3}(1 - 3\cos^2 a) \qquad (7)$$

According to eq 7, when the bases are directly on top of each other (*i.e.*, $a = 90°$, $\cos a = 0$), then the H value is the highest; when a is near 55° ($3\cos^2 55° \approx 1$), H is near zero, and when a is from 50 to 0° (at 0° the bases are lying horizontally side by side), H will have a negative value (or the interaction leads to hyperchromism instead). Therefore, it is clear that hypochromism is sensitive to lateral movement of the two bases relative to each other. In summarizing the eq 3–7, which describe the dependence of circular dichroism and H (hypochromism in ultraviolet absorbance) measurements on the geometrical relationship between two parallel bases in a homodimer (especially adenine), we can make the following comparison. (1) In terms of rotation or angle θ, circular dichroism is very sensitive to both the sign and the magnitude of this parameter, while H is not. (2) In terms of the distance or R_{12}, circular dichroism is dependent on $|R_{12}|^{-2}$ and H is dependent on $|R_{12}|^{-3}$. (3) In terms of the lateral movement or cos a, then circular dichroism is not sensitive to this term other than the effect on $|R_{12}|^{-2}$ while H is dependent on $1 - 3\cos^2 a$ in addition to the effect on $|R_{12}|^{-3}$. The overall evaluation is that the circular dichroism value is more dependent on the angle between the bases, and the H value is more dependent on the distance between the two bases. In utilizing these relationships, the assumptions and the approximations should be clearly kept in mind. For instance, this analysis is for the in-plane π–π^* transition and not for out-of-plane n–π^* transition, etc. These approaches are also limited by the inherent problems in the dipole approximation. Recently, circular dichroism spectra of nucleic acids have been calculated on the bases of monopole–monopole interactions (Johnson and Tinoco, 1969).

We shall now attempt to correlate the optical data (Table IX and Figures 1–9) and the relationships between the optical measurements and the geometry of the conformation (eq 4–7) to the conformational models established by the proton magnetic resonance studies (Figure 11). From the extent of the overlap and the proximity of the two bases of the five dimers shown in these models, the following order can be established: $A_{5'}p_{5'}A > A_{2'}p_{5'}A > A_{3'}p_{5'}A$; $A_{2'}p_{5'}C > A_{3'}p_{5'}C$. This order agrees with the order of the hypochromicity data (Table IX). This agreement is supported by the conclusion on the relationship of H with the geometry developed for the homodimer model that H values are strongly dependent on the distance between the bases. The correlation of these proton magnetic resonance models with the amplitudes obtained in the circular dichroism data is less certain, however. In the models for $A_{3'}p_{5'}A$ and $A_{2'}p_{5'}A$ (Figure 11), the bases have the same direction in their orientation, *i.e.*, the 6-NH_2 groups are pointing toward the same direction. Therefore, the angles between the geometrical principal axes of the bases are the same as the angle θ between the transition moments. In the present model, the θ for $A_{3'}p_{5'}A$ is about 60° and the θ for $A_{2'}p_{5'}A$ is about 80°. Since sin 60° cos 60° value is larger than sin 80° cos 80° value, therefore the circular dichroism amplitude arising from the base–base interaction (eq 5) of $A_{3'}p_{5'}A$ is larger than that for $A_{2'}p_{5'}A$ as observed. It should be noted, because of the uncertainty of the model proposed (*i.e.*, ±15°), the transition θ can be in the second quadrant in which the sin θ cos θ value is negative. This situation would require the circular dichroism of $A_{2'}p_{5'}A$ to be opposite in sign to that of the $A_{3'}p_{5'}A$ which is not observed, or conversely to have the handedness (left) of $A_{2'}p_{5'}A$ opposite the handedness (right) of the $A_{3'}p_{5'}A$. The present data on the H-1′ protons indicate that the predominant conformation is right handed (see Results). Therefore, the angle θ should remain in the first quadrant. Nevertheless, this discussion illustrates the uncertainty in the assignment of the handedness of the stack purely from the circular dichroism consideration on the geometry when the angle θ between the transition moments is close to $\pi/2$ or π. This is because the circular dichroism curve from the θ (90° + x) in the second quadrant for a left-handed stack is the same as that from θ (90° − x) in the first quadrant for a right-handed stack. For the $A_{5'}p_{5'}A$, the bases have opposite direction in their orientation, *i.e.*, the 6-NH_2 groups are pointing toward opposite directions. Under this situation, the angle θ cannot be obtained from the angle of the principal axes of the two bases and has to be obtained directly from the intersection of the two transition moments. Experimentally (Stewart and Davidson, 1963; Stewart and Jensen, 1964), the major component of the first long-wavelength transition of 9-methyladenine was found to be polarized along an axis 3° (toward N-7) with respect to the C-4–C-5 line (transverse axis). Adaptation of this polarization axis for the transition moment in the adenosine unit in the present conformational model for $A_{5'}p_{5'}A$ (Figure 11) will give a rather small value of θ. From this consideration, the circular dichroism amplitude of the $A_{5'}p_{5'}A$ should be smaller than that of $A_{3'}p_{5'}A$, in agreement with the experimental findings (Figures 1 and 3). Since both $A_{2'}p_{5'}A$ and $A_{5'}p_{5'}A$ have a θ value close to $\pi/2$ or π, the difference between θ and π (or $\pi/2$) probably is smaller than the variation range in the conformational model based on proton magnetic resonance data, and it would not be meaningful to compare the circular dichroism amplitudes of these two isomeric ApA(s). However, it would be instructional to ask whether the circular dichroism of the $A_{5'}p_{5'}A$, is expected to have the same sign as the other two isomers observed, in view of the fact that the two adenines have an opposite direction in this model. In this case, the vector direction of the transition moment has to be assigned in a constant manner to the adenines in all three ApA(s). From this operation, it was found that a small variation (±10%) in the vector direction of the transition moments and a small variation (±15°) of the angle of the intersection of the principal axes of the

two neighboring adenines will change the θ from one quadrant to the next (such as from quadrant II to quadrant III in the case of $A_{5'}p_{5'}A$). The change of the θ from one quadrant to the other will change the sign of the circular dichroism curve and consequently the argument for the handedness of the stack as well. Thus, analyses on the present conformational model of $A_{5'}p_{5'}A$ based on the existing circular dichroism theory and knowledge presented above agree with the observed results that the circular dichroism magnitude shall be relatively small. However, in a manner analogous to the situation of $A_{2'}p_{5'}A$, the analysis does not provide definitive information about the sign of the circular dichroism curve, consequently the handedness of the stack. As for the ApC dimers, from the transition moments of adenine and cytosine recently calculated (Johnson and Tinoco, 1969; Berthod *et al.*, 1966), it is also possible that the θ is closer to 0° in $A_{2'}p_{5'}C$ than in $A_{3'}p_{5'}C$. Nevertheless, since ApC is a heterodimer and the spectra of the two isomers have different positions for the peak and the trough, a comparison of the amplitudes between the circular dichroism curves of these two isomers may not yield meaningful results. In general, we may conclude that the conformational models for these five dimers derived from the proton magnetic resonance studies are in agreement with the results from the hypochromicity data and are not in apparent disagreement with the results from the circular dichroism studies. Again at this juncture, we wish to defer a discussion of the temperature effect on the optical properties in relation to the temperature-induced change of these conformational models.

There are two additional problems concerning the circular dichroism studies. The first one is the possible existence in the population of some dimer molecules which have a left-handed turn for their screw axis in the stack, even though the circular dichroism and the proton magnetic resonance studies indicate that a major portion of the population should be right-handed conformers. The existence of a certain percentage of the left-handed conformers in solution would have a very large effect on the amplitude of the circular dichroism curves; it would have negligible effect on the $\Delta\delta_D{}^l$ for the base protons but would have an effect on the $\Delta\delta_D{}^t$ for the H-1′ protons (Ts'o *et al.*, 1969a). The effect on the $\Delta\delta_D{}^l$ value of the H-1′ proton, however, is difficult to evaluate quantitatively. This problem will be directly discussed in the studies on L-$A_{3'}p_{5'}A$ and $A_{2'}p_{5'}A$ (Tazawa *et al.*, 1970). The results indicate that there may exist a considerable percentage of left-handed conformers in the population, especially in the case of $A_{2'}p_{5'}A$. The CPK models of $A_{2'}p_{5'}A$ or $A_{2'}p_{5'}C$ suggest that the stereochemical hindrance for the formation of the left-handed stack may be rather small (Ts'o *et al.*, 1969a). This situation may be partially responsible for the small amplitudes of the circular dichroism curves observed for the 2′–5′ dimer. The second problem concerning the circular dichroism studies is the second peak and trough region around 210 mμ (Bush and Scheraga, 1969). In the following paper there is an indication that the peak at 220 mμ is larger for the $A_{2'}p_{5'}A$ than for the $A_{3'}p_{5'}A$. This situation is not understandable from the present considerations discussed above. Obviously, more knowledge about the electronic origin of this circular dichroism peak and trough at 200-mμ region is needed.

There is one very interesting observation on the interaction of $A_{5'}p_{5'}A$ with poly U. In solutions of 0.01 M $MgCl_2$–0.01 M Tris (pH 7.5) (for detailed conditions see the following paper, Tazawa *et al.*, 1970), $A_{3'}p_{5'}A$ will form a complex (1:2) with poly U with a T_m of about 13° as measured by ultraviolet absorbance or by circular dichroism. Under the identical conditions, however, no complex formation between $A_{5'}p_{5'}A$ with poly U could be found. This preliminary observation can be explained from the conformational model of $A_{5'}p_{5'}A$ (Figure 11) discussed above. The hydrogen bonding sites (NH_2 and N-1 groups) of each of the two adenines in the dimer are facing a different direction from each other. Attempts to build a 2:1 complex between poly U and $A_{5'}p_{5'}A$ with this conformation were unsuccessful, which is in agreement with the experimental findings.

When this manuscript was nearly completed, we received an informative preprint from Warshaw and Cantor (1970) concerning the circular dichroism studies on dinucleoside monophosphates. They have also studied $A_{2'}p_{5'}A$, $A_{3'}p_{5'}A$, $A_{2'}p_{5'}C$, and $A_{3'}p_{5'}C$. Their experimental findings are comparable with ours. They also concluded that 2′–5′ dimers "have a substantial degree of base stacking at room temperature. The geometry of the stacked conformation is different from that of either the normal ribo dimer or the deoxy dimer."

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