STUDIES OF RAMAN SPECTRA OF WATER SOLUTIONS OF ADENOSINE TRI-, DI-, AND MONOPHOSPHATE AND SOME RELATED COMPOUNDS

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ABSTRACT Using a He-Ne CW laser source together with a digital photon counting system, we have obtained well resolved Raman spectra for adenosine mono-, di-, and triphosphate (AMP, ADP, ATP) in aqueous solution. Spectra of these compounds were studied as a function of pH from pH = 0.5 to 13.5 and between 550 and 1700 cm^{-1}. It was found possible to distinguish spectroscopically between the three phosphates over the pH range studied. A qualitative analysis of vibrational modes responsible for various spectral lines is given. Lines at about 960 and 1100 cm^{-1} were found to be good indications of the degree of ionization of the terminal phosphate group.

I. INTRODUCTION

An understanding of the structure and conformation of biological molecules in solution generally requires spectroscopic data. Vibrational spectra can give detailed information regarding the molecular conformation and can be obtained either by direct absorption or Raman spectroscopy. Strong limitations are placed on the use of direct absorption by the properties of water in the infrared.

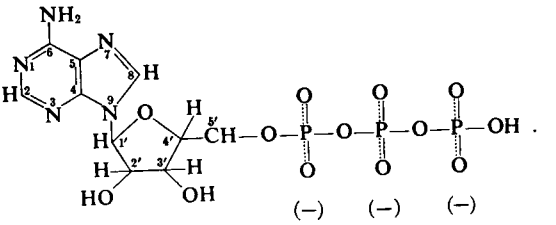
There are a number of drawbacks to traditional Raman spectroscopy using mercury arc excitation, among these are strong background fluorescence, spurious lines from the mercury arc source, and difficulties in achieving good polarization. The use of an He-Ne red laser source together with a digital photon counting (DPC) system of essentially infinite dynamic range overcomes most of these difficulties, since monochromaticity and polarization of the laser source are, for all practical purposes, ideal. Previously conventional Raman spectra have been reported for a number of bases and corresponding ribonucleosides. (1, 2)

Adenosine and its three phosphate derivatives play a central role in biological energy transfer reactions. Infrared and nuclear magnetic resonance (NMR) spectra

have been used (3–9) to study the conformational changes associated with pH and metal ion complexes. We have undertaken the complete study of the Raman spectra of these compounds as a function of pH and metal ion concentration using a laser–Raman DPC spectrometer. We here report on the first part of this research comprising the pH dependence of the spectra of the three adenosine phosphates.

In studying the Raman spectra as a function of pH, and therefore of the degree of dissociation for the various functional groups, one finds marked changes in some regions of the spectrum. As a given dissociation proceeds certain lines will decrease while others will increase in intensity. Previous chemical evidence of which particular group is undergoing ionization enables one to ascertain the assignment of these vibrations. This is the basic method of identification used here.

The numbering convention employed in referring to ATP is



In the range of pH that we have investigated (0.5–13.5) the primary phosphate protons are assumed to be ionized as shown. Primary phosphate protons on ADP and AMP are assumed to be ionized as well (5, 10, 11). A pK of 4.0 for ATP (10, 11) (3.95 for ADP and 3.74 for AMP) corresponds to an ionization on the adenine moiety generally assumed to be the 1-nitrogen (4). The pK's corresponding to the ionization of the terminal phosphate proton are 6.95 in ATP to 6.88 in ADP (12) and 6.05 in AMP (11). Raman data in these pH ranges will be used in the discussion of the phosphate group vibrations. A significant observation in this work is that it is possible to make *spectroscopic* distinction between ATP and ADP in aqueous solutions. This finding may have important applications in the study of ATP reactions.

Section II deals with experimental methods, Section III with the data and their interpretation; and in Section IV the more important conclusions are summarized.

II. EXPERIMENTAL

Our measurements (in contrast to the earlier work (1) were performed using as a source a He-Ne laser in the 6328 A Ne transition, at a power level of about 50 mW The incident light was collimated on the sample as a small diameter parallel beam. The light scattered at 90° was focused on the entrance slit of a suitably modified double pass grating monochromator (Perkin-Elmer Model 99) and detected by a cooled RCA-7265 photomultiplier. The sample had a volume of 0.25 ml and the length of the source beam was about 1.2 cm. With a beam diameter of 0.05 mm, the useful active sample volume was in the order of 2×10^{-5} ml. In many instances the

relatively weak Raman spectrum has to be recorded in the presence of a large background (tail of Rayleigh line and possible fluorescence background). Because such effects were much less troublesome with the red light excitation than with excitation by a 5145 A line of an argon laser, the He-Ne source was used. Even then, in many samples scattered light intensity required much larger dynamical range than that offered by the conventional detection system with a synchronous amplifier-detector; this was also the case in the observation of weak Raman lines near transitions of much higher intensity. Large dynamic range is usually obtainable using a digital data processing system.

A block diagram of our apparatus is shown in Fig. 1. For the present observations synchronous detection had to be retained, since the second pass radiation in the spectrometer had to be chopped in order to distinguish it from first pass radiation which reached the exit slit. We used the following system: The output of the photomultiplier (PM) was fed into a wide-band linear amplifier height discrimination system. Pulses generated at the PM by the photons were thereby selected from background noise and brought to a constant amplitude at the output of the discrimination system. From there the pulses were passed through a variable countdown (divide) circuit feeding a bidirectional (up-down) counter that was switched between the add and subtract mode synchronously with the second pass chopper (the synchronous detector feature of the system). The number accumulated in the up-down counter was transferred to a shift register by the action of a synchronizing pulse occurring every 2^mth chopper cycles. This pulse also cleared the bidirectional counter. The register held this number until the occurrence of the subsequent synchronizing pulse. Contents of the shift register were converted to an analog voltage in a digital to analog (DA) matrix.

The signal countdown at the counter input allowing $(\frac{1}{2})^n$ of the PM pulses to be

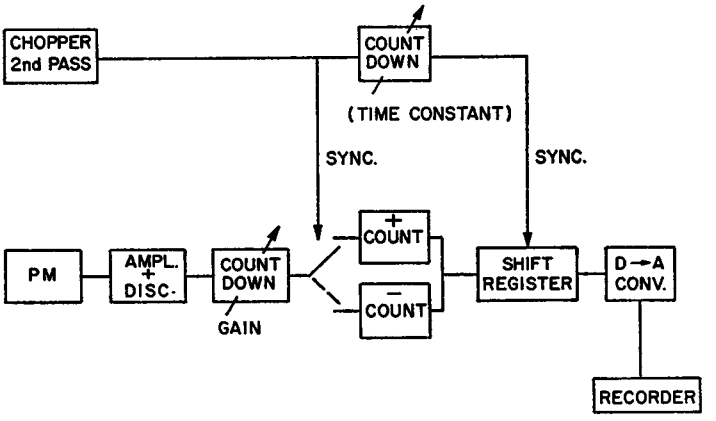


FIGURE 1 Block diagram of the electronics for the digital photon counting system. (*PM*, photomultiplier; ampl. + disc., amplifier height discrimination system; sync., synchronous; and D → A conv., digital to analog conversion.)

counted, acted as an amplitude control when n was varied. Similarly, varying m and therefore the rate of the register transfer pulse was equivalent to changing the integrating time. Digital integration eliminates the problem of asymmetric line distortion found in conventional resistor and capacitor (RC) smoothing circuits.

The polarization character of the transitions was determined by taking spectra with the incident polarization both parallel and perpendicular to the scattered light wave vector. This is somewhat unorthodox but avoids the signal attenuation which is unavoidable with the use of an analyzer–polarization scrambler combination.

Most data were obtained on 0.5 M solutions; in a few cases other concentrations were examined (0.1 to 1.0 M) and in this range no concentration dependence was observed.

Adenosine mono-, di-, and tri-phosphates were purchased from the Nutritional Biochemical Corporation, (Cleveland, Ohio). Following an initial pH adjustment with NaOH or HCl, the nucleoside solutions were cleared of insolubles by vacuum filtration or shaking with CCl_4 or both

The basic independent variable in the present measurements is the H^+ ion concentration as indicated by the pH. We report here in some detail on studies of the spectra of the phosphates around the pH corresponding to the terminal proton ionization.

Another point of interest is the possibility of distinguishing by this technique solutions of the various phosphates. Such an application would have direct bearing on the study of actual biological interactions involving ATP degradation. The results in this area seem rather encouraging.

III. RESULTS

In the phosphates we can classify the most important transitions into three groups: those unaffected by any pH change, those whose intensity is affected by pH changes around pH $\sim$ 4 (associated most probably with the adenine component), and those changing markedly around pH $\cong$ 6.5, corresponding therefore to the phosphate group. In this report we shall deal with vibration frequencies in the range of 550 to 1700 cm^{-1} (C—C, CN stretch, C—H, N–H deformation, etc.) The high frequency range involving the X–H stretches will be discussed in later publications. A study of the spectrum of ribose indicates the presence of a series of very weak lines in this low frequency region, none of which corresponds to any of the vibrations to be discussed here.

Fig. 2 shows a sample of data obtained on the three phosphates, at pH = 6, where they are quite evidently distinguishable. The strong line at 727 cm^{-1} is essentially unaffected in both intensity and frequency by the pH, and is also present in adenine and adenosine. Therefore, it seems to be a good standard for relative intensity measurements.

In Table I we summarize the results on vibrations associated with the phosphate

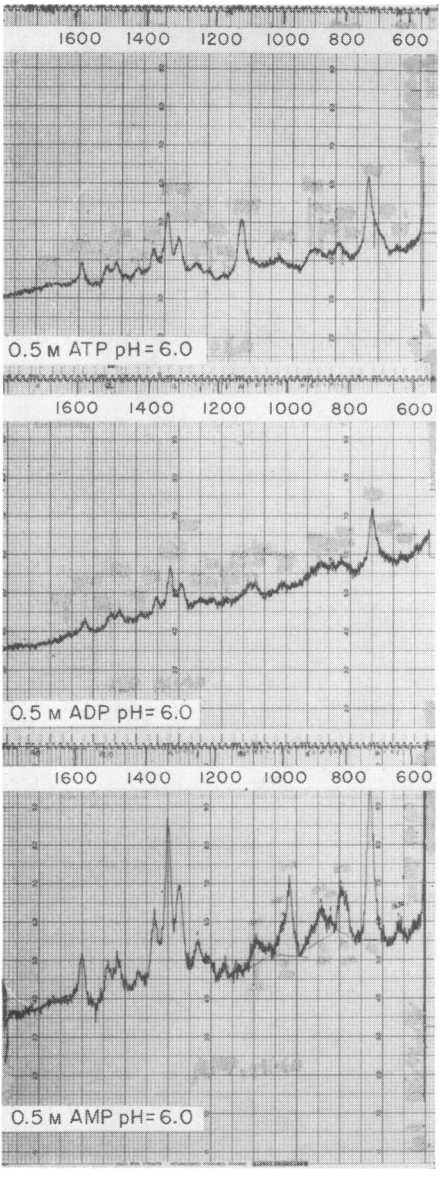


FIGURE 2 Sample of the actual data recordings.

complex either by their pH dependence or by their absence in the spectrum of adenosine. ADP and ATP are easily distinguished by the measurement of the relative intensity, frequency, and shape of the strong lines in the neighborhood of 1125 cm^{-1}. The spectral differences in the range 800–920 cm^{-1} are subtle but exhibit distinguishing features. The AMP spectrum is strongly differentiated from the polyphosphates. In Fig. 3 we illustrate the pH dependence of the spectra in the region 600–1200 cm^{-1}, through the pK corresponding to the loss of the terminal (secondary) proton in the

TABLE I

LINES ASSIGNED TO PHOSPHATE VIBRATIONS

AMP		ADP		ATP	
pH: 5.5	7.5	5.5	7.5	5.5	7.5
817*	817*	826	828	920	§
858	850	860		830	§
	882				
1082¶	980‖	1108¶	1090¶	1125¶	1112¶
1005‡	1005‡	1005		1008	
	1123**				
	1170**				
		710		680‡‡	

* This line is stronger in low pH.

‡ This line is stronger in high pH.

§ The structure of the lines in this region changes in a complicated way with pH.

‖ Symmetric stretch of PO_3 group.

¶ Symmetric stretch involving combinations of O=P=O.

** Twofold degenerate asymmetric PO_3 stretch, split by lower symmetry in the molecule.

‡‡ A collective mole.

three phosphates and for ADP through the pK for protonation of the adenine component.

Fig. 4 also shows the pH dependence of the intensity of some lines in the AMP spectrum (measured relative to the line at 727 cm^{-1}). From data such as these one is able not only to assign this group of lines to vibrations mainly associated with the phosphate group, but also one discovers which line belongs to a given state of ionization. (For pH > 0.5 the primary or side protons on the polyphosphate complex are all to be considered ionized). [4, 10, 11] Thus, vibrations at 817 and 1082 cm^{-1} are associated with the protonated form, whereas the lines at 882, 980, and 1005 cm^{-1} correspond to transitions within the ionized groups. We should mention also that the lines at 980 and 1082 cm^{-1} are strongly polarized. These results when viewed in conjunction with previous work on infrared (IR) spectra of these compounds and inorganic and simple organic phosphates (3, 4) allow us to make the following assignments for the PO symmetric stretch vibrations involving a resonant double bond in both configurations of AMP: ionized form, PO_3 at 980 cm^{-1} and protonated form (PO_2) OH at 1082 cm^{-1}.

The asymmetric stretch, which would be doubly degenerate in the highly symmetric ionized form of the PO_3 group, will presumably be split by the lowering of symmetry due to the ribose and adenosine moieties. The two weaker lines at 1170 and 1123 cm^{-1}, characteristic of the ionized form, may well be related to this asymmetric stretch. No line due to the phosphae group could be found for the protonated species in the region around 1230 cm^{-1}. Tnis is the expected frequency of the antisymmetric stretch for the $P\langle^{O}_{O}$ subgroup. The absence of a well defined Raman

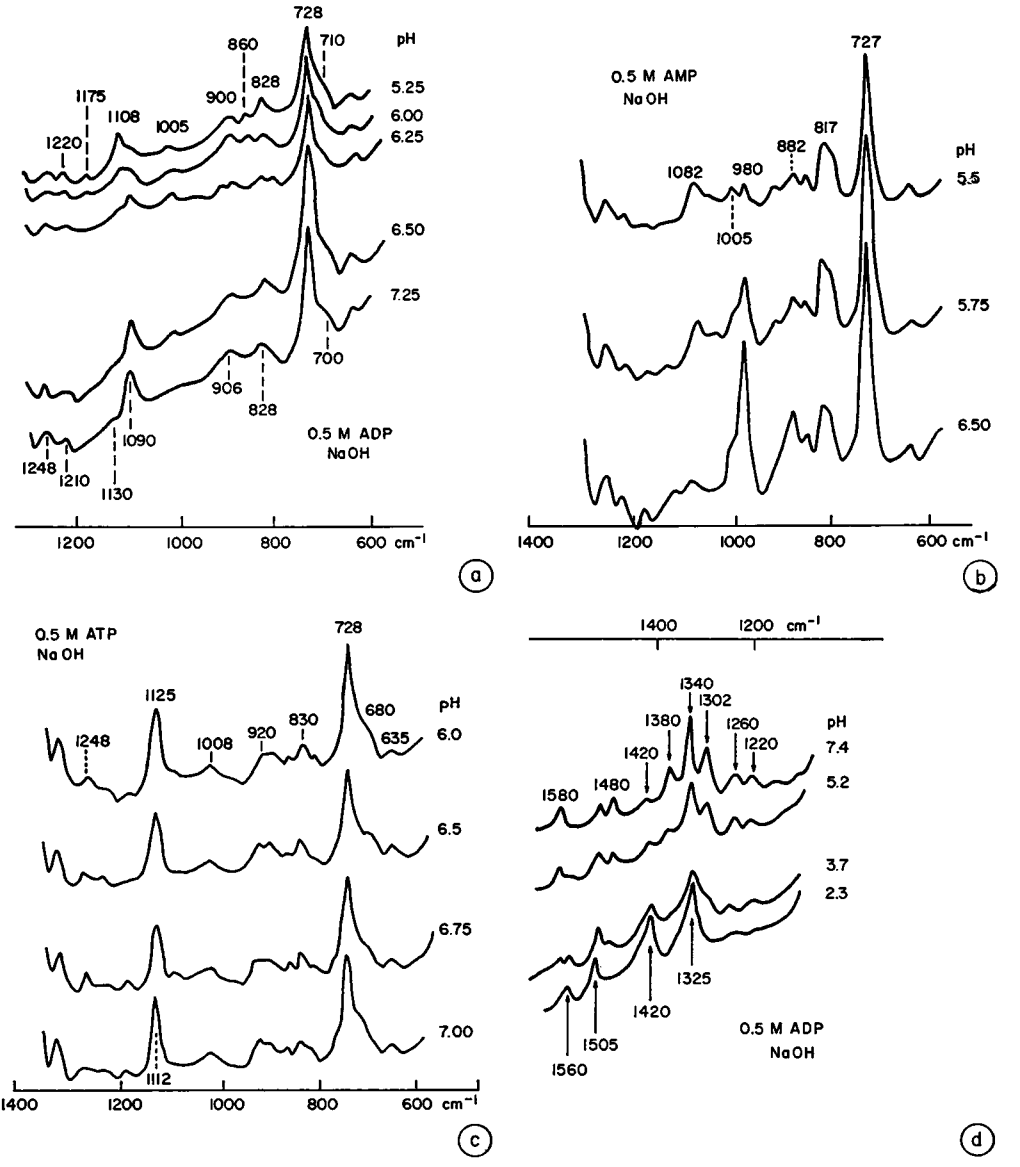


FIGURE 3 (a) ADP around the pK_2 for terminal phosphate proton ionization. (*b*) AMP around the same pK_2. (*c*) ATP around the same pK_2. (*d*) ADP around the pK_1 for ionization of the adenine component.

line associated with this vibration is probably a result of the inherent weakness of the associated Raman transition and the presence of nearby adenosine lines.

In ADP, as the data of Fig. 3 show, there is again a pair of conjugate lines, at 1108 and 1090 cm^{-1}, which we have found to be well polarized. Again the lower frequency line corresponds to the fully ionized variety. The analogous lines in ATP

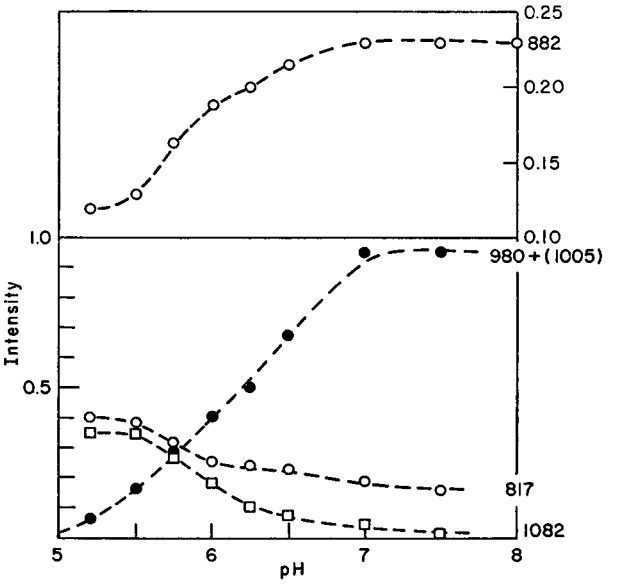
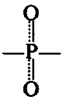


FIGURE 4 Relative intensities in arbitrary units for the transitions at 817, 882, 980, and 1082 cm^{-1} in AMP around the pK_2 for terminal phosphate proton ionization.

appear at 1126 and 1112 cm^{-1} respectively, below and above $pK_2 = 6.5$ (Fig. 3). These pairs of lines are probably both associated with a symmetric stretch involving the various groups of the form



in the polyphosphates. However, these are modes of the chain as a whole, i.e., they are linear combinations of displacements involving all the groups; it is thus understandable that the effect of the loss of the terminal proton is less than in the monophosphates.[1] The shift of the 1100 cm^{-1} phosphate line upon ionization decreases as we go from the mono- to the triphosphate as 100 cm^{-1}, 18 cm^{-1}, 13 cm^{-1}, just as expected from the above consideration.

The frequency in the protonated form remains approximately constant for all three phosphates. This can be explained if we assume that these vibrations involve mainly the P = O double bond. These double bonds resonate between the two oppositely located side oxygen in each phosphate group in the protonated form. The motion in this vibration would correspond to in phase displacements of the P

[1] It should be pointed out that an absorption band at 995 cm^{-1} in the IR spectrum of ionized ATP has been attributed to the nonprotonated terminal phosphate symmetric stretching mode. (4) It is not easily explained why a strong polarized Raman line was not observed near the same frequency.

atoms against the oxygens. The frequency of such a mode is expected to be relatively insensitive to the number of groups involved.

The fact that in ADP the two lines corresponding to the protonated and ionized species can be well resolved at pH = 5 indicates that the exchange frequency for this terminal proton is considerably less than their separation of 18 cm^{-1} (5.4×10^{11} sec^{-1}). We could not resolve this doublet in ATP at the intermediate pH mainly due to the width of the individual lines being comparable to their separation. One can, from these experiments, set an upper limit of 3×10^{11} to this terminal proton exchange frequency.

The study of Fig. 3 shows that, in contrast to AMP, both ADP and ATP show a shoulder on the line at 727 cm^{-1}. This shoulder corresponds to a transition at 710 cm^{-1} in ADP and 680 cm^{-1} in ATP. Due to its absence in the monophosphate, and its decreasing frequency with increasing number of phosphate groups, we tentatively assign this line to a collective vibration of the polyphosphate chain.

In all the adenosine phosphates two transitions near 1258 and 1220 cm^{-1} seem to be associated with the adenosine component since they increase markedly in intensity around pH = 4 (which is the pK corresponding to a proton ionization in adenine). Nevertheless, in both ADP and ATP there seems to be a marked perturbation of the spectrum in this region when the pH approximates the pK for the ionization of the terminal phosphate. This perturbation may well be attributed to the Raman activity of the antisymmetric-PO_2-stretching frequency. However, one may also conjecture that this indicates the presence of some type of association between the protonated terminal phosphate and a hydrogen bonding nitrogen site of the adenine.

Fig. 3 *d* shows spectra of ADP as a function of pH around the pK of 4 corresponding to the ionization of the adenine moiety.

IV. CONCLUSIONS

In summary, we report detailed Raman spectra and their pH dependence in the adenosine phosphates. Some assignments are made of vibrations of the phosphate complex, and the possibility of distinguishing ADP from ATP by this technique is substantiated. A wide dynamic-range DPC spectrometer system is also described.

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