*Articles*

# Raman Spectroscopic Measurement of Base Stacking in Solutions of Adenosine, AMP, ATP, and Oligoadenylates†

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ABSTRACT: Measurements of the colligative properties of nucleosides and their derivatives have shown that bases form transient aggregates in solution [Ts'o (1967) *J. Am. Chem. Soc. 89*, 3612–3622]. Aggregation of nucleotides cannot be measured by osmometry due to the presence of counterions. Sedimentation measurements are difficult to obtain and have been complicated by differences in pH [Ferguson et al. (1974) *Biophys. Chem. 1*, 325–337]. Raman studies of oligonucleotides have shown that the intensities due to base vibrational modes depend on the extent of base stacking, but this dependence has not been quantitated. We have measured this dependence by relating changes in the Raman spectra of nucleotides and nucleosides with previous measurements of colligative properties. Visible Raman spectra of ATP, AMP, and adenosine, taken over a range of concentrations from 1 to 1000 mM, show that the peak intensity ratio $(I_{1305} + I_{1380})/I_{1340}$ varies linearly with the log of the concentration for all three bases. This concentration-dependent change correlates with published molal osmotic coefficient data for functionally similar bases with a correlation coefficient of 0.99. In contrast, UV resonance Raman spectra of the same bases show changes that vary linearly with concentration.

An understanding of the interaction and association of bases, nucleosides, nucleotides, and oligonucleotides in solution has grown out of the application of several physical techniques. Ts'o and colleagues have used vapor pressure osmometry (VPO)[1] to measure interactions of bases and nucleosides in aqueous solution (Ts'o et al., 1963; Broom et al., 1967; Ts'o, 1969). From these measurements they have developed a mathematical model of indefinite self-association that postulates an equal association constant for each step and have calculated the distribution of aggregate sizes at different concentrations (Ts'o & Chan, 1964). For example, in a solution of purine at 1.0 M, 11% base is calculated to be free monomer. The rest is in aggregates, some being five bases and longer. However, although osmometry has given direct information about the interactions of uncharged bases and nucleosides, it cannot be used to study the interactions of nucleotides because of the presence of counterions.

Ts'o and colleagues have studied interactions in both nucleosides (Broom et al., 1967; Ts'o, 1969) and nucleotides (Ts'o et al., 1969) using proton nuclear magnetic resonance (NMR) to measure concentration-dependent changes in the chemical shift of base hydrogens. These studies show that changes observed for nucleotides are different from changes observed for nucleosides. This difference is not interpreted to indicate a significant difference in the association constants for nucleotides and nucleosides, however. Ts'o and colleagues have also studied adenosine dimers with different covalent linkages and conclude that the type of overlap is strongly affected by the type of linkage (Ts'o, 1970; Kondo et al., 1970). Other NMR studies of covalent dimers and trimers have shown that 2′,5′ oligomers have a greater tendency to stack than the corresponding 3′,5′ oligomers (Doornbos et al., 1981). This observation is supported by studies using circular dichroism (CD) (Doornbos et al., 1981; Warshaw & Cantor, 1970; Kondo et al., 1970; Brahms et al., 1967) and (most convincingly) by gel filtration (Sussman et al., 1973).

Sedimentation measurements of the association of ATP in solution have indicated that ATP associates with a much higher affinity than those calculated by Ts'o and co-workers for bases and nucleosides (Ferguson et al., 1974). These results have also shown that interactions are limited to oligomers no larger than trimers.

Raman spectroscopic studies of short oligonucleotides and polynucleotides in solution also detect the presence of base stacking interactions. These interactions are indicated by differences in the intensities of several bands when compared with spectra of mononucleotides or as a function of temperature (Prescott et al., 1974; White et al., 1987; Small & Peticolas 1971a,b; Turpin et al., 1987; Thomas & Peticolas, 1983b). However, interpretations of these differences in terms of the extent of base stacking have been qualitative. There is a need for more quantitative information about the vibrational spectra of nucleotides in stacking interactions. In this paper we describe measurements of concentration-dependent changes in the Raman spectra of adenosine, AMP, ATP, and oligoadenylates. We observe a linear relationship between these changes and those obtained by osmometry, indicating

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[1] Abbreviations: pA2′pA2′pA, 5′-*O*-monophosphoryladenylyl-2′,5′-adenylyl-2′,5′-adenosine; d(pA3′pA3′pA), 5′-*O*-monophosphoryldeoxyadenylyl-3′,5′-deoxyadenylyl-3′,5′-deoxyadenosine; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CD, circular dichroism; NMR, nuclear magnetic resonance; VPO, vapor pressure osmometry.

that Raman can be used in empirical measurements of the colligative properties of nucleic acids. We observe no significant differences in the association constants for adenosine and ATP, in contrast to results from sedimentation. Our results for oligoadenylates are consistent with those from other methods.

## Materials and Methods

The visible Raman instrument has been previously described (Williams, 1986). Briefly, solutions in capillary tubes were irradiated by an argon ion laser operating at 514.5 nm. Most spectra were collected by a Princeton Instruments Model EIRY-1024 diode array detector attached to a Spex Model 1403 spectrometer equipped with 600 groove/mm ruled gratings. Otherwise, the spectrometer was equipped with 1800 groove/mm holographic gratings and an RCA 31034 photomultiplier tube.

The UV Raman instrument consists of a Spectra-Physics Model DCR-2A Nd-YAG laser operating at 30 Hz with a 2-ns pulse width, a Model HG-2 fourth harmonic generator for 266-nm light, a Spex Model 1269 1.25-m spectrometer equipped with an 1800 or 2400 groove/mm holographic grating, a Princeton Instruments Model IRY 700/05 diode array detector, and a Model ST-110 detector-controller interfaced to a Cromemco Z80 based microcomputer. Other wavelengths (1064 and 532 nm) were diverted by a Pellin-Broca prism (Spectra-Physics). Light energy at the sample was about 0.17 mJ/pulse. The sample solution of about 0.7 mL was recirculated by a Cole-Parmer peristaltic pump to flow by gravity between two stainless steel wires 1.5 mm apart to produce a flat capillary film. The incident laser light was focused on the sample film to a spot less than 1 mm in diameter. The laser beam intercepted the sample surface at an angle −37° from a horizontal line normal to the collection lens. Scattered light was collected by a 1-in. lens and focused on the entrance slit of the spectrometer.

Calibration of the spectrometer and detector was performed by taking the spectrum of the 266.04-nm laser line at spectrometer settings of 264.60, 265.00, 265.60, 266.62, and 266.60 nm. A plot of the observed laser peak diode number for each of these spectra versus spectrometer position was linear (correlation = 0.99) and showed that the spectrometer setting corresponded to a diode number of 411.44.

Data were analyzed by using a Cromemco 68020 based computer as follows. The detector dark signal was subtracted from the raw spectra to produce a level base line. All spectra were 9 point smoothed by using the algorithm of Savitsky and Golay (1964), and the base lines were adjusted to bring them to near zero at 1050 and 1450 $cm^{-1}$. The UV Raman spectra for ATP were normalized between 1070 and 1540 $cm^{-1}$, AMP spectra were normalized between 1250 and 1390 $cm^{-1}$, and adenosine spectra were normalized between 1295 and 1400 $cm^{-1}$. For the UV Raman spectra, peak frequencies, heights, and widths were determined by using a Gaussian–Lorentzian curve-fitting program described previously (Williams, 1986). Average shift in UV peak frequency was determined as the mean of the difference between the frequency for 0.0001 M AMP and the frequency for each concentration of AMP for peaks at 1308, 1340, 1480, and 1510 $cm^{-1}$. Peak heights for visible Raman data were taken directly from the final adjusted data file. Least-squares regressions and confidence limits for figures were produced on a DEC VAX 8200 using GPLOT from SAS Institute Inc. (Box 8000, Cary, NC 27511-8000).

All monomeric bases were obtained from Sigma Chemical Co. (St. Louis, MO) and were used as supplied. AMP was the sodium salt, ATP was the disodium salt, and poly(A) was the potassium salt. pA2′pA2′pA was from Pharmacia Chemical Co. (Piscataway, NJ), lot 00661-14. d-(pA3′pA3′pA) was a gift from Dr. L. M. S. Chang. All samples were adjusted to a pH of 7.0 in 0.14 M NaCl unless otherwise indicated. The molar extinction coefficient for d(pA3′pA3′pA) was taken to be 34.5, so that a 0.001 M solution gave $A_{260}$ = 11.4 (Cassani & Bollum, 1969). The molar extinction coefficient for pA2′pA2′pA was also taken to be 34.5, although these solutions were made by weight. The pH of very small samples (1–5 μL) was measured by using a Model MI-415 6-cm combination electrode from Microelectrodes, Inc. (Londonderry, NH).

## Results

The visible spectra of ATP, AMP, adenosine, and polyadenylic acid are shown in Figure 1, parts A, B, C, and D, respectively. The high concentrations are represented by the solid line and the lowest by the dashed line. The spectra of ATP and AMP at high concentration (near 1.0 M) are very similar except for the phosphodiester peak at 1115 $cm^{-1}$. The high-concentration spectrum of adenosine (0.05 M) appears different due to its limited solubility. These spectra show a number of concentration-dependent changes. The measured change showing the smallest uncertainty is the ratio of the sum of peak heights at 1305 and 1380 $cm^{-1}$ divided by the height of the 1333-$cm^{-1}$ peak. Figure 2 shows that this ratio changes linearly with the log of the concentration of the base. This linear relationship is not significantly different for the nucleotide triphosphate, monophosphate, and the nucleoside. Also shown in Figure 2 are the intensity ratios for pA2′pA2′pA and d(pA3′pA3′pA). These data indicate that pA2′pA2′pA, with intensity ratios equal to those for monomers at 10 times the concentration, is more stacked than d(pA3′pA3′pA) and that d(pA3′pA3′pA) shows the same extent of base stacking as an equimolar (by base) solution of monomer.

The average aggregate length at each concentration was calculated by using an association constant $K$ = 4.5 and assuming indefinite self-association as determined by Ts'o and co-workers (Ts'o et al., 1963; Broom et al., 1967) in their model of base–base interactions. Figure 3 shows the relationship between the calculated average aggregate length and the intensity ratio. The inset shows the distribution of the fraction of bases expected to be found in any given aggregate length. This is calculated for several different concentrations, indicating that according to this model a significant percentage of the bases are stacked at concentrations of 0.1 M and above.

Figure 4 shows the UV resonance Raman spectra for ATP and AMP at high (1.0 M) and low (0.0001 M) concentrations. The spectrum for adenosine (not shown) is similar to those of ATP and AMP, but no concentration-dependent changes are observed, probably because the upper limit of solubility for adenosine (50 mM) is below the observed lower limit of the range of concentration-dependent changes for the other bases (Figure 5). Concentration-dependent changes in the peak intensity ratio seen in the visible Raman spectra are not seen in the UV resonance Raman spectra of the same nucleotides. The most reliable concentration-dependent change in the UV Raman spectra is frequency shift, shown in Figure 5. This figure shows that no changes are seen below a concentration of 0.1 M. These changes are linear with respect to concentration (inset of Figure 5). The concentration-dependent changes in normalized peak height and width also appear to be linear over the same concentration range, but the uncertainty in these measurements is higher (not shown).

Figure 6 shows the relationship between the molal osmotic coefficient ($\phi$) for 2′-*O*-methyladenosine and the visible Raman

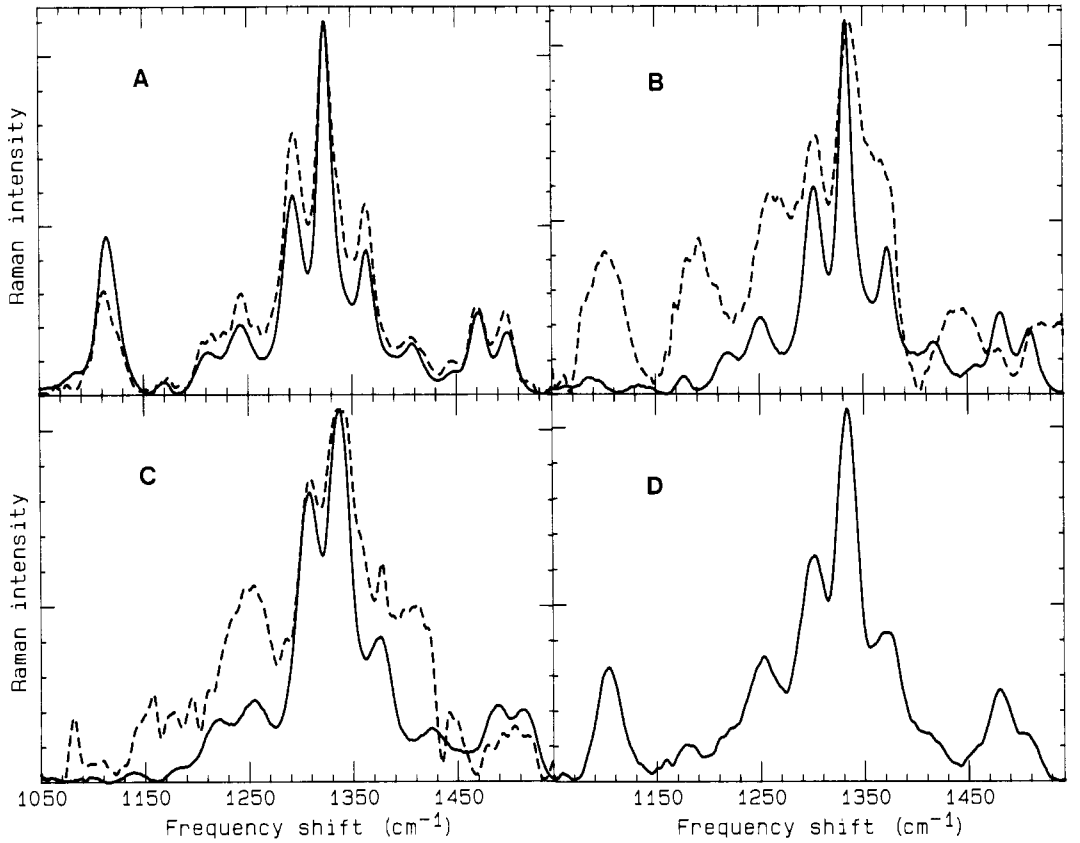


FIGURE 1: Effect of concentration on visible Raman spectra. (A) ATP: Solid line 0.91 M, dashed line 0.001 M. (B) AMP: Solid line 1.0 M, dashed line 0.001 M. (C) Adenosine: Solid line 0.05 M, dashed line 0.001 M. (D) Polyadenylic acid: 0.01 M in base. The spectra of all intermediate concentrations for (A)–(C) fall between the two spectra shown and have been omitted here for clarity. Data from the omitted spectra are represented in Figures 2 and 3.

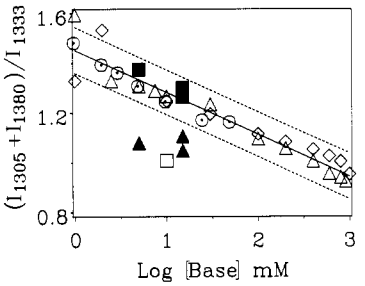


FIGURE 2: Effect of concentration on the visible Raman peak height ratio $(I_{1305} + I_{1380})/I_{1333}$. (Triangles) ATP; (diamonds) AMP; (circles) adenosine; (square) polyadenylic acid, 0.01 M in base; (solid squares) 3′,5′-A trimer, 0.015 and 0.005 M in base; (solid triangles) 2′,5′-A trimer, 0.015 and 0.005 M in base. The solid line is the linear regression for all of the points of ATP, AMP, and adenosine. Dashed lines show a 90% confidence interval around the regression line.

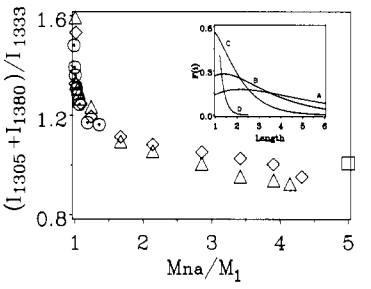


FIGURE 3: Relationship between calculated aggregate length, $M_{na}/M_1$, and visible Raman peak height ratios. (Triangles) ATP; (diamonds) AMP; (circles) adenosine; (square) polyadenylic acid, 10 mM in base. (Inset) Effect of concentration of base on the fraction of bases $F(i)$ expected to be found at a given aggregate length, $i$, in bases: (A) 1.0 M; (B) 0.4 M; (C) 0.1 M; (D) 0.001 M. $F(i)$ is calculated from eq 10 of Ts'o et al. (1963). Length = $M_{na}/M_1$, where $M_{na}$ is the number-averaged molecular weight and $M_1$ is the molecular weight of the monomer.

intensity ratio. We have used the data for 2′-*O*-methyladenosine because the molal osmotic coefficients of adenosine and 2′-*O*-methyladenosine are nearly identical over the solubility range of adenosine. 2′-*O*-Methyladenosine is more soluble than adenosine, and its osmotic coefficients are known to 0.3 M (Broom et al., 1967). The correlation coefficient between these two parameters in the range between 0.025 and 0.3 M is 0.99. These results show that, for concentrations above 0.025 M, the change in the intensity of the visible Raman bands is directly related to base stacking.

To measure the effect of salt on the Raman spectrum and base stacking of nucleotides, we collected spectra of 0.1 M AMP with NaCl concentrations of 0.0, 0.14, and 1.14 M. The respective $(I_{1305} + I_{1380})/I_{1333}$ ratios for these samples were 1.17, 1.11, and 1.09, indicating that the change in ionic strength due to changes in AMP concentration from 1 to 1000 mM has a minimal effect on base–base interactions under these conditions.

## Discussion

A decrease in the Raman intensity of base vibrational bands occurs when nucleic acids interact. This decrease, which is called a hypochromism, is due to a change in preresonance Raman effects as UV absorption intensities also decrease (Peticolas, 1984). A complete quantum mechanical explanation of this change has not been worked out, partly because ab initio calculations of Raman intensities have not been available. We have measured this change and observe that there is an empirical linear relationship between the relative intensity of visible Raman bands and the osmotic coefficient

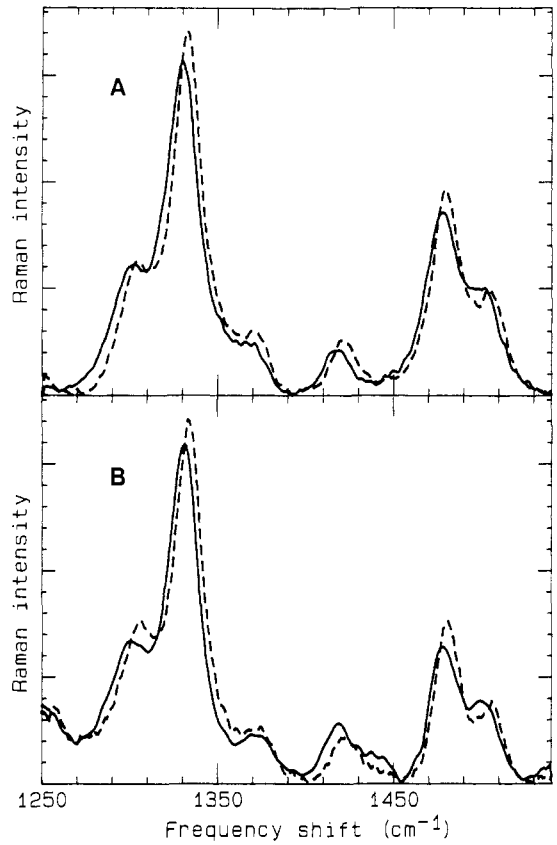


FIGURE 4: Effect of concentration on UV resonance Raman spectra. (A) ATP: (solid line) 0.91 M, (dashed line) 0.001 M. (B) AMP: (solid line) 1.0 M, (dashed line) 0.0001 M. The spectra of all intermediate concentrations for (A) and (B) fall between the two spectra shown and have been omitted here for clarity. Data from the omitted spectra are represented in Figure 5.

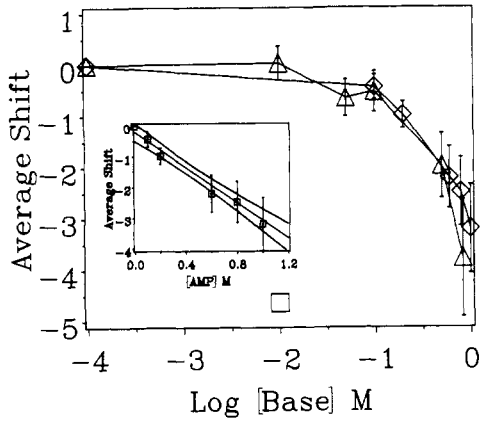


FIGURE 5: Effect of concentration on UV resonance Raman peak frequency, showing that this relationship is significantly different from changes observed in the visible Raman spectra. Each point represents the average shift in peak frequency. Average shift = $[(\nu_{0.001} - \nu_c)_{1308} + (\nu_{0.0001} - \nu_c)_{1333} + (\nu_{0.0001} - \nu_c)_{1480} + (\nu_{0.0001} - \nu_c)_{1510}]/4$, where $\nu_{0.0001}$ is the frequency of each peak at 0.0001 M AMP and $\nu_c$ is the frequency of each peak at concentration $c$. Error bars show 1 standard deviation around the mean. (Triangles) ATP; (diamonds) AMP; (square) polyadenylic acid, 0.01 M in base. (Inset) Linear plot of the mean frequency shift for AMP. The dashed line shows a 95% confidence interval around the regression line.

that may be useful in the study of base–base interactions. These results may also be useful for an evaluation of theoretical calculations.

We observe a linear relationship between the visible Raman peak intensity ratio and the log of base concentration in the range from 0.001 to 1.0 M that is virtually the same for ATP, AMP, and adenosine. This is consistent with results from

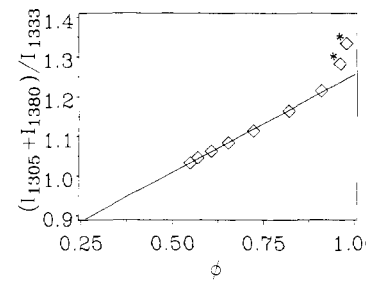


FIGURE 6: Relationship between visible Raman intensity ratio and molal osmotic coefficient ($\phi$). The osmotic coefficient data (Broom et al., 1967) are from 2′-*O*-methyladenosine, in concentrations from 0.005 to 0.3 M. The Raman intensity ratio values are from the linear regression line in Figure 2 for the same concentration range. Values marked with an asterisk are from an extrapolation below the lowest actual data point (0.025 M) to the theoretical zero point by Broom et al. and do not represent actual data. The line is the linear regression for values from 0.025 to 0.3 M. The correlation coefficient for values in this range is 0.99.

Broom et al. (1967) indicating that modification of the ribose does not affect stacking. Measurements of base–base interactions using vapor pressure osmometry and proton NMR over a range of 0.025–1.0 M show similar concentration-dependent changes (Ts'o, 1969). At very low base concentrations the concentration of salt used here is essentially 0.14 M. As suggested by a reviewer, a complete charge neutralization under these conditions would account for the similarity in base stacking effects for both nucleosides and nucleotides. At base concentrations much greater than 0.14 M the concentration of counterions is essentially determined by the base concentration and can be near 1 M. However, these much higher concentrations of salt do not appear to significantly affect base stacking. We are unable to measure the influence of the pentose group on stacking since the solubility limit of adenine is about 0.003 M, near the low limit for nonresonance Raman spectroscopy. Our results for purine (not shown) show no repeatable concentration-dependent change from 0.04 to 1.0 M, perhaps indicating that the intensity ratio changes we observe in the spectra of nucleotides and adenosine are caused by electrostatic interactions of the C-6 amine. The face to face stacking interaction proposed by Ts'o and co-workers (Ts'o, 1970; Kondo et al., 1970) shows two bases superimposed but rotated about 45°. This arrangement brings into alignment five pairs of opposite electrostatic charges around the edge of the ring (Bonaccorsi et al., 1972) in which the C-6 amine figures prominently.

Our UV resonance Raman measurements are inconsistent with our visible Raman results, as well as with results from osmometry and NMR (Broom et al., 1967). No change is seen in the UV Raman spectra below 0.1 M base. We suspect that this is an artifact of the cell-free backscattering design of the sample recirculating apparatus. This design is required because of the high optical density of the sample solution to both the incident and Raman scattered light. As a consequence, a large fraction of the scattered light reaching the detector is from the liquid sample surface, where nucleotides may tend to partition and where the structural equilibrium is likely to be different from that in the bulk solution. (The visible Raman measurements are taken from a laser beam passing through the bulk solution in a glass capillary.) A reviewer has suggested that the inconsistency between the normal and resonance Raman results may be due to disruption of the stacked monomers by turbulence resulting from the pumping of the sample. However, the pump is used only to maintain a small reservoir from which the sample flows very slowly by gravity. The sample crosses the laser beam several seconds after being

pumped. The capillary film produced by this arrangement would not in our view produce turbulent flow, since there are virtually no surfaces near the laser spot to produce adequate friction or lamellar flow on a time scale comparable with the (probably) rapid base stacking equilibrium.

The generation of excited states due to the high power of the pulsed laser could also affect our UV Raman results. To test this possibility, we measured the spectrum of 1 and 100 mM ATP at power levels ranging from 23 mJ/pulse, the highest energy available at 266 nm, to less than 0.2 mJ/pulse, an energy just above the laser threshold and barely adequate to produce a spectrum after about 30 min of data collection. These spectra were identical. The total energy received by the sample also had no effect on the spectrum.

While our visible Raman results for AMP and ATP are consistent with results from osmometry for purine and nucleosides, they appear to be inconsistent with results from a hydrodynamic study of ATP self-association. Ferguson and co-workers (1974) have shown that, in solutions of ATP ranging from 1.5 mM at pH 4.7 to 30 mM at pH 3.0, self-association below 10 mM is much stronger than observed by others in solutions of purine and nucleosides (Ts'o, 1969). They further observe that no further association occurs in solutions above 10 mM ATP. We attribute these observations to the titration of the C-6 amine, which has a p$K$ of 4.2.

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**Registry No.** ATP, 56-65-5; AMP, 61-19-8; pA2′pA2′pA, 61172-40-5; d(pA3′pA3′pA), 14258-27-6; adenosine, 58-61-7; poly(adenylic acid), 24937-83-5; 2′-*O*-methyladenosine, 2140-79-6.

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