SELECTIVE ALKALI METAL ION COORDINATION IN THE SELF-ASSEMBLY OF 5'-GUANOSINE MONOPHOSPHATE

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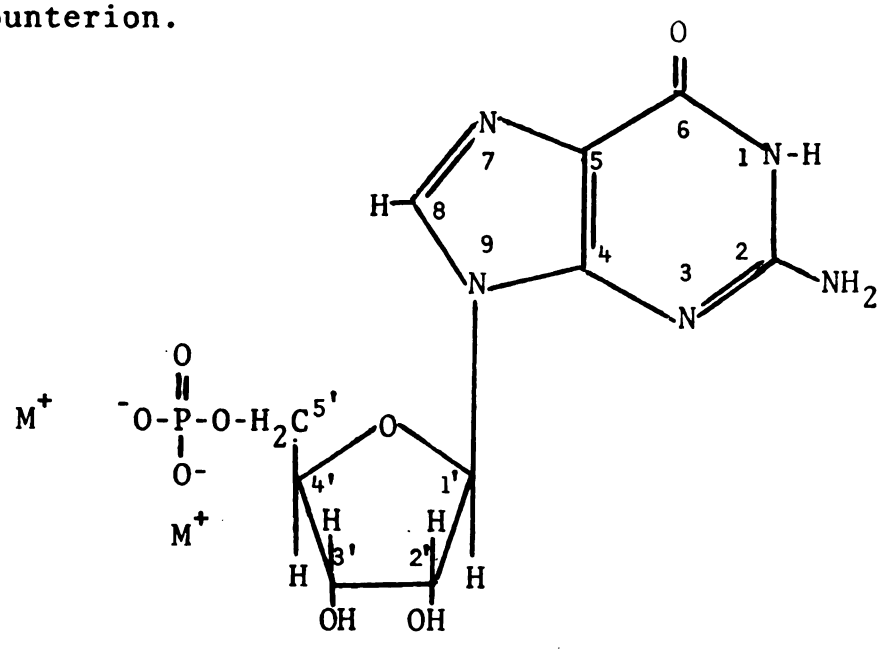
ABSTRACT

SELECTIVE ALKALI METAL ION COORDINATION IN THE SELF-ASSEMBLY OF 5'-GUANOSINE MONOPHOSPHATE

By

Christopher L. Marshall

Variable temperature NMR and IR studies have shown that the spontaneous intermolecular self-assembly of 5'-guanosine monophosphate [1] (5'-GMP) in aqueous solution (pH = 7.0 - 8.0) is dramatically dependent on the alkali metal counterion.



[1]

The type of structure, degree of structuring, and thermal stability of the formed structures have been shown to be dramatically different for each of the alkali metal cations.

These results show for the first time that selective alkali metal coordination by a nucleic acid in aqueous solution does occur, and that the coordination interaction can be important in the formation of regular, ordered structures involving intermolecular association of nucleotides.

The 220 MHz proton NMR spectra indicate that approximately three structures exist in solution for the potassium salt, two for rubidium, and at least one for cesium. The lithium salt shows no evidence for self-structuring ability. In all structuring cases, changing the alakali metal ion showed a difference in the number and chemical shift of the H(8) resonances observed. Previously reported self-assembled Na_2(5'-GMP) forms one or two self-structures[1] which are different from those of the remaining alkali metal ions.

Infrared studies have shown the melting temperature (T_m) for concentrated solutions of each structuring salt to be Na^+, 19°C; K^+, 29°C; Rb^+, 18°C; and Cs^+, 10 to 15°C. The results indicate that thermodynamic stability of structures is at a maximum when the counterion is K^+ and decreases as the charge to radius ratio is either increased or decreased.

The results indicate a size dependent coordination of the alkali metal ion by the nucleotide. This type of interaction is suspected of having a greater stability than that normally observed for ion dipole interactions between

the metal ion and the anionic phosphate group. Due to the similarities between the IR spectra of Na_2(5'-GMP) and the remaining structuring salts, it is likely that self-structuring in all salts involves formation of associated nucleotide units through hydrogen bonding between O(6) and N(7) as acceptors and N(1) and N(2) as donors. The hydrogen-bonded nucleotide units, perhaps tetramer units, then coordinate with selected alkali metal ions to form a regular, ordered structure that is slow to undergo chemical exchange. Although the physical data do not allow identification of the coordination mechanism, theoretical consideration of possible coordination at O(6) shows that base stacking should be selective in the size of cation allowed to coordinate.

REFERENCES

1. T. J. Pinnavaia, H. T. Miles, and E. D. Becker, J. Am. Chem. Soc., 97, 7198(1975).

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Christopher L. Marshall

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To Sue

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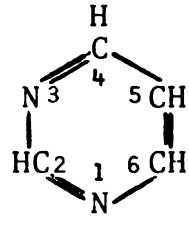
INTRODUCTION

Mononucleotides serve as the building blocks of the nucleic acids DNA and RNA and thus participate in the molecular mechanisms by which genetic information is stored, replicated, and transcribed throughout the cell. Nucleotides have also been shown to participate in intermediary metabolism, to act as factors to certain enzymatic processes, and to take part in energy transforming reactions. Mononucleotides are composed of three characteristic components: 1) a nitrogenous base, 2) a five carbon sugar, and 3) phosphoric acid.[1]

The nitrogenous bases are aromatic heterocyclic compounds which are classified as either pyrimidines [1] (six-membered rings) or purines [2] (pyrimidines with fused imidazole rings).



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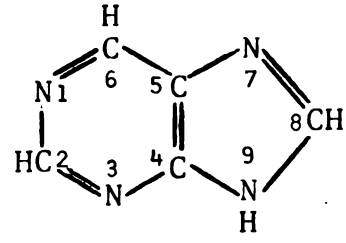
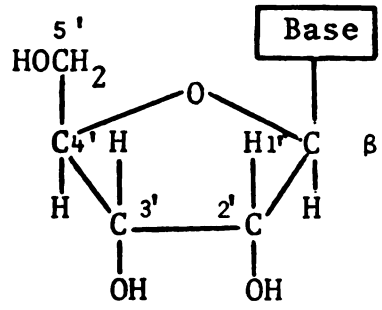


[2]

Cytosine, thymine, and uracil are the most important pyrimidines while the purines are represented by adenine and guanine. With the exception of thymine which is found only in DNA molecules and uracil which is found only in RNA, the bases are found in both forms of cellular polynucleotides. The aromatic rings may exist in one or more tautomeric forms. The nitrogenous bases alone have only limited water solubility.

Nucleosides are N-glycosides of the pyrimidine or purine bases, in which carbon atom 1 of the pentose (normally ribose) is glycosidically linked to nitrogen atom N(1) of a pyrimidine or to nitrogen atom N(9) of a purine.



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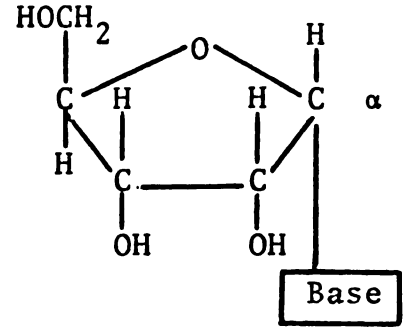


[4]

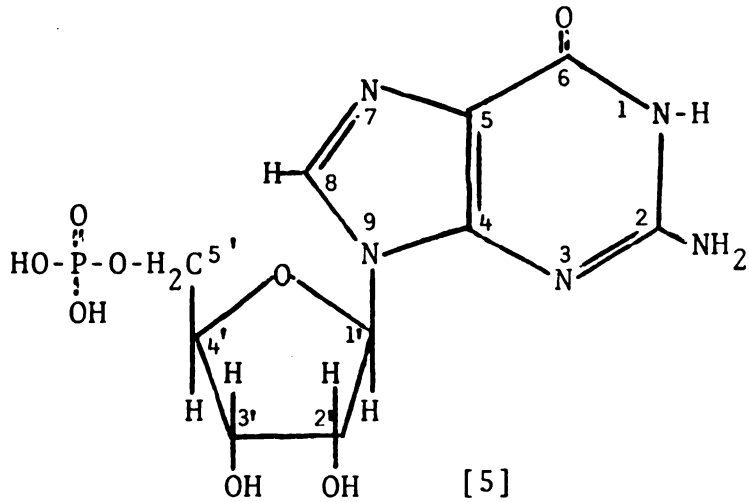
In the naturally occurring nucleosides the glycosidic linkage is always to the β epimer [3] (the α [4] has never been observed), and the pentose is always present in the furanose form. Nucleosides can be classed into two major types: the ribonucleosides, with D-ribose as the sugar

components, and the 2'-deoxyribonucleosides, which contain 2-deoxy-D-ribose. The most common ribonucleosides, adenosine, guanosine, cytidine, thymidine, and uridine are more water soluble than their parent bases.

Nucleotides are phosphoric acid esters of nucleosides in which phosphoric acid is esterified to one of the hydroxyl groups on the pentose. Although the phosphate group may be bound to the 2', 3', or 5' position on the pentose, the 5' isomer is by far the most abundant in the cell. The mononucleotides are strong acids with two dissociable protons on the phosphoric acid group having pK_a values of approximately 1.0 and 6.2. At neutral pH the free nucleotide is totally dissociated.

Mono-nucleotides are able to form hydrogen-bonded complexes between complimentary base-pairs as was first proposed by Watson and Crick for the double stranded helix formation of DNA.[2] This base-pairing is highly specific and has been studied for nucleosides by infrared spectroscopy in non-aqueous solvents.[3-6] Evidence for complimentary base hydrogen-bonding has also been shown using NMR spectroscopy in aqueous solution by following the change in chemical shifts of the amino and aromatic ring protons involved in the hydrogen-bonding.[7-9] Concentration and temperature dependent downfield chemical shifts of the hydrogen-bonded amino protons compared with the unassociated monomer were reported.

Guanylic acid (GMP) [5] and its derivatives have shown the unique ability to spontaneously form a regular, ordered, hydrogen-bonded polymeric structure in aqueous solution.



[5]

This ability has not been observed to date by any other nucleic acid component. The nature of this structuring is manifested in two forms.

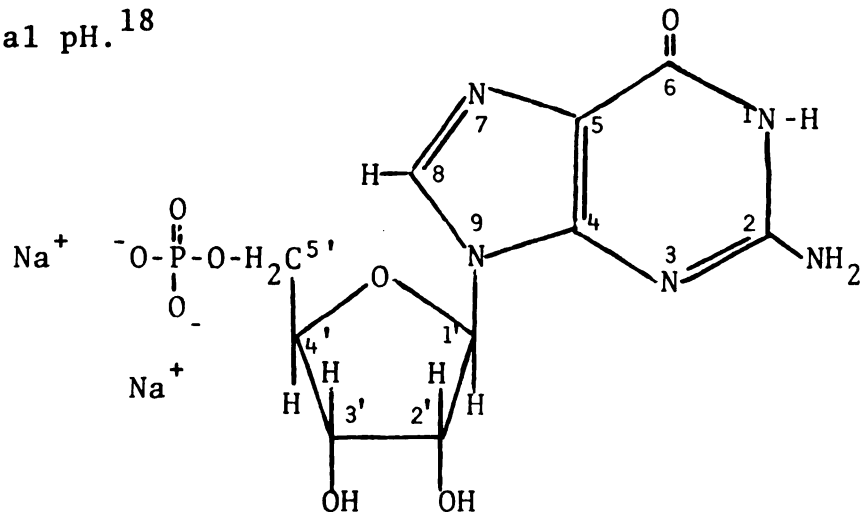
Bang reported in 1910 that concentrated solutions of guanylic acid, in acidic solution, formed a gel.[10] Several authors have investigated the structure of this gel (which is formed at pH ~ 5) by infrared spectroscopy,[11,12] optical rotatory dispersion (ORD),[13] and x-ray fiber diffraction.[14,15] Investigation of the fibrillar structure upon drying of the gel indicated that at pH = 5, helix formation of guanylic acid was occurring. A hydrogen-bonded structure of the bases in the gel was proposed. Based on these x-ray data, structures were proposed in which 5'-guanosine monophosphate (5'-GMP) consisted of continuous helixes with 15 nucleotide units in four turns[15] and 3'-guanosine monophosphate (3'-GMP) consisted of planar tetramer units (Figure 1)

Figure 1. Proposed structure of the hydrogen bonded tetramer unit in the 3'-GMP gel (ref. 14).

stacked in a helical array.[14] In both of these structures, nitrogen (1) and nitrogen (2) were proton donors, while oxygen (6) and nitrogen (7) were proton acceptors.

Miles and Frazier in 1972 reported ir evidence for a regular, ordered structure of 5'-GMP in neutral or slightly alkaline aqueous solution (pH ~8).[16] At this pH, both protons of guanylic acid are dissociated (pK_{a_2} = 6.1).[17] This structuring differed from that found in acidic solution for 5'-GMP by the lack of gel formation and different ir properties. Due to the similarities between the ir spectra of 3'-GMP in acidic media[11] and 5'-GMP in neutral or slightly alkaline media they postulated the structure of the 5'-GMP to be stacked planar tetramer units (Figure 1).

Pinnavaia, Miles, and Becker have recently shown that the proton NMR of Na_2(5'-GMP) [6] shows evidence for a regular ordered structure with slow chemical exchange at neutral pH.[18]



[6]

The latter property is unique to GMP in that all previously reported complexes involving monomer nucleotides, including mixed complexes of complimentary base pairs, undergo rapid chemical exchange and give time-averaged NMR spectra.[7-9] Pinnavaia, et al. found that at 19°C, a 0.23 M solution of Na_2(5'-GMP) gave a spectrum that was the same as that which had been preivously reported for the unassociated monomer.[9] Specifically, they observed that the H(8) resonance was a sharp singlet with a chemical shift of 8.17 ppm and that the ribose protons showed characteristic chemical shifts and splitting patterns. As the concentration was increased the self-assembly of the Na_2(5'-GMP) could be observed by the broadening of the H(8) monomer line and at 0.42 M by the appearance of two new H(8) resonances at 8.55 and 7.25 ppm. The chemical shift of these new peaks was independent of concentration indicating slow chemical exchange between these two new H(8) environments. At 0.83 M the H(8) resonance at 8.17 ppm had vanished and four well-resolved lines at 8.55, 8.26, 7.53, and 7.25 ppm were observed. The assignment of these resonances as being due to H(8) was confirmed by a decrease in their absolute intensities after exchange with D_2O under conditions where guanosine H(8) but not ribose C-H protons are replaced by deuterium.[19] An analogous behavior of the H(8) resonance was found with decreasing temperature. They stated that the upfield shift of the highest field H(8) resonance was due to the ring current shielding by the

purine bases which resulted from intermolecular base stacking of the tetramer plates. This upfield shift of 0.95 ppm, however, is quantitatively higher than those predicted from isoshielding contours calculated by Geissner-Prettre and Pullman.[20,21] The downfield shifts of the third and fourth highest field H(8) lines were presumed to be due to electric field effects caused by the nearness of the phosphate group (doubly ionized in Na_2-(5'-GMP) to the H(8) protons. The authors estimated that the highest and lowest field H(8) lines were from different proton environments within stacked tetramer plates due to the fact that the intensities, spin-lattice relaxation times (T_1), and line relaxation with added paramagnetic ion were nearly identical for the two resonances. They estimated the amount of tetramer stacking to be three which would yield a dodecamer. The nature of the other two H(8) resonances was not as clear but due to the fact that they had similar T_1's, they were assigned to the same structured unit, but most likely one that was different from the structure giving rise to the highest and lowest field H(8) lines. This would indicate the existence of either one or two types of structures in concentrated solutions of Na_2(5'-GMP) in aqueous solution at neutral pH.

Recently, Mettler has shown that the metal counter-ion has a complex forming role in the structure formation of M_2(5'-GMP) in neutral solution.[22] In her work, M was equal

to Li^+ or K^+. The 60 MHz proton NMR of aqueous 0.5 M Li_2(5'-GMP) shows no evidence of structure formation with change in temperature. The splitting of the ribose protons is unchanged unlike the structured spectrum of Na_2(5'-GMP), and the H(8) line remains a sharp single peak. The potassium salt, however, at 0.26 M and 32°C shows evidence of self-structuring which is different from that observed for the sodium salt. At this same temperature and concentration Na_2(5'-GMP) was completely unassociated.[18] In the limiting spectra of 1.13 M, four incompletely resolved H(8) lines were observed at 7.60, 7.30, 7.10, and 6.50 ppm. This is in contrast to the 0.83 M solution of Na_2(5'-GMP) in which four well-resolved lines at 8.55, 8.26, 7.53, and 7.25 ppm are observed.

This is direct evidence for a structure forming role of the metal ion in neutral solutions of M_2(5'-GMP). Mettler states that if the only role of the alkali metal were that of a counter-ion then we could expect only a slight modification of the chemical shift with structure formation. However, the NMR spectra of Li_2(5'-GMP), Na_2(5'-GMP), and K_2(5'-GMP) are too significantly different to rule out this possibility. The lithium salt shows no evidence for structure formation while the sodium and potassium salts differ in the nature and stability of their structures. This implies that the structure formation of M_2(5'-GMP) is dependent upon coordination of the metal and that the nucleotide is selective in its choice of

coordinating metal. In her work, Mettler suggested that the selectivity of complexation is controlled by the ionic radius of the alkali metal.[22] Lithium (r = 0.68 Å) would appear to be too small for complexation and sodium (r = 0.97 Å) and potassium (r = 1.33 Å) have significantly different ionic radii, affecting the nature and thermodynamic stability of their respective complexes.

Mettler's work has indicated that the metal ion is an integral part of the structuring of M_2(5'-GMP). The present study is aimed at investigating further which metal ions are able to form these complexes and what their relative stability is with respect to each other. If size is a limiting factor in this complexation, it might be expected that a metal cation too large for complexation may be found to prove this assumption.

EXPERIMENTAL

A. Materials, Techniques, and Preparation

5'-guanosine monophosphate was purchased as the disodium salt monohydrate (Na_2-5'-GMP) from P-L Biochemicals, Inc. and from Calbiochem, Inc. The nucleotide was used without further purification. Paramagnetic ion inpurities were removed from the distilled water by a Millipore Corporation, Milli-Q water purification system. Exchange of metal ions was accomplished using a Dowex 50W-X8 (100-200 mesh) cation exchange resin obtained from the Dow Chemical Company. The resin has an exchange capacity of 1.7 meq/ml[23] and a ten-fold excess of the replacing cation was used to ensure complete exchange. The wet resin was prepared by washing with: 1) 3 N hydrochloric acid to remove soluble impurities and to ensure that all of the resin is in the H^+ form, 2) 10^{-3} M disodium ethylenediamine-tetraacetate to remove any dissociable paramagnetic impurities, and 3) 1 N solution of the desired metal ion hydroxide in order to exchange the cationic resin with the desired metal ion. An aqueous solution of Na_2-5'-GMP was passed through a column packed with the exchanged resin. Flow rate was controlled to 3 ml/min to ensure complete metal ion exchange. The exchanged M_2-5'-GMP was lyophilized

to a dry powder, then re-lyophilized twice from a 0.1 M solution of the salt in 99.5% D_2O obtained from Matheson, Coleman, and Bell, Inc. to ensure that D_2O had been exchanged for the hydrated water. NMR and IR samples were prepared using 100% D_2O purchased from Aldrich Chemical Company. Sodium 2,2,3,3,-d_4-3-trimethylsilylpropionate (NaTSP) purchased from Merck and Company, Inc. was added to all NMR samples as an internal reference (0.1 wt%). A 3×10^{-3} M NaTSP solution had a pH of 7.90.

B. Instrumentation

1. Nuclear Magnetic Resonance Spectroscopy

Initial proton magnetic resonance spectra were obtained with a 60 MHz Varian A56/60 spectrometer. Probe temperature was regulated with a Varian V-6040 temperature controller. The probe temperatures were measured by the chemical shift differences between the proton resonances of methanol (0-40°C) and ethylene glycol (40-90°C) using the Van Geet equations.[24] Maximum radio-frequency field strengths and spectrum amplitudes were necessary to maximize spectra resolution. Due to the low resolution of the 60 MHz spectra, PMR spectra were also taken on a 220 MHz Varian Associates HA-220 proton NMR spectrometer. The spectra were provided by Dr. C. Fisk of the National Institutes of Health, Bethesda, MD. Probe temperatures were determined by the same method as described above.

2. Ultra-Violet Absorption Spectroscopy

Concentrations of specific metal ion 5'-guanosine monophosphate solutions were determined by UV absorption using a Gilford model 252 UV-visible spectrometer which is an updated version of a Beckman DU. Solution concentrations were determined using Beer's Law:[25]

$$A = \varepsilon bc$$

where A is the absorption, ε is the molar absorptivity in $\underline{M}^{-1}$ cm^{-1}, b is the cell length in centimeters, and c is the concentration in moles per liter. The molar absorptivity (ε) for Na_2-5'-GMP is 1.37×10^4 $\underline{M}^{-1}$ cm^{-1} at its absorption maximum of 252 nm.[26] The molar absorptivity was assumed to be constant while the metal ion was changed. The length of the quartz cells used was one centimeter.

3. Infrared Spectroscopy

Infrared spectra were taken on a Perkin-Elmer 225 Grating Infrared Spectrometer. Matched and sealed CaF_2 cells purchased from Perkin-Elmer Company with a path length of 15μ were modified with a water jacket to enable the temperature of the cell to be controlled. Water jacket temperature was controlled by a Forma Scientific model 2095 Bath and Circulator and was monitored using a copper-constantan thermocouple mounted on the cell window.

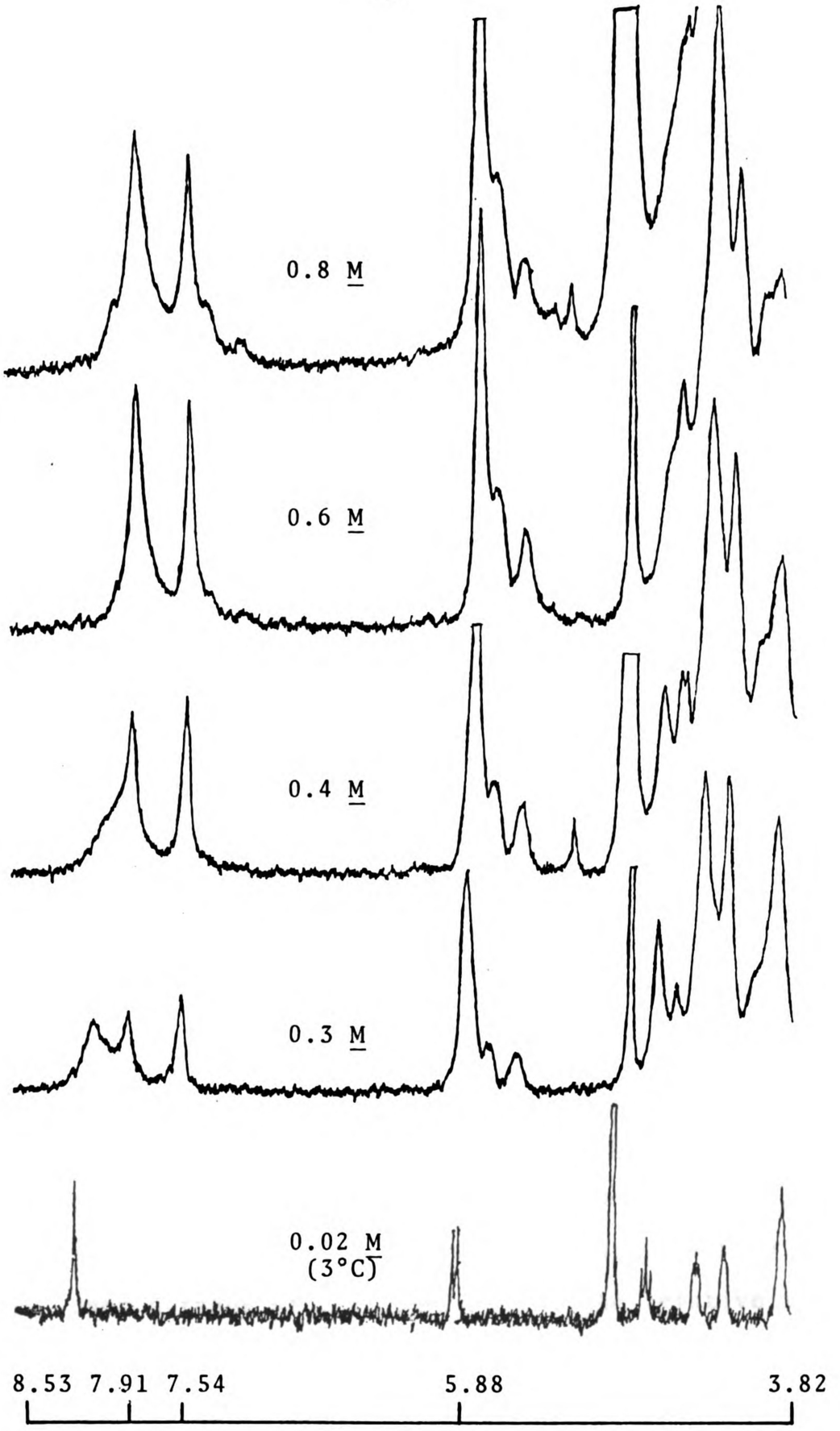
RESULTS

A. NMR Investigation

1. K_2(5'-GMP)

Figure 2 shows the concentration dependence of the 220 MHz proton NMR spectrum of K_2(5'-GMP) at 18°C. The spectrum of the unassociated monomer at 0.02 M and 3.0°C is the same as that which has been previously noted for the unassociated monomer of Na_2(5'-GMP).[18,19] The H(8) line is a sharp singlet with a chemical shift of 8.17 ppm ($\nu_{1/2}$ = 3.1 Hz) and the ribose region of the spectrum (3.9 - 5.9 ppm) shows a characteristic simple splitting pattern for H(1') at 5.88 ppm (doublet, J = 5.9 Hz), H(2') at 4.75 ppm (triplet, J = 5.4 Hz), H(3') at 4.45 ppm (triplet, J = 3.5 Hz), H(4') at 4.28 ppm (doublet, J = 3.0 Hz), and 2 H(5')'s at 3.94 ppm (a pair of superimposed doublets J ≈ 2.5 Hz). As the concentration increases and structure formation begins, the proton resonances in the ribose region become much broader and the splitting pattern becomes obscured. In the H(8) region at 0.3 M the monomer peak at 8.17 ppm has broadened ($\nu_{1/2}$ ≈ 39.5 Hz) and two new lines of approximately equal intensity have appeared at 7.91 and

Figure 2. Concentration dependence of the proton NMR spectrum of K_2(5'-GMP) in D_2O at 18°C. The chemical shift scale is in ppm downfield from NaTSP as internal reference.


0.8 M
0.6 M
0.4 M
0.3 M
0.02 M
(3°C)
8.53 7.91 7.54
5.88
3.82

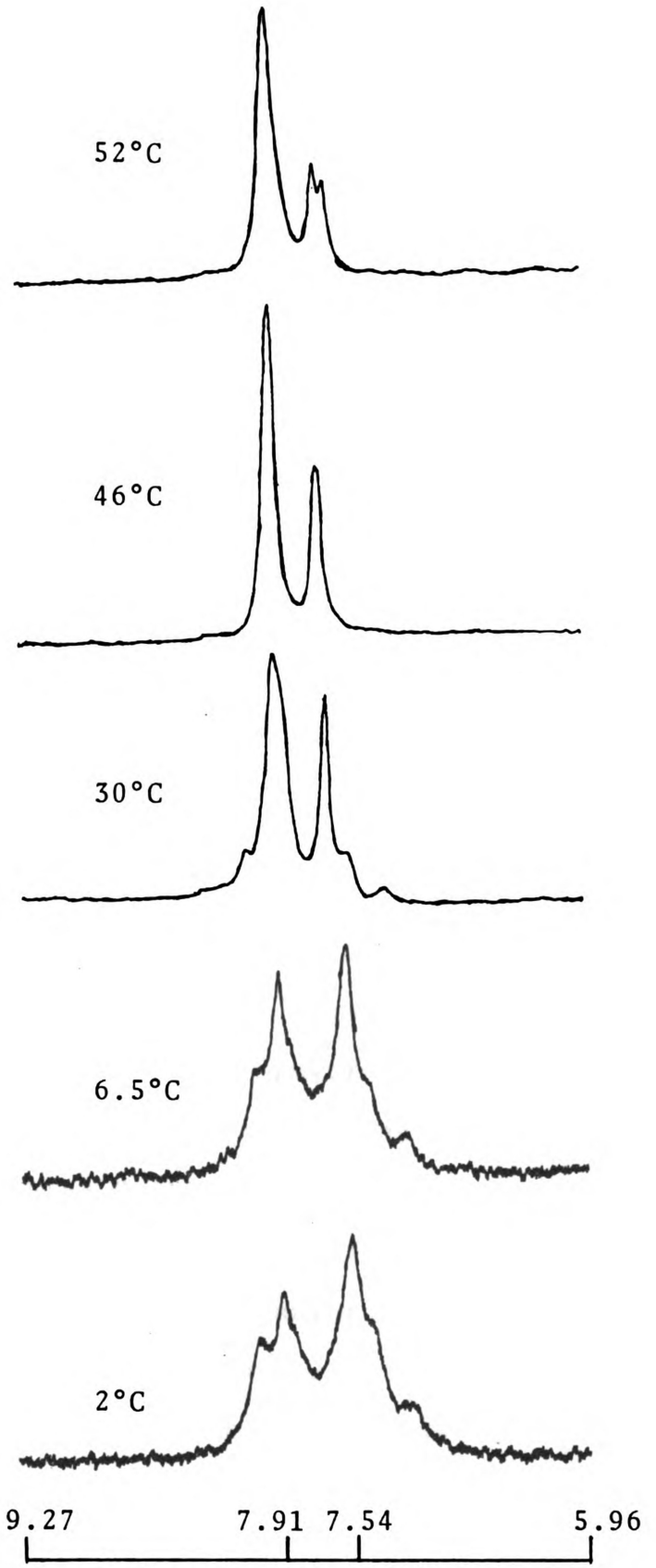
7.54 ppm. It should be noted that at this concentration and temperature the sodium salt is completely unstructured.[16,18] When the concentration is further increased to 0.4 M the monomer H(8) line has almost completely disappeared and the peaks at 7.91 and 7.54 ppm have sharpened and increased in intensity. At 0.6 M the monomer line has totally vanished, the structured peaks at 7.91 and 7.54 ppm have become more intense, and three new incompletely resolved resonances at 8.04, 7.44 and 7.23 ppm have appeared. The spectrum of the most concentrated solution (0.8 M) that is shown for this temperature shows little difference from the spectrum at 0.6 M except for the fact that the peaks at 8.04, 7.44 and 7.23 ppm are now slightly better resolved. There is also a possible proton resonance on the upfield side of the 7.91 ppm but its intensity is weak.

This confirms what Mettler[22] observed in her 60 MHz NMR investigation. It is readily apparent that the potassium salt is quite different in its behavior as compared to the sodium salt. The limiting spectrum of the sodium salt exhibits four H(8) lines over a chemical shift range of 1.30 ppm whereas the spectrum of the potassium salt shows at least five H(8) resonances over a range of 0.81 ppm. Also, the relative intensities of the H(8) lines differ in the two complexes. Pinnavaia, et al.,[18] estimated the number of

different complexes in Na_2(5'-GMP) to be either one or two. Inspection of the spectra with changing concentration for K_2(5'-GMP) suggests that it contains two different complexes also, but with different overall structure from Na_2(5'-GMP). The two intense central peaks at 7.91 and 7.54 ppm seem to behave identically and have nearly the same peak area. These could arise from one type of structure with two non-equivalent H(8) environments. The three remaining peaks at 8.04, 7.44 and 7.23 ppm appear simultaneously and could arise from three other non-equivalent H(8) environments in a second type of structure. However, inspection of the temperature dependence of the K_2(5'-GMP) complex (Figure 3) suggests that more than two different structures are probably present.

Figure 3 shows the H(8) region of the proton NMR spectra for a 0.94 M solution of K_2(5'-GMP) between 52° and 2°C. At 52°C, a strong peak is observed at 7.85 ppm and two less intense and incompletely resolved resonances occur at 7.60 and 7.55 ppm. The latter two lines collapse to a single line at 46°C. Two important observations can be gathered by comparing this spectrum with the spectra reported in Figure 2. First, the chemical shifts in the potassium complex, unlike those of Na_2(5'-GMP), are temperature dependent, and second, the two most intense lines noted in the concentration dependent spectra (Figure 2) are of unequal intensity.

Figure 3. Temperature dependence of the H(8) resonance of 0.94 M K_2(5'-GMP) in D_2O. Chemical shift scale is in ppm downfield from NaTSP as an internal reference.


52°C
46°C
30°C
6.5°C
2°C
9.27
7.91
7.54
5.96

This would imply that these two lines are not from the same structure in K_2(5'-GMP). Decreasing the temperature to 46°C results in a sharpening of the 7.85 ppm resonance and the pseudo-unresolved doublet (7.60 and 7.55 ppm) becomes a much sharper singlet with a chemical shift of 7.61 ppm. At 30°C the downfield peak broadens and remains in the same position but the 7.61 ppm peak has moved upfield to 7.58 ppm and has sharpened. Also, the resonances at 8.01, 7.43 and 7.24 ppm have appeared. A further decrease in temperature to 6.5°C results in significant changes in relative intensities and small chemical shift changes. Most noteworthy is the inversion of relative intensities for the two most intense lines near 7.91 and 7.54 ppm. It can be seen that the equal intensities observed for these two lines at 18°C in the concentration-dependence studies (cf. Figure 2) was fortuitous. The 2°C spectrum shows little change from that at 6.5°C.

With this information the number of different structures present in an aqueous solution of self-assembled K_2(5'-GMP) can be estimated. The peak at 7.91 ppm, due to its different behavior from the peak at 7.54 ppm with decreasing temperature, has been assigned to a structured unit with a single H(8) environment. A separate structure with two H(8) environments is assigned to the 7.54 ppm resonance. The presence of

two H(8) environments is supported by the splitting observed at 52°C. Finally, the peaks at 8.01, 7.44 and 7.24 ppm which behave identically with change in concentration (Figure 2) and with change in temperature (Figure 3) have been assigned a third structural unit with three inequivalent H(8) environments. This indicates that for K_2(5'-GMP) there are at least three separate types of structures that can exist in aqueous solution versus a maximum of two as reported by Pinnavaia, et al. for Na_2(5'-GMP).[18]

2. Rb_2(5'-GMP)

The temperature dependence of the proton NMR of 0.67 M Rb_2(5'-GMP) is shown on Figure 4. At 22.5°C, Rb_2(5'-GMP) has the characteristics of the random monomer that has been previously observed in the unassociated form of sodium and potassium 5'-GMP. The H(8) singlet appears in the normal 8.17 ppm position and the ribose C-H protons are the normal multiplets with the usual chemical shifts. As the temperature is lowered to 19°C, the ribose lines again begin to broaden and splitting is obscured. The H(8) line has remained at 8.17 ppm but has broadened appreciably from a $\nu_{1/2}$ value of 5 Hz (22.5°C) to 35 Hz (19°C). At 2°C the monomer H(8) peak has broadened further ($\nu_{1/2}$ = 85 Hz) and two new resonances at 7.66 and 7.22 ppm have also appeared. An analogous behavior is

Figure 4. Temperature dependence of the H(8) resonance of 0.67 M Rb_2(5'-GMP) in D_2O. Chemical shift scale is in ppm downfield from NaTSP as an internal reference.

found when the temperature is held constant and the concentration increased.

It is readily apparent that the structures formed from complexation of Rb^+ are different from either Na^+ or K^+. One obvious anomaly is that in the 2°C spectrum the monomer peak is still strong and more intense than any other H(8) peak. This could indicate that while the cation is capable of complexing the nucleotide, the majority of the 5'-GMP remains as the random, unstructured salt. On the other hand, the broadening of the line could mean that it arises from a structured form which happens to have the same chemical shift as the unassociated monomer. The two peaks at 7.66 and 7.22 ppm have different areas and therefore could arise from two different structures, each having only one distinguishable H(8) environment. These results indicate the presence of at least two and perhaps three structures in the Rb_2(5'-GMP) system.

3. Cs_2(5'-GMP)

The spectra of 0.6 M Cs_2(5'-GMP) at 21°C and 2°C are shown in Figure 5. The high temperature spectrum shows the same characteristics noted previously for the unstructured monomer. The 2°C spectrum shows a strong, sharp H(8) monomer line with broad resonances at 8.28, 7.66 and 7.18 ppm. Splitting of the H(1') signal (5.81 ppm) is still seen, which further confirms

Figure 5. Temperature dependence of the proton NMR spectrum of 0.6 M Cs_2(5'-GMP) in D_2O. Chemical shift scale is in ppm downfield from NaTSP as an internal reference.

the assignment of the most intense H(8) peak to unassociated monomer. In all other cases in which structure formation by the metal ion has been noted, the ribose splitting is lost by the broadness of the line. This could be just a function of the small amount of complex formed in the Cs_2(5'-GMP) solution. Cs_2(5'-GMP), therefore, is able to form a small amount of at least one structure which is different from any previously observed. A closer estimate as to the number of different structures for this salt will have to await more data.

4. Li_2(5'-GMP)

The analysis of Cs_2(5'-GMP) brought up a question as to the ability of Li_2(5'-GMP) to form a self-structure. In her work at 60 MHz, Mettler found no evidence for structure formation.[22] Since weak structure peaks similar to those found at 220 MHz for Cs_2(5'-GMP) could be obscured by the poorer resolution at 60 MHz, the lithium salt was reinvestigated at 220 MHz to verify that there was no self-structuring of Li_2(5'-GMP).

Figure 6 shows the spectra at 52°C and 1°C of a 0.65 M solution of Li_2(5'-GMP). Except for a slight line broadening and with it an obscuring of the ribose splittings, the 1°C spectrum shows no evidence for structure formation, in agreement with Mettler's findings.[22]

Figure 6. Temperature dependence of the proton NMR spectrum of 0.65 M Li_2(5'-GMP) in D_2O. Chemical shift scale is in ppm downfield from NaTSP as an internal reference.

5. Other Monovalent Cation Salts of 5'-GMP

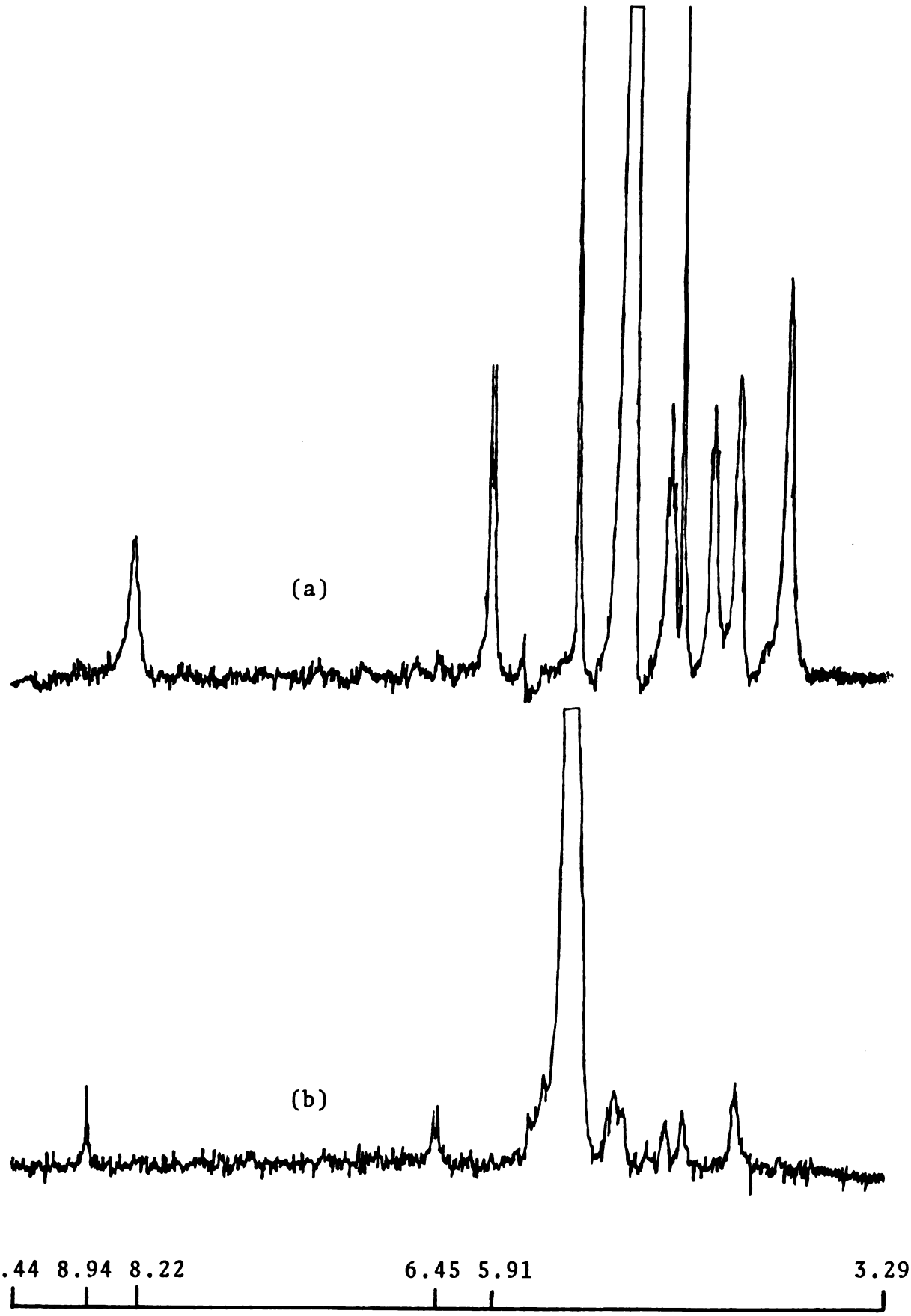
With the above information, a search was conducted for other monovalent cations which might also form self-structures, adding support to this theory. (Mettler found the divalent Mg^{2+} and Ca^{2+} salts to be too insoluble to give resonable spectra[22]). Ag^{+} and Tl^{+} were investigated along with the non-metallic cations NH_4^{+} and $N(CH_3)_4^{+}$.

The ammonium ion was too acidic a cation for 5'-GMP. It protonated the phosphate group (pKa_2 = 6.1), giving a characteristic gel which was inadequate for NMR investigation. The tetramethylammonium ion showed a slight line broadening ($\nu_{1/2}$ for H(8) of 12.5 Hz) which was attributed to increased viscosity but showed no other evidence for structure formation at a concentration of 1.0 M and a temperature of 1°C.

Figure 7 shows the 2°C spectra of saturated solutions of the thallium and silver salts of 5'-GMP. The thallium salt, which was saturated at 0.09 M, shows only a single monomer H(8) peak occurring at 8.22 ppm with the significant peak broadening ($\nu_{1/2}$ = 15 Hz) due to the solution's high viscosity. The lack of expected structure formation is attributed to the low solubility of the salt.

The silver salt gave an insoluble gel with a pH of 3.4. A search of the literature revealed that silver is believed to selectively coordinate to the monomer

Figure 7. Proton NMR spectra of a) 0.09 M Tl_2(5'-GMP) and b) 0.17 M Na_2Ag(5'-GMP) in D_2O at 2°C. Chemical shift scale is in ppm downfield from NaTSP as an internal reference. Both solutions are saturated.


(a)
(b)
9.44 8.94 8.22
6.45 5.91
3.29

at O(6) and N(7).[27] The mechanism proposed (Figure 8) requires the loss of the N(1) proton, which would make the solution sufficiently acidic to protonate the ribose phosphate and induce gel formation. This system should remain a monomer in neutral solution due to the fact that both of the hydrogen bond acceptors for the tetramer (O(6) and N(7)) are coordinated to the silver (see Figure 1). The gel was back-titrated with NaOH to neutrality and the spectrum of the mixed salt (formula Na_2Ag(5'-GMP)) was taken. This showed only a single H(8) resonance as would be expected, although the peak was shifted downfield to 8.94 ppm.

6. Summary of NMR Data

Table I summarizes the NMR data that has been collected on M_2(5'-GMP). It is readily apparent upon inspection of the right hand column of every structure forming metal that there is a noticeable difference between the types of structures formed upon changing the metal ion.

B. Infrared Investigation

The IR of the unstructured monomer for a 0.64 M solution of Na_2(5'-GMP) at 50°C is shown on Figure 9. This corresponds well with the spectrum reported by Miles and Frazier[16] for the same salt. The band assignment has been

Figure 8. Proposed mechanism for coordination of Ag^+ to guanosine derivatives (cf. ref. 27).

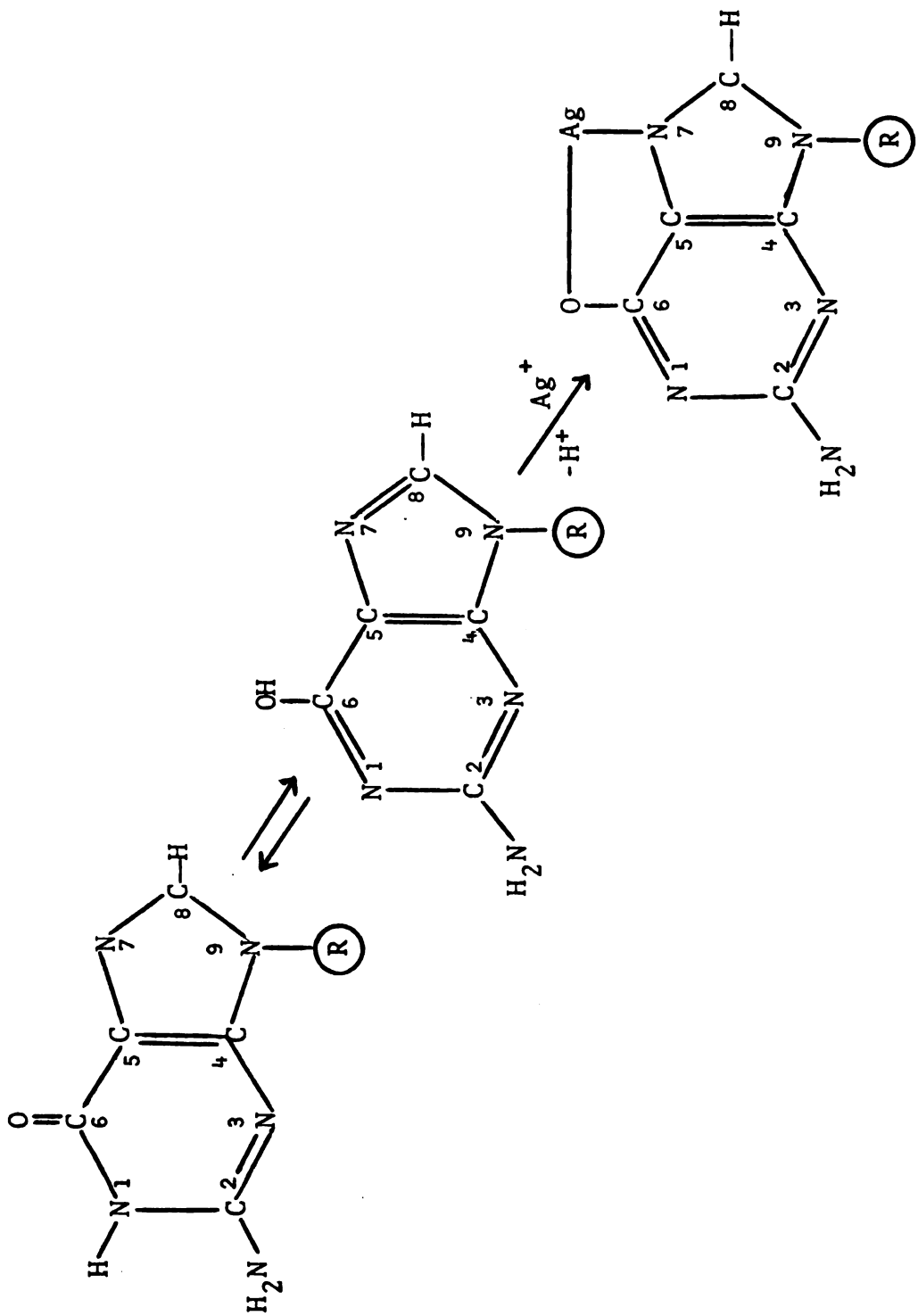


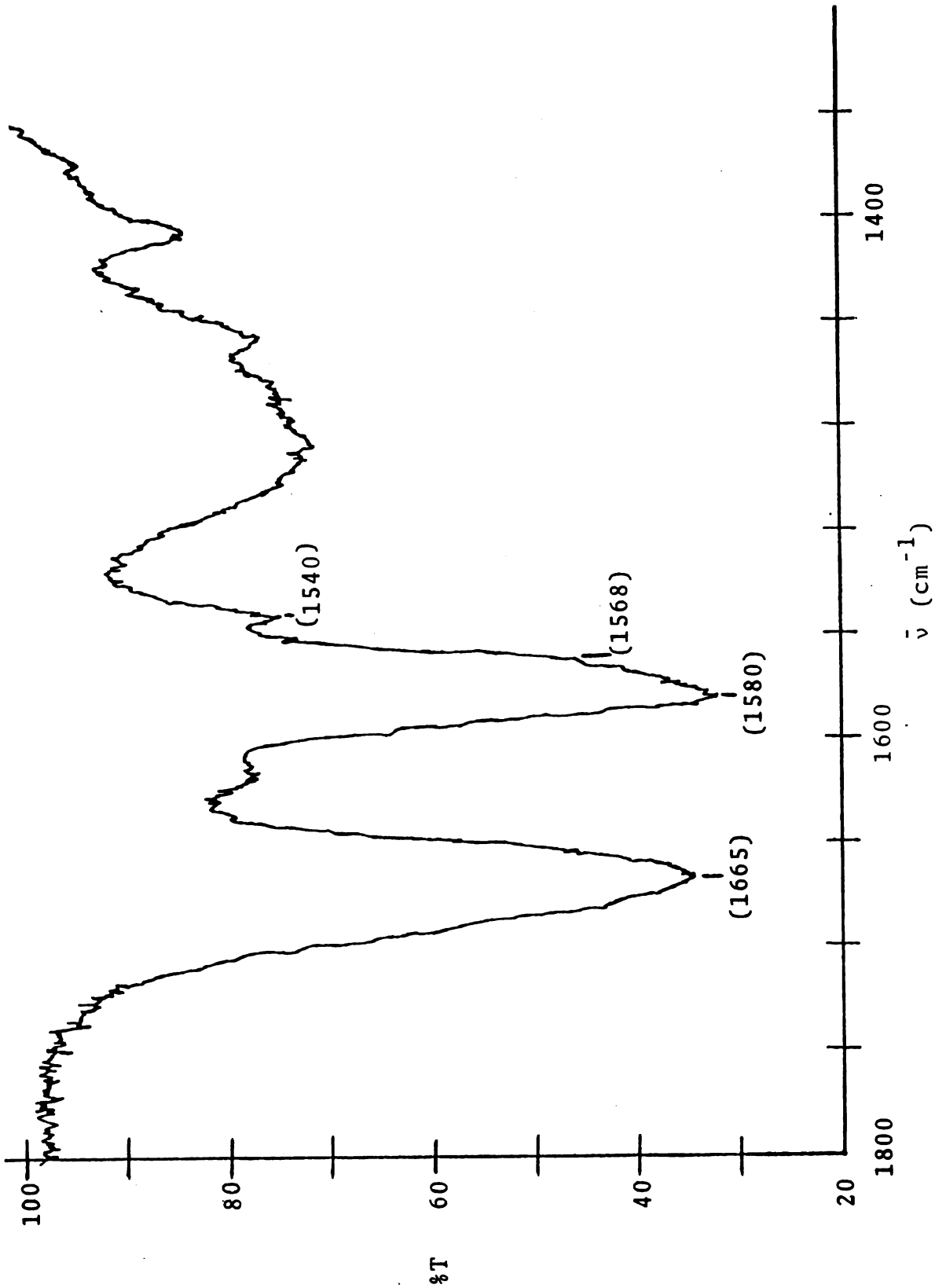
Table I

Chemical Shifts of H(8) and H(1') Resonances of M_2(5'-GMP) Salts in D_2O Solutions

M^+ =	Li		Na*		K*		Rb*		Cs*		Ag		Tl	
TEMP[a] =	53	1	19	2	3	2	2.25	2	21	2	22	2	22	4.7
CONC[b] =	0.65	0.65	0.23	0.60	0.02	0.94	0.67	0.67	0.60	0.60	0.17	0.17	0.09	0.09
H(8) Resonances[c]	8.14	8.14	8.17	8.55 8.26 7.53 7.25	8.17	8.01 7.91 7.90 7.54 7.44 7.23	8.17	8.17 7.66 7.22	8.17	8.28 8.16 7.66 7.18	8.94	8.94	8.22	8.22
H(1') Resonances	5.86	5.86	5.88	5.93 5.86 5.70 5.57 5.49 5.45	5.88	5.79 5.64 5.41 5.11	5.89	5.89 5.45	5.81	5.94 5.93 5.81	6.45	6.45	5.91	5.91

* Structuring Salts. [a] Temperature is in degrees centigrade. [b] Concentration is in molarity units. [c] All chemical shifts are in ppm downfield from NaTSP as internal standard.

Figure 9. Infrared spectrum of 0.64 M Na_2(5'-GMP) in D_2O at 50°C (unstructured).

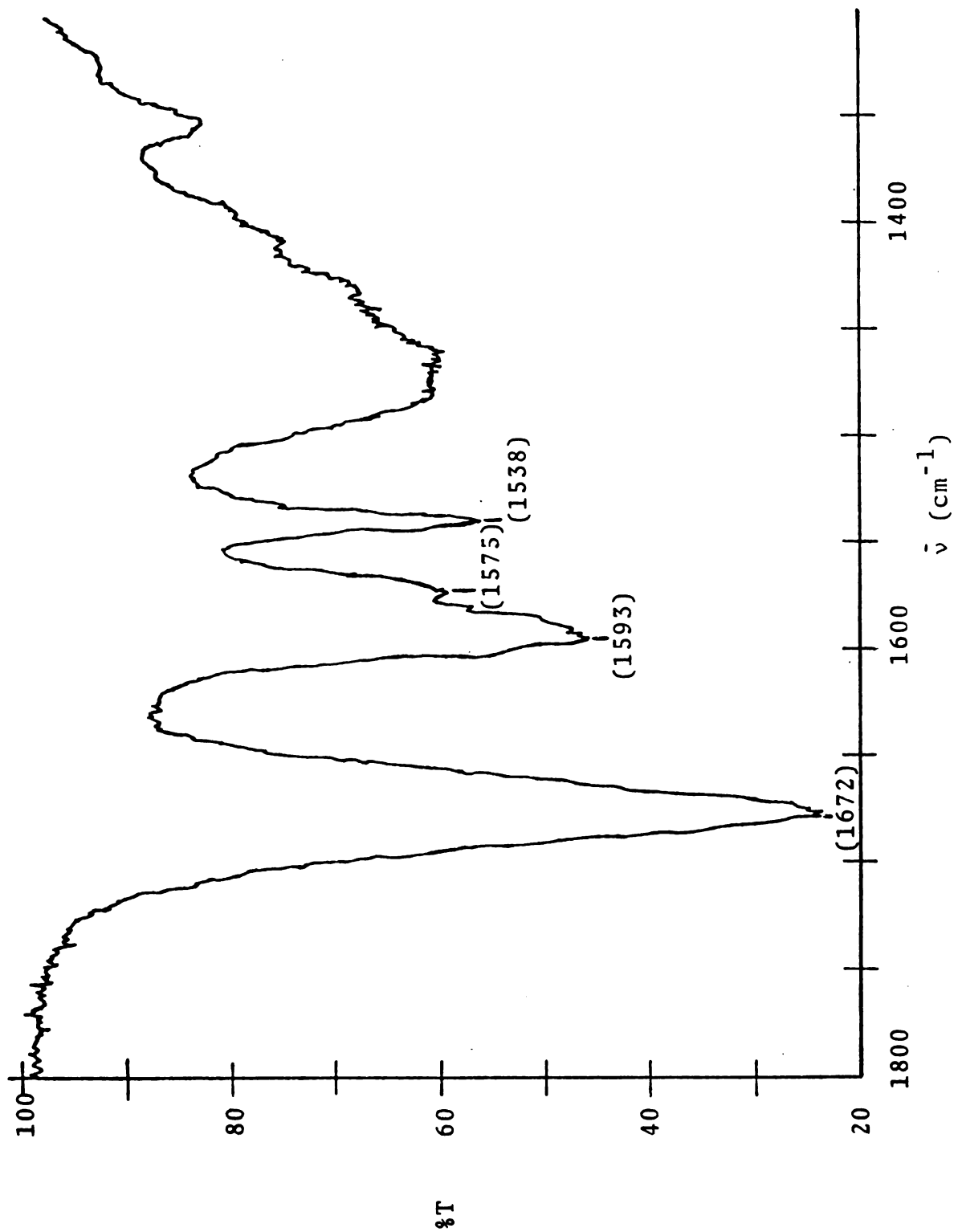

100
80
60
40
20
%T
1800
1600
1400
$\bar{\nu}$ (cm^{-1})
(1665)
(1580)
(1568)
(1540)

discussed quite extensively in the literature.[12,13,16,28] The absorption at 1665 cm^{-1} is assigned to a carbonyl stretching vibration and the shoulder at 1568 cm^{-1} to a normal mode which involves, to a much smaller extent, some motion of the carbonyl oxygen. The intense band at 1580 cm^{-1} is due largely to the C(4) = C(5) stretching vibration in the ring and the 1540 cm^{-1} absorption has its major contribution from a C(8) = N(7) vibration.

As the temperature is lowered and the nucleotide begins to self-assemble and the spectrum changes due to hydrogen bonding. The limiting spectrum is shown on Figure 10. This spectrum shows a marked change from the unassociated nucleotide. The carbonyl band at 1672 cm^{-1} becomes sharper ($\Delta\bar{\nu}_{1/2}$ from 28 cm^{-1} to 23 cm^{-1}) and has shifted to higher energy. The vibrations between 1550 cm^{-1} and 1600 cm^{-1} undergo a large intensity decrease upon structuring and have maxima at 1593 cm^{-1}, 1585 cm^{-1}, and 1570 cm^{-1}. The C(8) = N(7) ring vibration at 1540 cm^{-1} in the unassociated nucleotide is intensified in the ordered form and is shifted slightly to 1538 cm^{-1}.

Miles and Frazier[16] plotted temperature profiles of infrared absorbances in order to find out two pieces of information, the T_m(melting temperature) and the degree of cooperativity of the self-structuring process. The degree of cooperativity is defined by the temperature range over which the nucleotide goes from structured to unstructured form. The smaller the temperature range, the greater the

Figure 10. Infrared spectrum of 0.64 M Na_2(5'-GMP) in D_2O at 10°C (structured).


100
80
60
40
20
%T
1800
1600
1400
$\bar{\nu}$ (cm^{-1})
(1672)
(1593)
(1575)
(1538)

cooperativity in structure formation. This corresponds to a large positive value of ΔH upon solution "melting" of the self-structure. The T_m is defined as the temperature at which half of the molecules are unstructured for a given solution. Miles and Frazier[16] found T_m = 9°C for a 0.1 M solution of Na_2(5'-GMP) and Pinnavaia, et al.[18] arrived at a value of 13.5°C for a 0.26 M solution.

Figure 11 shows a plot of absorption at 1579 cm^{-1}, the most intense absorption in the unassociated nucleotide, and at 1672 cm^{-1}, the most intense absorption in the associated nucleotide, versus temperature. The degree of cooperativity is the temperature range between the upper and lower plateau and the T_m is the temperature at the mid-point of the curve. From Figure 11, for 0.64 M Na_2(5'-GMP), the T_m is found to be 19°C.

These types of melting curves are useful if there is only one structure type formed in the solution, or, if there is more than one, if the molar absorptivity for the investigated band does not depend on the nature of the self-structure. This criterion has been met for Na_2(5'-GMP).

Because of the information that can be obtained from these variable temperature IR studies about the thermodynamic stability of the nucleotide complexes, an examination of the infrared spectra of the structuring salts was undertaken. Figures 12, 13, and 14 show the IR spectra of structured solutions of K_2(5'-GMP), Rb_2(5'-GMP), and Cs_2(5'-GMP) respectively. The spectrum of Li_2(5'-GMP) at low

Figure 11. Plot of absorbance at 1579 cm^{-1} (•) and 1672 cm^{-1} (■) bands of 0.64 M Na_2(5'-GMP) in D_2O as a function of temperature.

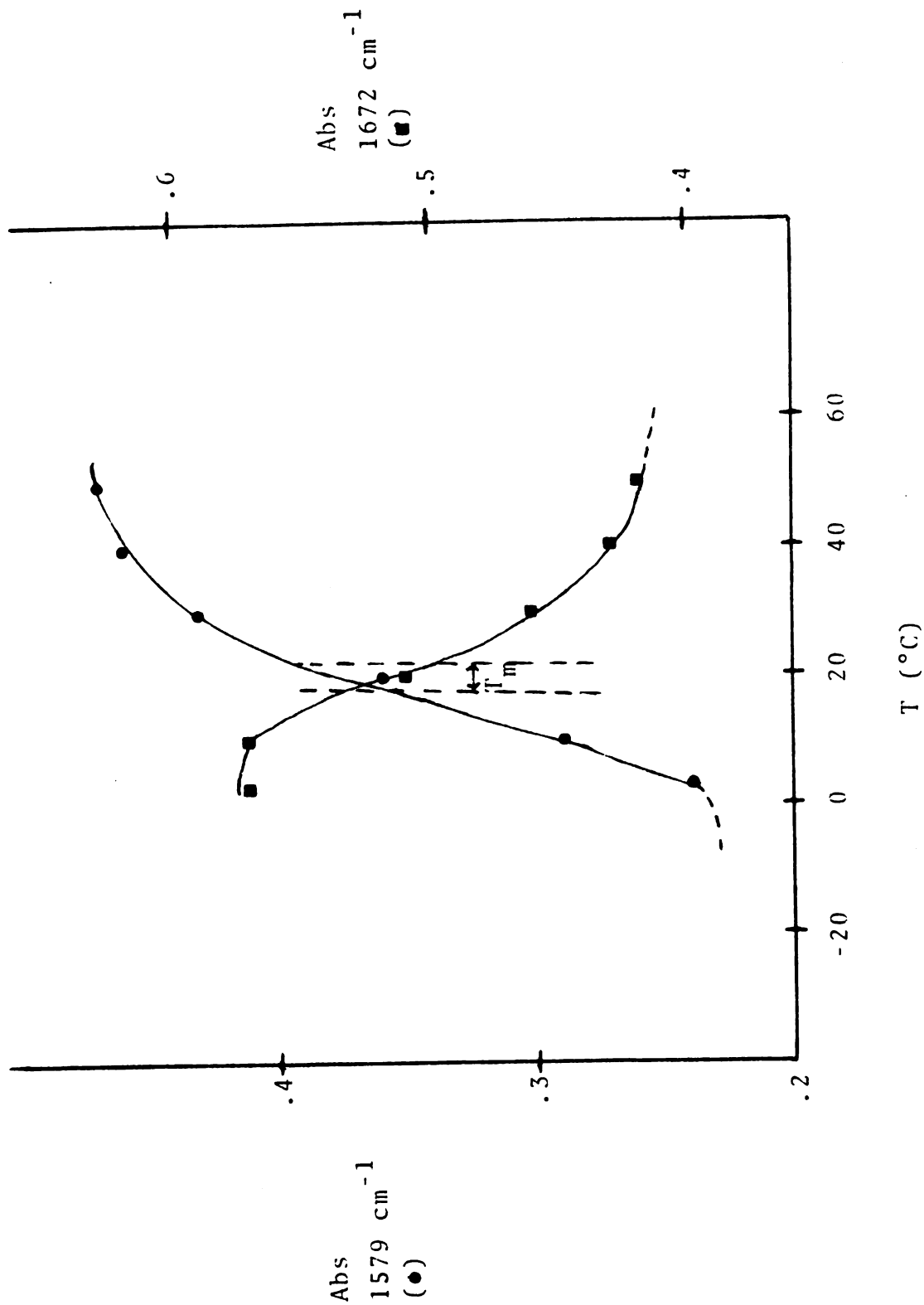

Abs
1672 cm-1
(■)
.6
.5
.4
Abs
1579 cm-1
(●)
.4
.3
.2
-20
0
20
40
60
T (°C)
Tm

Figure 12. Infrared spectrum of 0.59 M K_2(5'-GMP) in D_2O at 10°C (structured).

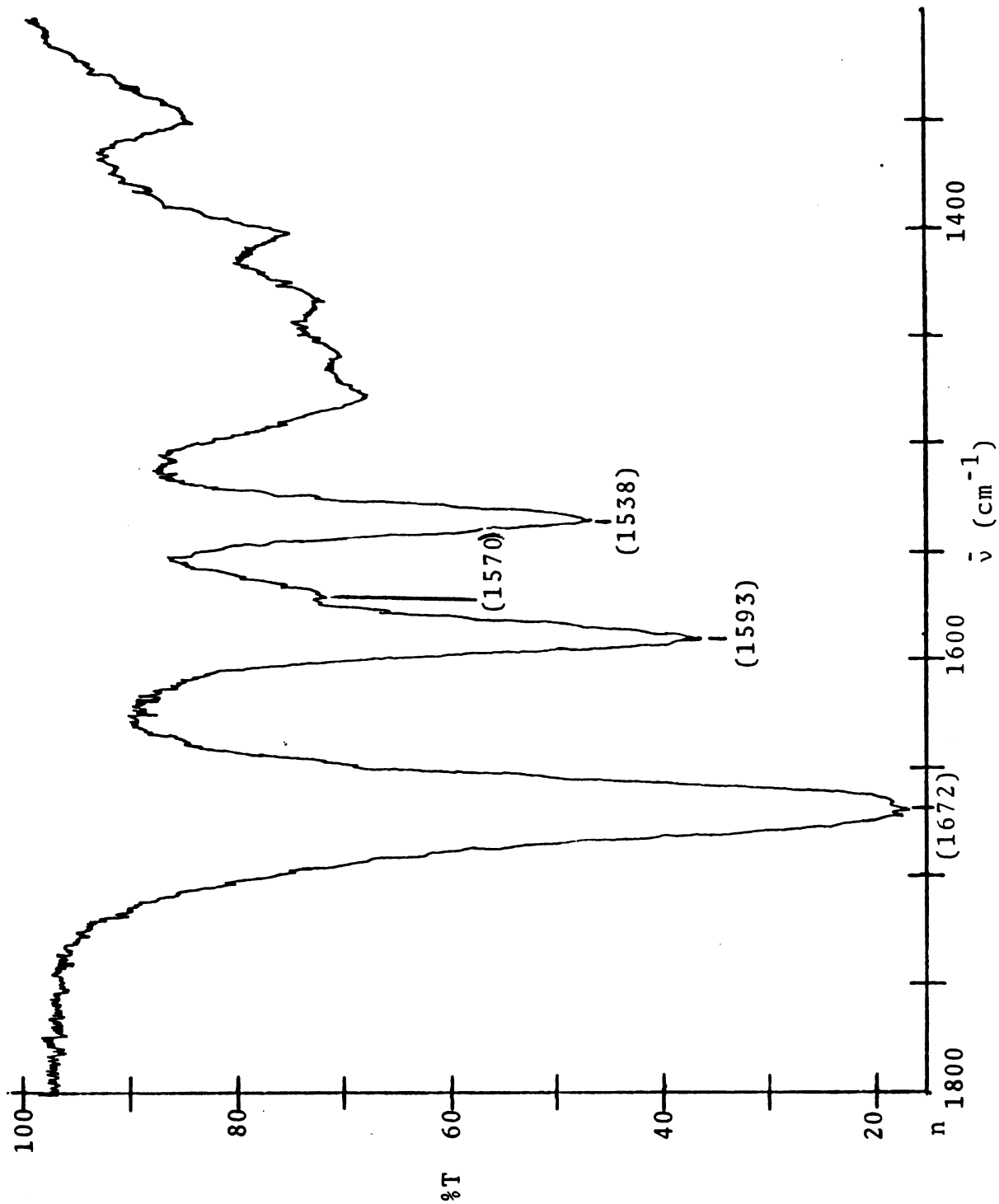

100
80
60
40
20
%T
1800
(1672)
1600
1400
$\bar{\nu}$ (cm^{-1})
(1593)
(1570)
(1538)

Figure 13. Infrared spectrum of 0.41 M Rb_2(5'-GMP) in D_2O at 10°C (structured).

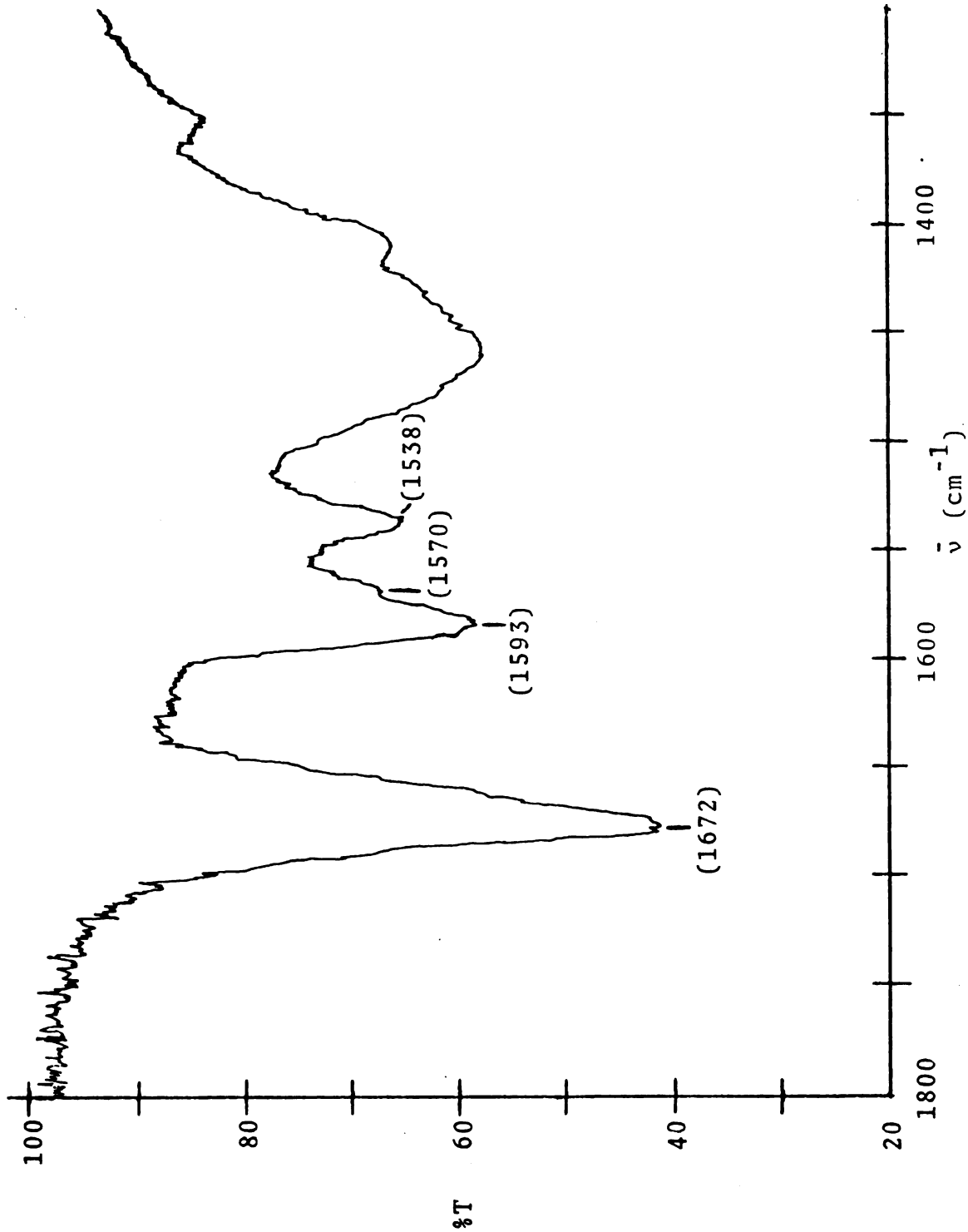
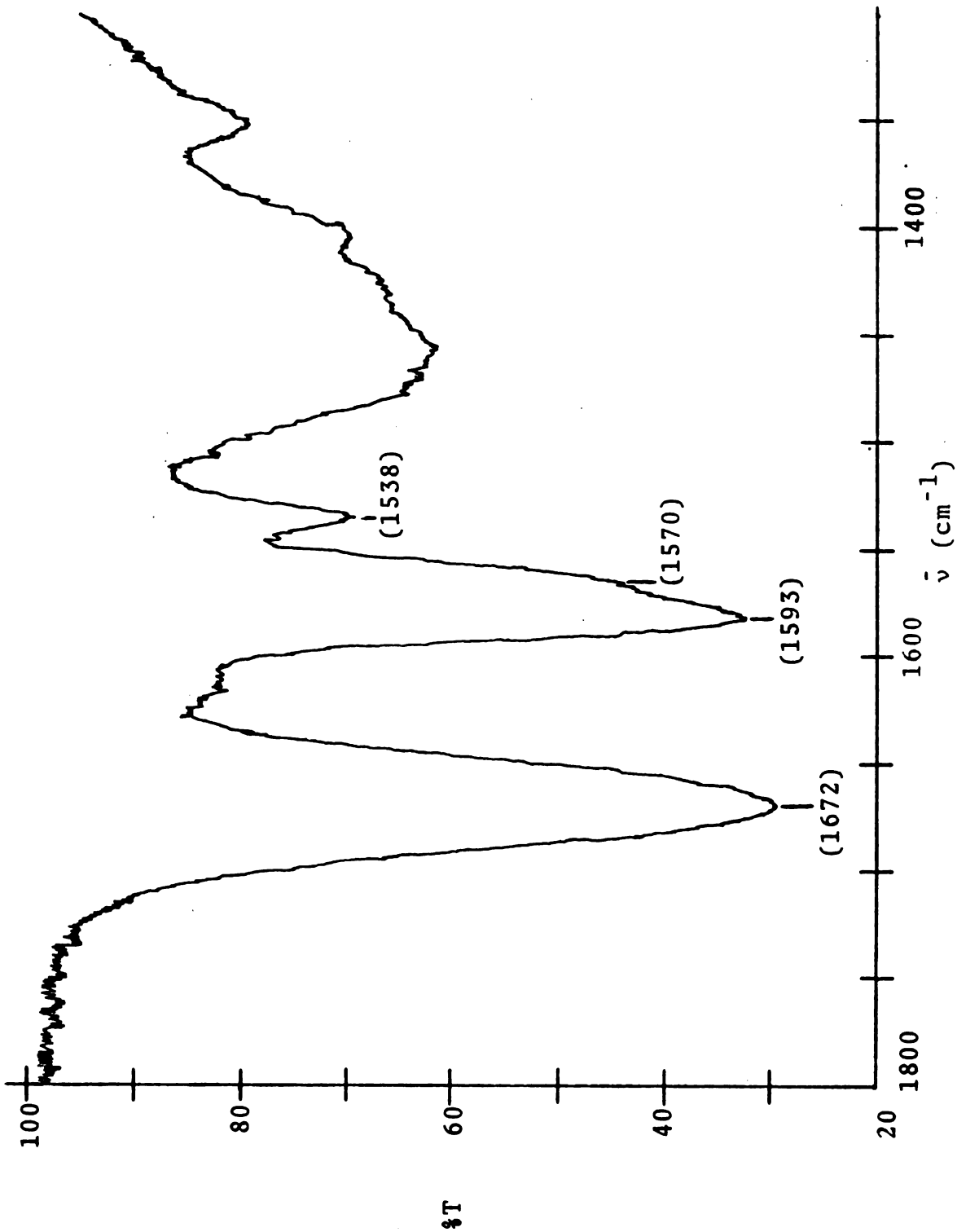

100
80
60
40
20
%T
1800
1600
1400
$\bar{\nu}$ (cm^{-1})
(1672)
(1593)
(1570)
(1538)

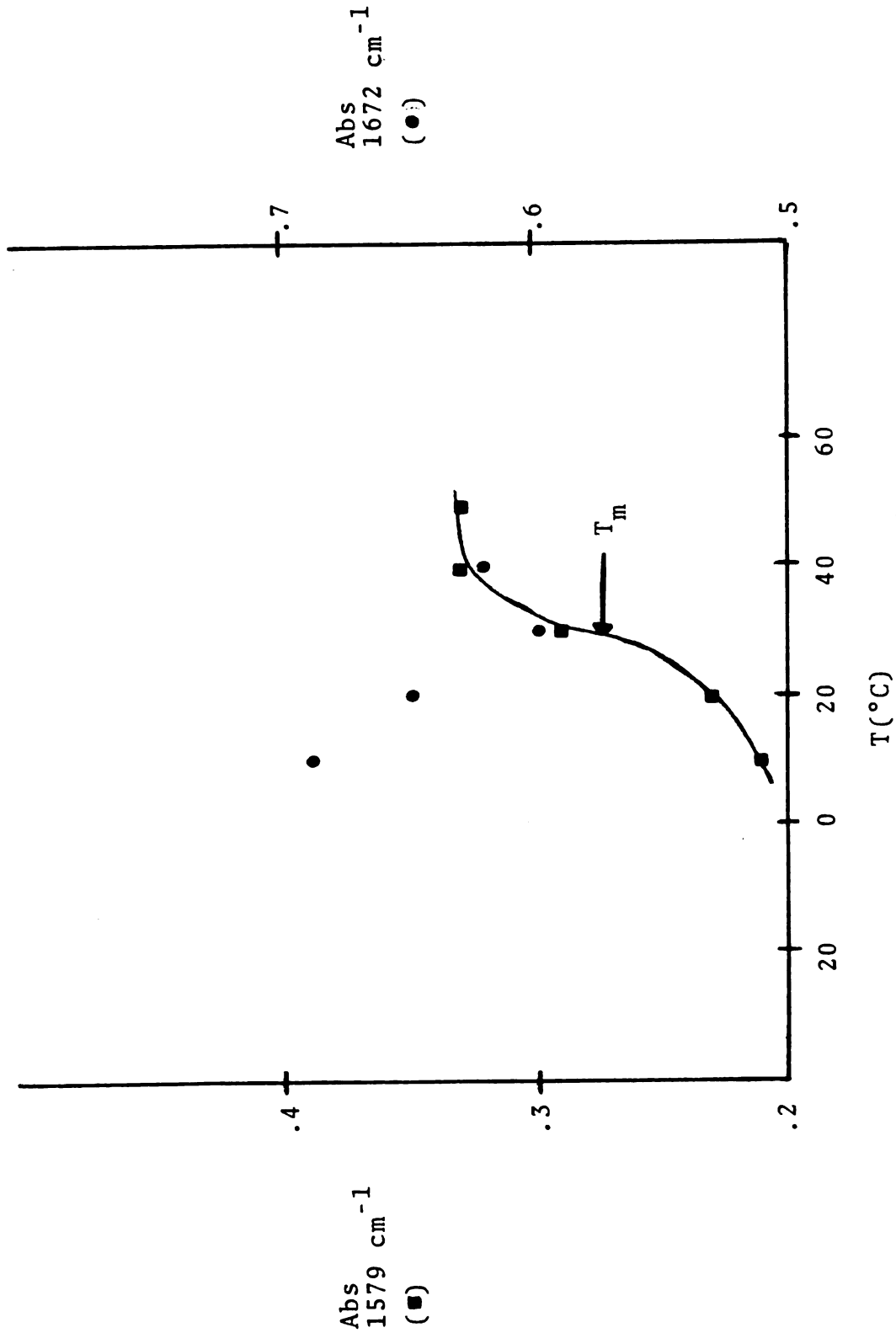
Figure 14. Infrared spectrum of 0.40 M Cs_2(5'-GMP) in D_2O at 10°C (structured).


100
80
60
40
20
%T
1800
1600
1400
$\bar{\nu}$ (cm^{-1})
(1672)
(1593)
(1570)
(1538)

temperature was nearly identical to unstructured Na_2(5'-GMP) which supports evidence for lack of structure formation by the lithium cation. In all cases the unstructured and structured solutions had the same qualitative appearance as Figures 9 and 10 respectively, regardless of the metal ion present, the small differences attributed to variation in the amount of structuring taking place. This would indicate that the metal ion either is not complexing to the purine ring or, if it is, the vibrations are insenitive to the nature of the structure-forming ion. With the exception of Na_2(5'-GMP), the criterion of constant molar absorptivity is not met for each of the IR bands. Figure 15 shows, for example, a plot of temperature versus absorption at 1672 cm^{-1} and 1579 cm^{-1} for a 0.59 M solution of K_2(5'-GMP). The 1579 cm^{-1} band gives a smooth curve but the band at 1672 cm^{-1} is not as smooth. From the inflection point of the 1579 cm^{-1} a value of T_m = 29°C was obtained, which makes this clearly a more stable complex than the sodium complex. It should be pointed out that qualitatively the K_2(5'-GMP) is not melted out at 50°C. The carbonyl stretch at 1672 cm^{-1} is still stronger than the 1579 cm^{-1} band. This adds additional support to the idea that more than one type of structure exists for the potassium complex.

A 0.41 M solution of the rubidium complex gave a T_m of 18°C. No low temperature plateau could be observed for any IR band for a 0.40 M solution of the cesium complex but an estimate was made that T_m would be between 10°C and 15°C.

Figure 15. Plot of absorbance at 1579 cm^{-1} (•) and 1672 cm^{-1} (■) bands of 0.59 M K_2(5'-GMP) in D_2O as a function of temperature. Note that the 1672 cm^{-1} plot does not give a sigmoidal curve.


Abs
1672 cm-1
(●)
.7
.6
.5
Abs
1579 cm-1
(■)
.4
.3
.2
Tm
20
0
20
40
60
T(°C)

This gives a thermodynamic stability range for M_2(5'-GMP) of

$$Cs^+ < Rb^+ \simeq Na^+ < K^+$$

which is in agreement with the proton NMR results.

DISCUSSION

The results clearly indicate that spontaneous self-assembly of 5'-guanosine monophosphate is highly dependent on the metal ion. This dependence must be due to a coordinate bond between an electron donor position on the 5'-GMP molecule and the metal ion. If the effect of the metal ion was only to shield phosphate charge through ion pairing, then qualitatively similar proton NMR spectra with small chemical shift differences would be expected due to the differences in the metal ions ability to neutralize charge. However, the NMR results indicate a far greater metal ion dependence on the type and extent of structure formation. It must be concluded that the metal ion plays a principle structure forming role in the self-assembly of GMP.

With the discovery of alkali metal coordination to the 5'-GMP dianion comes the question of the site of the metal binding. A quite obvious area to consider would be the phosphate moiety. At the pH investigated (~8.0) the phosphate group is a dianion with three oxygens available for electron donation. Several authors have investigated the binding of alkali metal cations to various forms of phosphate.

Smith and Alberty have studied the stability constants of Li^+, Na^+, and K^+ binding to 5'-adenosine-n-phosphate where

n is mono-, di-, tri-, or tetra.[29] Stability constants were evaluated from pK_a values. For all anions investigated the value of the stability constants always followed the pattern

$$Li^+ > Na^+ > K^+.$$

Strauss and Ross using polyphosphate[30] and Ross and Scruggs using calf thymus DNA[31] studied the binding of Li^+, Na^+, K^+, and Cs^+ to the phosphate group by the gel electrophoresis method. The values of the stability constants were calculated using the assumption that the greater the metal binding to the phosphate, the less negative charge carried, and the less the mobility in the gel. Both papers found the order of stability constants to be

$$Li^+ > Na^+ > K^+ > Cs^+.$$

Working with ATP and pyrophosphate, Botts found the same ordering of stability constants with the inclusion of Rb^+ between K^+ and Cs^+.[32]

Therefore, the strength of binding of an alkali metal ion to phosphate decreases with increasing ionic radius. This implies that the self-structuring of M_2(5'-GMP) probably does not involve metal ion binding to oxygens of a single phosphate group. If the strength of the M-O-P bond were the only driving force towards complexation then lithium would be expected to form the most stable complex. NMR and IR spectroscopy, however, show no evidence for Li_2(5'-GMP) structuring. These arguments, based on the stability of

the metal-phosphate bond, do not preclude the possibility of binding to two phosphates or of bridging a phosphate and some other donor atom on the ribose or purine rings, a process which might be more dependent on cationic size.

Further evidence that a metal-phosphate bonding is not exclusive in GMP self-structuring is shown by Chantot and Guschlbauer's work with 8-bromoguanosine (8-BrG).[33] 8-BrG is a nucleoside with a bromine in the place of H(8) and no phosphate group. The authors found that addition of alkali metal salt was able to affect the T_m of the gel formation at acidic pH values. Furthermore, at constant ionic strength it was found that the metal ion present had a direct effect on the melting with the highest T_m found for K^+. The fact that this alkali metal dependence is found even in the absence of a phosphate group would imply that the phosphate may not be the coordination site for the metal.

The findings support the work by Mettler[22] that the structuring ability of a given alkali metal ion is dependent on its ionic (or possibly hydration) radius. A metal ion which is either too small or too large to efficiently be coordinated by the nucleotide would not be expected to show any structuring. The metal ion size would appear to have a lower limit of about 0.99 Å, the ionic radius of Na^+, and an upper limit of 1.60 Å, the ionic radius of Rb^+.[34] The ionic radius of Li^+ (0.59 Å) appears to be too small for structure formation and the Cs^+ radius (1.70 Å) seems to limit the extent of structuring. Tl^+ with a radius of

1.60 Å would be expected to behave in a manner analogous to Rb^+. However, the unsuccessful attempts to observe self-assembly of Tl_2(5'-GMP) may be related to its lower solubility. Rb_2(5'-GMP) at the same concentration and temperature shows no self-structuring. The silver ion with a 1.30 Å radius coordinates to the hydrogen bond acceptors thereby precluding any structuring of the form indicated in Figure 1.

In view of this size dependence of the metal ion a search was made for a coordinating site on the molecule which might be more selective to cationic size. Several recent crystal structures of derivatives of guanosine, thymidine, and uridine have shown preferential coordination of sodium by the carbonyl oxygen on the base ring.[35-38] From these structures a sodium to carbonyl oxygen bond distance has been found to be: 1) 2.35 Å for sodium adenyl-3'-5'-uridine,[35] 2) 2.36 Å for sodium deoxyguanosine-5'-phosphate,[36] 3) 2.35 Å for sodium urate,[37] and 4) 2.41 Å for sodium thymidyl-5'-3'-thymidylate.[38] This gives an average sodium-oxygen bond length of 2.37 Å. If one assumes an ionic radius of 0.99 Å for Na^+,[34] this would give an effective coordination radius of a carbonyl oxygen of 1.38 Å.

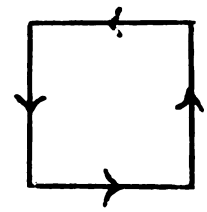
The tetramer structure of Na_2(5'-GMP) shown in Figure 1 has a hole in the center which is bordered by four carbonyl oxygens. These could act as a size-selective set of four donor atoms in a square planar array. Model building

studies have shown an O(6) - O(6) distance of 4.46 Å for oxygens on opposite corners. If a metal were to be located in the center of this square planar array of oxygens, the metal to oxygen radius therefore would be 2.23 Å. Subtracting out the effective radius of the carbonyl oxygen of 1.38 Å gives an optimum metal ion radius of 0.85 Å. The Na^+ radius of 0.99 Å although slightly larger than optimum would fit in this square planar array. The fit for Na^+ is better than for the remaining alkali metals. The Li^+ radius of 0.59 Å would be far too small and the K^+ radius of 1.30 Å would be too large.

The possibility also exists for the stacking of tetramer plates as suggested by Pinnavaia, et al.[18] If two plates are stacked on top of each other, an octamer results. The eight O(6) (four from each plate) can form the boundaries of a hole in either a cubic or square antiprismatic array. In either case the O(6)-M bond length would be 2.84 Å assuming a normal nucleotide base stacking distance of 3.50 Å.[2] Again subtracting the effective O(6) radius of 1.38 Å gives an optimum metal ion radius of 1.46 Å. The eight coordinate radii for the alkali metal ions are Na^+ (1.16 Å), K^+ (1.51 Å), and Rb^+ (1.60 Å). Clearly in this case the potassium ion would have the best fit for this eight coordinate system.

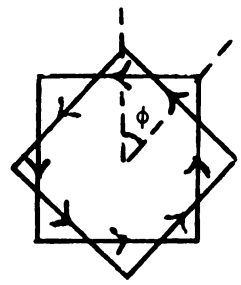
For stacking of tetramer plates, the symmetry of the stacking is quite important. The hydrogen bonding scheme for GMP has a pair of hydrogen bond donor atoms, N(1) and N(2), at nearly right angles with a pair of hydrogen bond

acceptors, O(6) and N(7). Arrows bent at a right angle, the head representing hydrogen bond donor atoms and the tail representing hydrogen bond acceptor atoms, can be used to represent a tetramer unit [7].

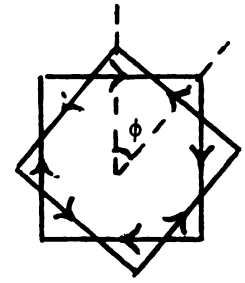


[7]

Due to the directional character of this type of bonding, when a pair of tetramers stack, this stacking can either be head-to-tail [8] or head-to-head [9].



[8]



[9]

The twist angle, ϕ, is defined as the angle caused by the projection of a line joining the center of a tetramer unit and a pair of hydrogen bonds with a similar line on the tetramer plate below. The optimum ϕ is obtained when a ribose-phosphate unit on one plate falls between a pair of ribose-phosphate groups on the second plate. This optimum ϕ of 45° gives a cubic arrangement of the eight O(6)'s

for head-to-head stacking and a square antiprismatic arrangement for head-to-tail stacking.

Consideration of the head-to-tail stacking in absence of the chiral ribose groups shows that the octamer unit is of C_{4h} symmetry when $\phi=0$ and S_8 symmetry when $\phi\neq0$. This means that the head-to-tail stacking of two planar tetramer units is achiral independent of ϕ before the addition of the ribose-phosphate. Since the unit is achiral, attaching the chiral ribose-phosphate groups give only one diastereomer. The point group now changes to C_4, regardless of ϕ and, therefore, the two plates are non-equivalent. This inequivalence means that two H(8) lines would be expected in the proton NMR.

In head-to-head stacking, the symmetry is D_4 in absence of the ribose-phosphate groups and independent of the value of ϕ. This means that the unit is chiral and the plates are equivalent. Ribose-phosphate attachment to this chiral unit should give two diastereomers, one for D-ribose attachment to the R enantiomer and one for D-ribose attachment to the S enantiomer. Each of the two diastereomers should give one H(8) line by proton NMR since the plates are equivalent, for a total of two lines for head-to-head stacking.

Therefore, the total number of H(8) lines expected by proton NMR for head-to-head and head-to-tail stacking would be four. This approach could explain the four H(8) resonances observed by Pinnavaia, *et al.*[18] for Na_2(5'-GMP),

but is insufficient in explaining the five lines observed for K_2(5'-GMP). From merely size consideration, the potassium ion is best suited for coordination to eight O(6) donors between two plates.

The metal - O(6) bonding while capable of accounting in part for the size dependence of metal ion bonding is not able to explain the IR data adequately. First, if the metal were bonded to the carbonyl oxygen, a difference in the carbonyl stretching frequency upon complexation would be expected. No change has been observed for all metals investigated (cf. Figures 10, 12, 13 and 14). Also, bonding to the carbonyl oxygen should weaken the carbonyl bond upon complexation due to a lowering of electron density. A corresponding shift to lower frequency should be observed for its IR absorption band. However, for all complexing metals the carbonyl stretch shifts from 1665 cm^{-1} in unstructured 5'-GMP to higher frequency at 1672 cm^{-1} for structured 5'-GMP. This shift, however, is also inconsistent with hydrogen bonding occurring at O(6) for which there is strong evidence from the N(1)-H and N(2)-H proton NMR spectra in H_2O.[18] No adequate explanation of this phenomena has yet been presented. One possible explanation could be that the carbonyl stretch is actually at lower frequency in the structured nucleotide but that it is coupled to a second vibration of the same symmetry and nearly the same energy. The effect, known as Fermi Resonance,[39] is that one absorption will shift to lower

frequency and the other will shift to higher frequency. This could cause the carbonyl to actually shift to higher frequency upon structure formation. If this is what is happening, the vibration which is Fermi coupled to the carbonyl stretch may now be occurring in the 1580 cm^{-1} region, wherein at least three peaks can be distinguished. This, however, is pure speculation and a test of its validity has not been attempted.

A second and possibly more viable idea is that in the monomer unit, hydrogen bonding can occur to one or two water molecules at the carbonyl. This should cause a low frequency shift of the carbonyl stretch. When complexation and self-structuring occurs, a weaker hydrogen bond is formed between O(6) on one molecule and N(1) on another. The weaker hydrogen bonding in the structured unit causes the carbonyl stretch to move to higher energy. The loss in hydrogen bond stabilization upon self-assembly could be more than compensated for by the base-stacking interactions. The important point is that the high frequency shift of the carbonyl is not well understood and therefore metal ion coordination to the carbonyl group cannot be eliminated.

CONCLUSION

The nature of the alkali metal ion has been proven to be important in the solution self-assembly of the 5'-guanosine monophosphate dianion. Each of the group IA metals exhibits a unique self-assembly behavior. This uniqueness is demonstrated by NMR and IR by the type of structure formed, extent of structuring, and structure stability. The results appear to be in agreement with Mettler's theory[22] on the size dependence of the metal ion. However, the present work has shown that as the ionic radius goes beyond that of K^+ (1.38 Å), the most stable alkali metal 5'-guanosine monophosphate complex, the extent and stability of structuring decreases. The stability of the nucleotide complex appears to be at a maximum for K^+, the T_m becoming lower for alkali metals having higher or lower ionic radii.

The structure forming role of the metal ion implies formation of at least one coordinate bond with the 5'-GMP molecule. This finding provides the first incisive evidence for selective metal ion coordination by a nucleic acid in solution. In Chantot and Guschlbauer's work with 8-bromo-guanosine[33] they were able to show that gelling of the molecule was greatly affected by the addition of a soluble salt. This may indicate a special coordination property of guanosine nucleosides and nucleotides. In general, the

role of the alkali metal in nucleic acid chemistry has been relegated to one of neutralizing the negative charge on the phosphate groups. Structure directing complexation of alkali metal ions is a novel idea in aqueous nucleotide chemistry. In this respect, the aqueous chemistry of M_2(5'-GMP) has a great deal of importance to biological systems. Even though the concentrations and temperatures employed are unrealistic in terms of living organisms, a thorough understanding of the mechanisms and equilibria involved in the complexation of 5'-GMP *in vitro* may give more insight as to the exact role of the metal ion *in vivo*.

FUTURE WORK

Further work in this area should concentrate on three major problems that have yet to be answered.

The first problem is to find out how many metal ions there are per structured unit. This should give some insight as to the type of structuring (tetrameric, octameric, etc.) that exists for any metal. NMR appears to be the best approach to this aspect of the problem. NMR titration curves could be obtained by the use of mixed salts of M_2(5'-GMP) in which one salt is a complexing one (e.g. Na_2(5'-GMP) and the other is a non-complexing one (Li_2(5'-GMP)). The titration curves would be obtained by monitoring either the ^{1}H or ^{13}C NMR while varying the molar ratio of the two salts. The point at which little or no change occurs in the spectrum will give the relative number of complexing metal ions per nucleotide.

This same experiment could be followed using alkali metal NMR (^{23}Na, ^{39}K, ^{85}Rb, ^{133}Cs) as a probe. As the metal becomes complexed, a broadening and/or change of the chemical shift of the line should occur. At the point at which all the metal is complexed, the broadening or changes in chemical shifts should cease. A plot of line width versus

mole ratio of M^+ to GMP^{2-} should give two curves which intersect at the point where most of the metal ion is complexed.[40]

The second major problem which should be approached is one of determining how many different structures there are in solution. Estimates of the number in each of the complexing salt solutions have been made but a more quantitative approach needs to be taken, including a determination of T_m values for each structure.

The third, and most interesting problem, is in locating the metal ion binding sites in the complex. To date, attempts at crystallization have shown no promise. Guanylic acid (pH ~ 5) has been crystallized but the neutral salts have not.

Both problems, therefore, must be approached in a more indirect manner. Again, NMR appears to be the best tool. With the recent advancements in instrumentation, the capability of examining other nuclei is greatly increased. An examination of other NMR nuclei (e.g. ^{15}N, ^{31}P) should increase information on both the number and type of structures occurring. ^{15}N NMR could be useful in this respect. If complexation is occurring at the purine ring, a chemical shift change should be observed in the spectrum. Also, if coordination occurs at a nitrogen, then line broadening due to quadrupolar interaction with the alkali metal nucleus should give information on both the binding site and the extent of covalent interaction.

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