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Conformation of Purine Nucleoside Pyrophophates as Studied by Circular Dichroism*

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ABSTRACT: The circular dichroism of dinucleoside 5′,5′-pyrophosphates of various purine nucleosides was studied for elucidation of the conformation. Pyrophosphates derived from adenosine, inosine, guanosine, and 8,3′-*S*-cycloadenosine were found to have a stacked symmetrical structure, in which bases are in anti conformation. 8,2′-*S*-Cycloadenosine 5′,5′-pyrophosphate cannot have a stacked conformation due to the restricted rotation of the bases. Pyrophosphates from 8-bromoadenosine, as well as 8-bromoguanosine, have a stacked conformation, in which bases are in syn form.

It is of importance to know the conformation of nucleic acids and their components in solution. For this purpose circular dichroism (CD) of nucleosides and nucleotides has been extensively studied (Miles *et al.*, 1969a,b; Ikehara *et al.*, 1971, 1972). Brahms *et al.* (1966, 1967, 1969) reported on the CD of oligonucleotides as models of nucleic acids. A theoretical study on optical rotatory dispersion (ORD) and CD of oligo- and polynucleotides was done by Tinoco (1964). It was shown that specific Cotton effects, which were different from those of monomers in origin, appeared by stacking of the component nucleotides. Michelson (1962) reported that P^1,P^2-dinucleoside 5′-pyrophosphates showed large hypochromicity and suggested that two bases in these molecules might be strongly stacked. In the preceding paper (Ikehara *et al.*, 1972) we have postulated conformations of various purine nucleoside 5′-monophosphates in solution as studied by CD. In this paper we describe results of the study of CD of P^1,P^2-dinucleoside 5′-pyrophosphates and discuss their conformation in solution. The pyrophosphates have symmetrical structure, and their degree of stacking is influenced by the torsion angle of the base moiety.

Materials and Methods

Synthesis of nucleoside 5′-monophosphates were described previously (Ikehara *et al.*, 1972). P^1,P^2-Dinucleoside 5′-pyrophosphates were synthesized from the appropriate 5′-monophosphate by condensation using dicyclohexylcarbodiimide (Smith *et al.*, 1961). All pyrophosphates run slower than the original monophosphate in paper electrophoresis performed in triethylammonium bicarbonate buffer (pH 7.5) and show smaller R_F values in paper chromatography in 1-butanol–acetic acid–water (5:2:3, v/v) system. Purification methods, uv absorption properties, R_F values in paper chromatography, and the mobility in paper electrophoresis are summarized in Table I.

CD was measured with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. Samples were filtered with Millipore filter and concentration of nucleotides was adjusted to 1–2 OD_{max}/ml. The measurement was performed at 15° in a 10-mm light-path cell. Calibration was made by *d*-10-camphorsulfonic acid. All runs were repeated at least twice until reproducible curves were obtained.

Uv absorption spectra were taken with a Hitachi EPS-3T spectrophotometer and phosphate analysis was made by a modified Allen's method (Allen, 1940). Solvents used were 0.001 N HCl–0.1 M phosphate buffer (pH 7.0) and 0.001 N NaOH. Molar extinction (ϵ) and molar ellipticity (θ) are presented as per residue values.

Nuclear magnetic resonance (nmr) spectra were taken with a Hitachi HA-100 spectrometer operated at 100 MHz. Samples (disodium salts) were dissolved in D_2O and lyophilized three times. Measurements were performed in D_2O solution and chemical shifts are given in parts per million relative to tetramethylsilane as the external standard.

Results

Absorption and CD Spectra of Pyrophosphates of Adenosine and Adenine S-Cyclonucleosides. For P^1,P^2-Diadenosine 5′-

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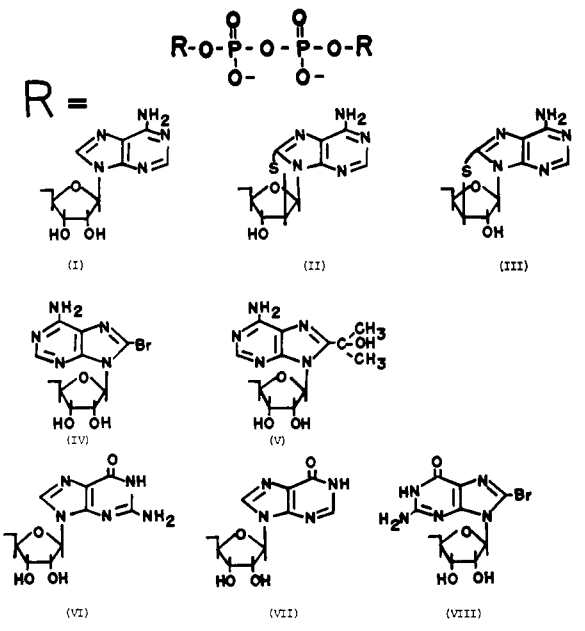
TABLE I: Properties of Different Pyrophosphates.

Compound	Uv γ_{max} (ϵ (P)) 0.001 N HCl	pH 7	0.001 N NaOH	Ppc[a] R_{AMP} (R_{MP}) Solvent A	Solvent B	Solvent C	Pe[b] R_{AMP}(R_{MP})	Ref[c]
Adenosine pyrophosphate (I)	258 (1.32)	261 (1.17)	261 (1.19)	0.36	0.87	1.05	0.87	A
8,2′-*S*-Cycloadenosine pyrophosphate (II)	278.5 (2.25)	276.5 (2.12)	276.5 (2.12)		0.48 (0.71)	0.82	0.70 (0.68)	B
8,3′-*S*-Cycloadenosine pyrophosphate (III)	282.5 (2.25)	282 (2.00)	283 (2.00)	0.16 (1.14)	0.50 (0.68)		0.62 (0.62)	C
8-Bromoadenosine pyrophosphate (IV)	262 (1.44)	264.5 (1.68)	265 (1.48)	0.42 (0.36)	1.16 (1.00)	1.26	0.85 (0.81)	B
8-Isopropanyadenosine pyrophosphate (V)	260 (1.56)	261.5 (1.40)	261.5 (1.40)	1.17 (0.72)			0.68 (0.74)	C
Guanosine pyrophosphate (VI)	256 (1.21)	252 (1.26)	256.5 (1.18)	0.07	0.34	0.74	0.91 (0.81)	C
Inosine pyrophosphate (VII)	250 (1.16)	250 (1.15)	254 (1.36)	0.23 (0.16)	0.59 (0.82)	1.02	0.99 (0.86)	B
8-Bromoguanosine pyrophosphate (VIII)	262 (1.36)	263 (1.40)	267 (1.31)	0.24 (0.21)	0.81 (0.68)	0.60	0.86 (0.82)	B

[a] Ppc stands for paper partition chromatography; R_{AMP}, mobility relative to 5′AMP, R_{MP}, mobility relative to corresponding MP. Solvent A, 1-butanol–acetic acid–water (5:2:3, v/v); B, ethanol–1 M ammonium acetate (7:3, v/v); C, isopropyl alcohol–concentrated ammonia–water (55:10:35, v/v). [b] Pe stands for paper electrophoresis. [c] A, purified by Dowex 50 (Cl^-) column; B, DEAE-cellulose column (bicarbonate); C, purified by paper electrophoresis at pH 7.5.

pyrophosphate (I) (see Scheme I) Michelson (1962) found a hypochromicity of *ca.* 25% at pH 7, and proposed a strong stacking of the adenine bases. As shown in Figure 1, the CD spectrum of I had two Cotton effects with almost the same amplitude but opposite sign on each side of the absorption maximum. According to Tinoco (1964), if two similar chromophores stack at certain angles, the transitions of these chromophores split and give Cotton effects having the same amplitude but opposite sign. It has been postulated by these authors that di- and polynucleotides having stacking of right handedness in screw axis will give a pair of bands positive and negative one from the long wavelength. Adenylyl-(3′–5′)-adenosine (ApA), in which two adenine residues are linked together with a phosphodiester bond, shows a hypochromicity of *ca.* 9% and a positive–negative (from the long wavelength) splitting at around 260 nm (Warshaw and Tinoco, 1966; Brahms *et al.*, 1966). Compound I also shows positive–negative splitting in B-band region (Clark and Tinoco, 1965; Bush and Tinoco, 1967; Miles *et al.*, 1969b). Comparison of the optical properties of I to those of ApA, showed that hypochromicity of I

SCHEME I



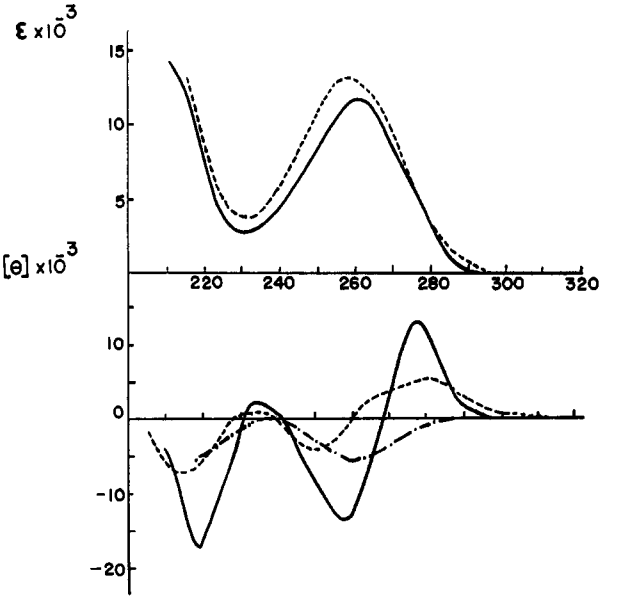


FIGURE 1: Uv and CD spectra of diadenosine pyrophosphate. (———) pH 7, (· · · · · · ·) 0.001 N HCl, and (–·–·–·–) AMP, pH 7.

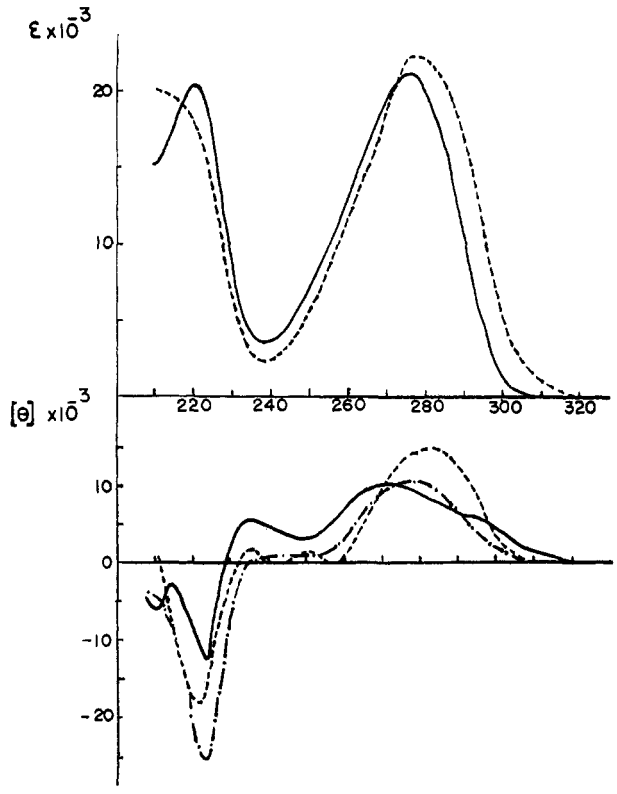


FIGURE 2: Uv and CD spectra of di-8,2′-*S*-cycloadenosine pyrophosphate. (———) pH 7, (· · · · · · ·) 0.001 N HCl, and (-·-·-·-) 8,2′-*S*-cycloadenosine 5′-monophosphate, pH 7.

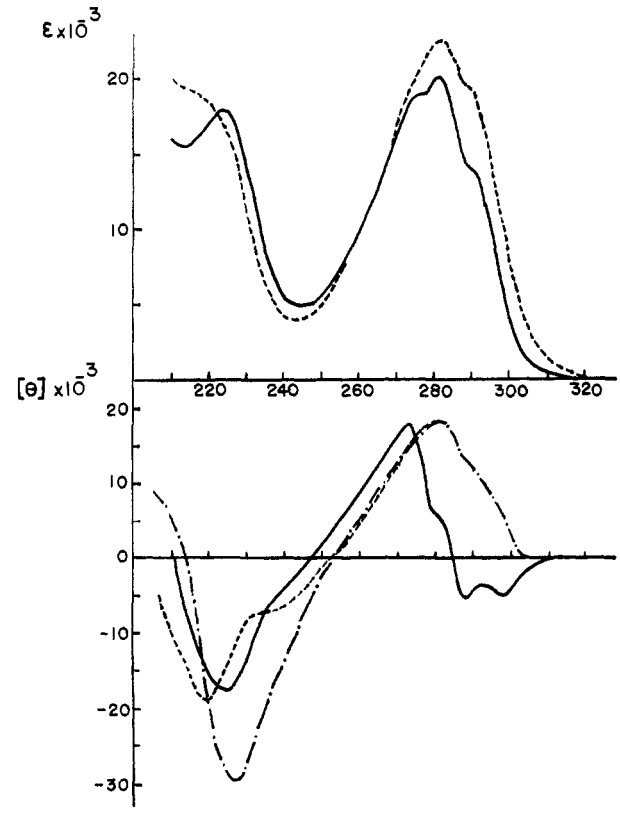


FIGURE 3: Uv and CD spectra of di-8,3′-*S*-cycloadenosine pyrophosphate. (———) pH 7, (· · · · · · ·) pH 3, and (-·-·-·-) 8,3′-*S*-cycloadenosine 5′-monophosphate, pH 7.

is twice as much as that ApA. Moreover, the absorption maximum of I exists in a longer wavelength region than ApA, in which λ_{max} shifted hypsochromically *ca.* 2 nm from pA. In the CD spectrum the Cotton effect amplitudes of I are smaller than those of ApA and the long-wavelength crossover point of I exists at 268 nm, which is bathochromically shifted from that of ApA (260 nm). Also a difference in the sign of the Cotton effect in the 220-nm region, negative for I and positive for ApA, was observed.

When uv spectra in 0.001 N HCl of I and ApA were compared, ϵ_{max} values increased significantly from those in neutral solution. Since ϵ of 5′-AMP decreased in an acidic solution, the large hyperchromicity of I and ApA seems to be due to a labilization of conformation in acidic conditions. This point was also supported by CD, in which the magnitude of Cotton effects decreased to less than one-half and the profile changed from that of the neutral solution. The same conclusion was reached by Scott and Zamecnik (1969).

It is almost established that adenosine and AMP have anti conformation in solution (Ts'o *et al.*, 1969; Miles *et al.*, 1968). The adenine residues in ApA are also in anti conformation (Ts'o *et al.*, 1969; Chan and Nelson, 1969). In order to investigate the conformation of adenosine in P^1,P^2-diadenosine 5′-pyrophosphate (I), we have synthesized two pyrophosphates derived from 8,2′-*S*-cycloadenosine (II) and 8,3′-*S*-cycloadenosine (III) (Ikehara and Tada, 1967) and compared their properties to those of I. Since in cyclonucleosides the conformation is firmly fixed in anti position ($\phi_{CN} = -108°$ for 8,2′-*S*-cycloadenosine and $-72°$ for 8,3′-*S* isomer) by the anhydro linkage, torsion angle could not change by formation of the pyrophosphate. P^1,P^2-Di-8,2′-*S*-cycloadenosine 5′-pyrophosphate (II) showed a CD curve closely resembling that of the monomer (Figure 2) and no hypochromicity in the uv spectrum. Therefore, we concluded that in II almost no stacking occurred. In contrast, P^1,P^2-di-8,3′-*S*-cycloadenosine 5′-pyrophosphate (III) showed a negative splitting at around 280 nm and this splitting disappeared in acidic solutions, although the nagnitude of the Cotton effect is similar to that of the monomer (Figure 3). In the uv absorption spectrum, III showed a hyperchromicity in acidic solutions, which suggested some stacking in neutral solutions. From this evidence it could be deduced that bases in II do not stack at all, whereas in III they stacked to a certain degree. A strongly stacked conformation of an ApA analog, 8,2′-*S*-cycloadenylyl-(3′–5′)-8,2′-*S*-cycloadenosine (Ikehara *et al.*, 1970), in which the two adenosines of ApA were replaced by 8,2′-*S*-cycloadenosines, suggested that the S atoms could not be the only reason for the weak stacking of bases in II and III.

Uv Absorption and CD Spectra of Pyrophosphates of 8-Substituted Adenosines. Next we measured the CD spectra of pyrophosphates derived from 8-substituted adenosines, in which syn conformation had been assigned (Ikehara *et al.*, 1972). Travale and Sobell (1970) reported that 8-bromoadenosine and 8-bromoguanosine have syn conformation ($\phi_{CN} = 120°$) in crystal lattice as studied by X-ray crystallography. As shown in Figure 4, P^1,P^2-di-8-bromoadenosine 5′-pyrophosphate (IV) showed Cotton bands two to three times larger than those of the monomer. This suggests that bromoadenine bases in IV stack as strongly as in I. However, the Cotton band in the B region showed a negative–positive splitting in contrast to I. In the uv absorption spectra IV had almost the same ϵ both in neutral and acidic solutions, whereas 8-bromoAMP showed hyperchromicity in acidic conditions. In accordance with this, the CD of IV at pH 3 showed an

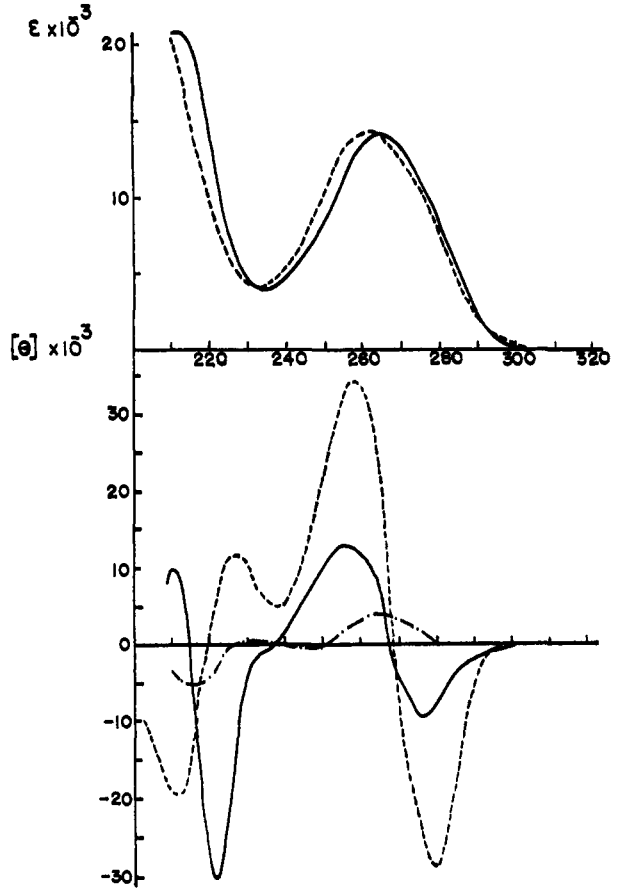


FIGURE 4: Uv and CD spectra of 8-bromoadenosine pyrophosphate. (———) pH 7, (·····) pH 3, and (-·-·-·-) 8-bromoadenosine 5′-monophosphate, pH 7.

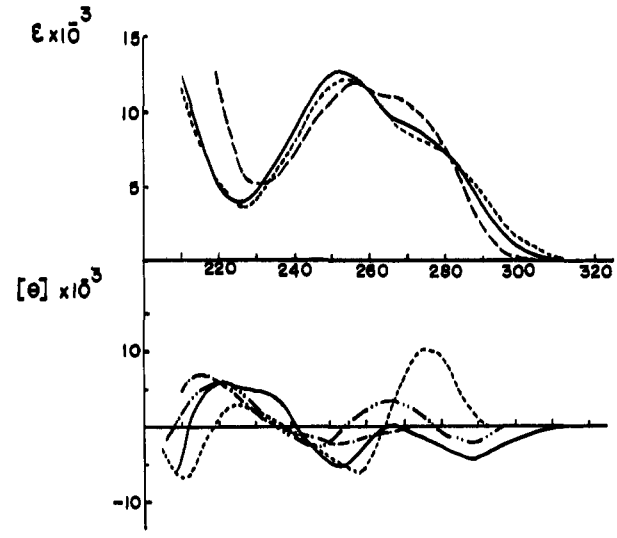


FIGURE 5: Uv and CD spectra of guanosine pyrophosphate. (———) pH 7, (·····) 0.001 N HCl, (— — —) 0.001 N NaOH, (-·-·-·-) pure water, and (-·-·-·-) guanosine monophosphate, pH 7.

increase in magnitude of the Cotton effects in the B region. These phenomena may be interpreted by a stabilization of the structure due to half-protonation and/or change of the structure by intermolecular association.

From these results it might be concluded that pyrophosphates of 8-substituted adenosine, which have syn conformation in the monomer form, can stack well at least in neutral solutions.

Absorption and CD Spectra of Pyrophosphates of Guanosine, 8-Bromoguanosine, and Inosine. As shown in Figure 5, P^1,P^2-diguanosine 5′-pyrophosphate (VI) has a characteristic CD curve in a neutral solution, which is clearly different from that of the monomer. In acidic conditions VI shows a positive–negative splitting at 265 nm, which is in the middle of two absorption bands. In order to exclude a possibility of intermolecular association, which has been reported in guanosine-containing nucleotides (Sarkar and Yang, 1965), the CD of VI was taken in pure water. The CD profile effectively changed from that in 0.1 M phosphate buffer, and a peak at 267 nm together with two troughs at 248 and 289 nm were observed. Crossing-over points were at 255 and 278 nm, which corresponded to B_{1u} and B_{2u} bands, respectively.

P^1,P^2-Diinosine 5′-pyrophosphate (VII), in which the 2-amino group of VI was missing, showed a positive–negative splitting at 250 nm. The position corresponded well to the uv absorption maximum (see Figure 6).

As shown in Figure 7, P^1,P^2-di-8-bromoguanosine 5′-pyrophosphate (VIII), in which 8-bromoguanosine was proved to be in the syn conformation, had three Cotton effects in the B-band region. These effects may be ascribed to an overlapping of the positive splitting of B_{1u} and negative splitting of B_{2u} bands and suggested a strong stacking of 8-bromoguanine residues in VIII. The uv absorption of VIII in 0.001 N HCl was similar to that in neutral solution and suggested a non-protonated form of VIII. The CD curve at acidic pH showed a decrease in magnitude of the 280-nm Cotton band compared to that in pure water.

Nmr Spectra of Dinucleoside Pyrophosphates. The results of the nmr spectra of several pyrophosphates are summarized in Table II together with those of their corresponding monophosphates.

Feldman and Agarwal (1968) assigned all proton signals in the nmr spectrum of 5′-AMP. In the nmr spectra of pyrophosphates, it was most profoundly observed that all corresponding signals of two nucleotide residues appeared at the same position. This suggests a symmetrical structure for the pyrophosphate. In the case of a 0.1 M nucleotide solution in

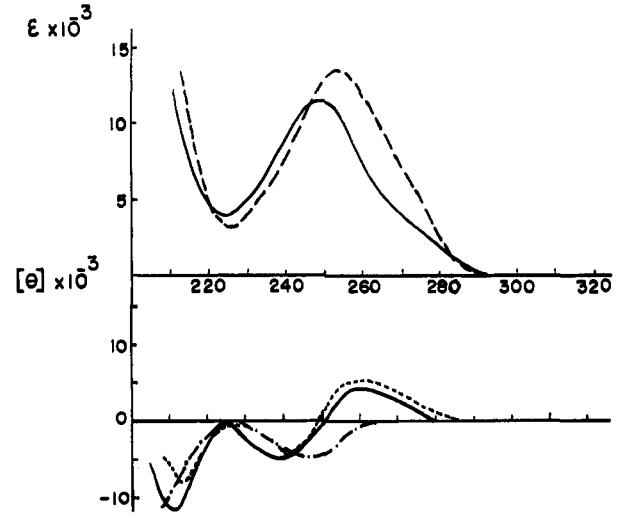


FIGURE 6: Uv and CD spectra of inosine pyrophosphate. (———) pH 7, (·····) pH 3, (— — — —) pH 11, and (-·-·-·-) inosine 5′-monophosphate, pH 7.

TABLE II: Chemical Shift of Base and Sugar Protons of Dinucleoside Pyrophosphates and Corresponding Monophosphates.[a]

Compound	8-H	2-H	1'-H	2'-H	5'-H	$J_{H_1'-H_2'}$
Adenosine pyrophosphate (I)	8.14	7.96	5.94		4.36	4.6
Adenosine monophosphate	8.62	8.25	6.21		4.22	5.0
8-Bromoadenosine pyrophosphate (IV)		8.14	6.09	5.27	4.38	6.5
8-Bromoadenosine monophosphate		8.27	6.22	5.40	4.26	5.7
8-Bromoguanosine pyrophosphate (VIII)			6.00	5.44	4.40	6.2
8-Bromoguanosine monophosphate			6.13	5.44	4.26	6.0

[a] Taken in D_2O as disodium salt of 0.1 M concentration, except for I (0.27 M). The chemical shift was presented in parts per million from the tetramethylsilane capillary. The coupling constant was represented as cycles per second.

D_2O, a 2'-H signal of 5'-AMP overlapped with HDO peak at 4.8 ppm. In contrast to this, all purine nucleoside pyrophosphates and 5'-monophosphates, in which syn conformation has been proposed, showed a well-resolved triplet peak of 2'-H at about 5.27–5.44 ppm (see Table II). This signal was observed also in the spectrum of 8-isopropanoladenosine 5'-monophosphate (Steinmaus *et al.*, 1969). Secondly, all 5'-H signals appeared at positions 0.12–0.14 ppm shifted toward lower field compared to those of the monomers. These shifts might be interpreted by taking a conformation, in which the sugar protons are affected by ring current of the base.

In I, 8-H, 2-H, and 1'-H protons shifted toward higher field due to stacking of these bases, and the shift is largest in 8-H. In 5',5'-diadenosine monophosphate the same tendency was reported (Kondo *et al.*, 1970). 8-Substituted adenosine pyrophosphates (IV and V) showed a high-field shift of 2-H of 0.09–0.14 ppm. In IV the 1'-H signal also shifted in the same amount as that of IV, but 2'-H showed no shift. These shifts of signals suggested, therefore, stacked conformations of various degrees for these pyrophosphates.

Coupling constants between the 1'-H and 2'-H of pyrophosphates were similar to those of 5'-monophosphates. This fact suggested that the conformation of the sugar residue might not be much different from that of monophosphates.

Discussion

Mode of Stacking of Diadenosine 5'-Pyrophosphate. As it was established that the adenine base in 5'-AMP is in anti conformation (Ts'o *et al.*, 1969), we can define a face of the base confronted to $C_{2'}$ and $C_{3'}$ as the A face, and another face confronted to lactol O atom as the B face. In ApA the two adenines stack with different faces, namely the A face of 5' end and the B face of 3' end nucleotide (Ts'o *et al.*, 1969; Chan and Nelson, 1969). In the CD spectrum of I, positive–negative splitting in the B region suggested that two adenines stacked in the screw axis of right handedness as in ApA. However, in I a symmetrical arrangement of the monomer unit is required. As shown previously, the two bases in di-8,2'-*S*-cycloadenosine 3'–5'-monophosphate stacked strongly (Ikehara *et al.*, 1970), whereas its pyrophosphate (II) could not stack at all. Therefore, the mode of stacking of dinucleoside 3'–5'-monophosphate and 5',5'-pyrophosphate (I) would be entirely different. When we examine Corey–Pauling–Koltun (CPK) models constructed for a symmetrically stacked form of the pyrophosphate, there exist two kinds of structures having A–A and B–B stacking (see Figure 8, (1) and (2)). If we define these structures as A and B structure, respectively, A structure has carbohydrate $C_{2'}$ and $C_{3'}$ folded inside of the molecule. In contrast, the B structure has lactol O inside and

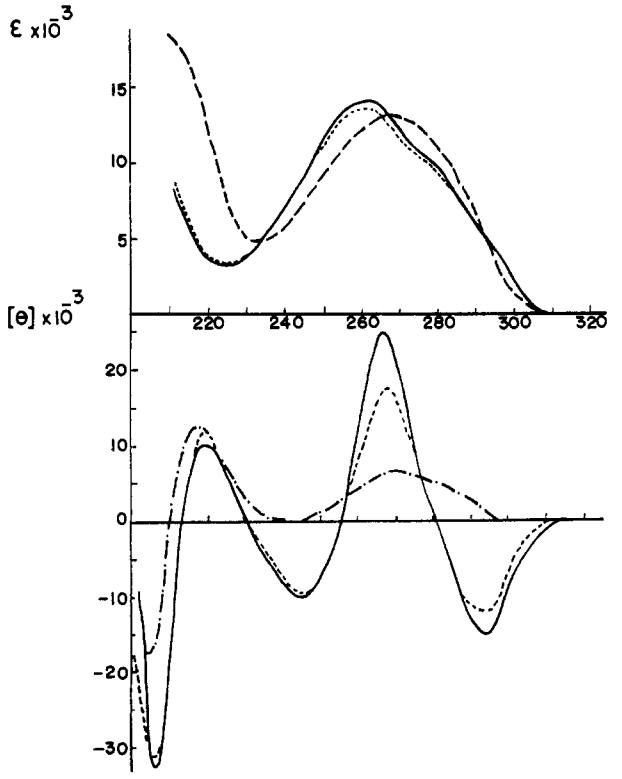


FIGURE 7: Uv spectra and CD spectra of 8-bromoguanosine pyrophosphate. (———) pH 7, (-------) 0.001 N HCl, (— — — —) 0.001 N NaOH, and (-·-·-·-) 8-bromoguanosine 5'-monophosphate, pH 7.

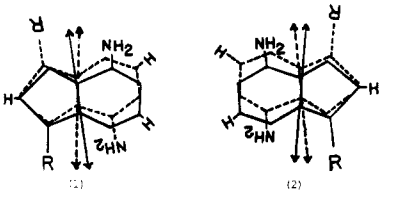


FIGURE 8: Schematical representation of stacked structure of pyrophosphate. (1) B structure and (2) A structure. Arrows shows transition moments. Solid line represents upper base and dotted line lower base.

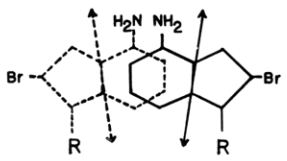


FIGURE 9: Schematic representation of stacked form of 8-bromoadenosine pyrophosphate. Arrows showed transition moments. Solid line represents upper base and dotted line lower base.

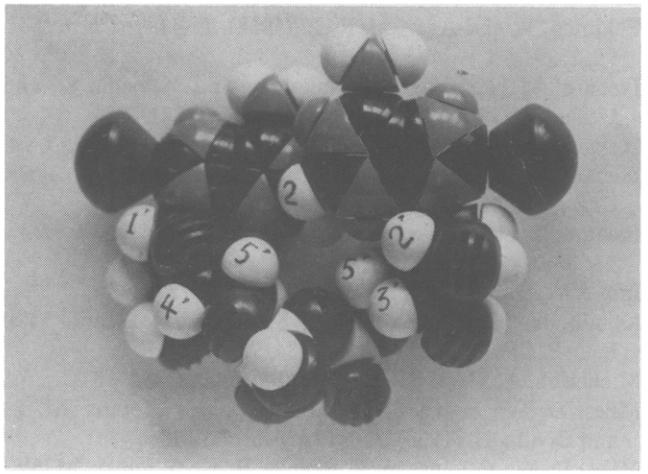


PLATE I: Stacked conformation of di-8-bromoadenosine pyrophosphate. Two bases are stacked in syn form showing bromo atoms exposed to right and left of the molecule.

relatively bulky cis diol group (2′- and 3′-OH) exposed toward the outside of the molecule. Therefore, if we consider steric hindrance, B structure seems to be more favorable than A structure. This structure is also supported by the fact that proton 8-H is affected by a larger shielding effect of the base than proton 2-H. The fact that high-field shift of 1′-H was almost the same as that of 2-H is also interpreted by this arrangement. Moreover, the low-field shift of 5′-H of all pyrophosphates could be interpreted by assuming a deshielding effect of the pyrophosphate group. These nmr data suggested also the B structure for I.

In the case of ApA, which has A–B stacking, the angle between transition dipoles must be the same as the angle formed by the bases. In contrast to this, diadenosine pyrophosphate (I), in which B form is to be, may have different directions of base angles and transition moments. Stewart and Davidson (1963) and Stewart and Jensen (1964) reported on the transition dipoles in 1-methylthymidine and 9-methyladenine. According to them, the intense long-wavelength band (275 nm) is short-axis polarized in 9-MeA. The weak band (225 nm) is long-axis polarized, and probably the second strong band (<230 nm) is also long-axis polarized. If we assume a slight declination of the transition moment of the 275-nm band toward N_7 as shown in Figure 9, the B band can split in positive–negative fashion (right handedness). This splitting is consistent with that observed in the experiment. As the hypochromicity of I is larger than that of ApA, the two bases must overlap over a wider region (Kondo *et al.*, 1970). The smaller magnitude of the CD of I compared to that of ApA (Scott and Zamecnik, 1969) suggested a smaller angle between transition dipoles in I.

Examination of CPK model showed that the two bases in I could be in a position, in which maximum overlap is possible, when the torsion angle is maintained between −30 and −40°. Compounds II and III, in which torsion angles are fixed showed only weak stacking. Especially, in II having a torsion angle of −108°, almost no stacking was possible. The CPK model of III could have an arrangement favorable for symmetrical stacking, though this form allowed only a small overlap for bases.

Mode of Stacking of Pyrophosphates of 8-Substituted Adenosine Nucleosides. For 8-bromoadenosine pyrophosphate (IV) we can construct a molecular model, in which the relative arrangement of sugar residues and phosphates is similar to that of I, but bases are rotated by 180° to the syn position as shown in Figure 9 and Plate I. If we assume that the direction of transition moments would be nearly the same as in I, negative–positive splitting of IV in this conformation could be interpreted, because the transition moments crossed oppositely to I. Although an alternative conformation, such as an anti-anti, left-handed one is feasible, CPK model building is rather difficult for this arrangement.

8-Bromoguanosine pyrophosphate (VIII), which has bulky 2-NH_2 groups must have a B structure. Assuming this structure has a transition moment similar to that of IV, the negative splitting of the B_{2u} band can be interpreted. The reason for a small change in the spectrum of VIII in 0.1 M phosphate buffer and in pure water may be explained by weaker basicity of the N^7 in 8-bromoguanine than in guanine.

From these results we can conclude that pyrophosphates having monomer residues with a syn conformation can exist in a stacked conformation. $\phi_{CN} = 140°$ is the most favorable angle for this structure as found in the CPK model.

Stacking of Other Pyrophosphates. P^1,P^2-Diinosine pyrophosphate (VII) showed positive splitting in the B-band region (Figure 6). Since inosine 5′-monophosphate has an anti conformation (Nagashima and Iitaka, 1968), the direction of the transition moment may be the same as in the case of adenosine pyrophosphate (I).

P^1,P^2-Diguanosine pyrophosphate (VI) showed a dramatic change in the CD profile in pure water in which it has a clear splitting from that taken in 0.1 M phosphate buffer at pH 7.0. In 0.1 M buffer, the CD profile of VI changed presumably due to self-association involving the N^7 atom. The small amplitude of the Cotton effect of VII is like that of GpG and IpI (Warshaw and Tinoco, 1966; Inoue and Satoh, 1969). This may be due to low stacking ability of bases and/or inadequate torsion angle.

Thus it might be concluded that the torsion angle of the monomer nucleotides strongly influences the three-dimensional structure and stacking tendency of pyrophosphates.

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Infrared Studies of Azide Bound to Myoglobin and Hemoglobin. Temperature Dependence of Ionicity†

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ABSTRACT: Recent developments in instrumentation and techniques have made possible studies of coordination of metal–protein complexes in aqueous solution by infrared spectroscopy. Thus early work with carbon monoxide complexes of hemoglobin, as well as preliminary studies with myoglobin (Mb) complexes, have now been quantitated and extended to the temperature-dependent ionicity of N_3^- bound to Mb. Titration of Mb with NaN_3 produces two peaks, one at 2046 cm^{-1} (ionic) and one at 2023 cm^{-1} (covalent). Both have narrow half-bandwidths (8 cm^{-1}) at N_3^-:Mb < 1, indicating that N_3^- is protected from the aqueous environment by the protein. The ratio of intensities is temperature dependent in agreement with the temperature-dependent spin state of Fe(III) reported by others. Thus high-spin Fe(III) is bound to ionic N_3^-, and low-spin Fe(III) to covalent N_3^-, in MbN_3 complexes. No intermediate states are detected by infrared spectroscopy. Ionic N_3^- in bulk aqueous solution has a broad half-bandwidth (25 cm^{-1}), but a frequency of maximum absorption similar to that of the bound ionic form. Titration spectra have been analyzed by comparison to computed sums of Lorentzian functions. A similar temperature dependence of ionicity is reported for the azide complex of human hemoglobin A, except that the complex is more covalent.

Techniques for infrared spectroscopy of metal proteins have been developed within the past several years (Alben and Caughey, 1966). Early experiments with carbon monoxide bound to the iron of hemoglobin or myoglobin (Alben and Caughey, 1968) indicated that the frequency of the CO stretching vibration is subject to the local molecular environment and effected by the electronic configuration and effective bond order of the carbon monoxide. The CO stretching frequency is the same for many hemoglobin derivatives but is observed at higher frequency when the distal histidine (E-7) is replaced by tyrosine or arginine in hemoglobin M_{Emory} or hemoglobin Zurich (Caughey *et al.*, 1969a) and at somewhat lower frequencies with carboxymyoglobin (Caughey *et al.*, 1969b). The Fe(II)–carbonyl and Fe(III)–cyanide complexes of hemoglobin or myoglobin are strong field ligands with low-spin iron and exhibit only one vibrational state of each ligand. We have recently studied the intermediate ligand field complexes of Fe(III)–azide which have been found by Beetlestone and George (1964) and by Iizuka and Kotani (1969a,b) to exhibit a temperature-dependent spin state of the Fe(III) by magnetic susceptibility measurements. The infrared spectra of azide bound to hemoglobin and myoglobin were first reported by McCoy and Caughey (1970) to consist of two bands which might be involved in such a temperature-dependent spin-state equilibrium. However, no quantitative studies were conducted. We have confirmed the observations of McCoy and Caughey and have obtained quantitative extinction coefficients for both of the infrared bands due to the azide bound to myoglobin. This has allowed us to demonstrate that the infrared

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