Intermolecular Interaction in Systems with Energy-Rich Phosphates, I

Stepwise Protonation of PO_4^{3-}, ADP and ATP Salts, IR Investigations

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ATP, Energy-Rich Phosphate, Hydrolysis Products of ATP, Proton Addition, Hydrogen Bonds Polarizable

IR spectra of aqueous solutions of PO_4^{3-}, ADP^{3-}, ATP^{4-}, $MgADP^-$ and ($MgADP^- + PO_4^{3-}$) (in two cases hydrated layers) were plotted. The parameter for the investigation was the mole percent of protons relative to the anions. The other cations were sodium or potassium. The purpose of our work was to obtain a basis for understanding the results regarding the changes to the interactions in ATP systems on hydrolysis. The addition of protons to the different acceptors is discussed on the basis of the bands of the phosphate groups and of the base ring vibrations.

The formation of easily polarizable hydrogen bonds could be demonstrated with the aid of an IR continuum. Such $OH^+ \cdots O$ bonds between hydrogen phosphate ions as well as $NH^+ \cdots N$ bonds form in the systems investigated. Thus the conditions for forming such bonds and the resulting intermolecular interactions will become evident in this work.

Introduction

The purpose underlying our work was to gain experimental results concerning the hydrolysis of the ATP system which would yield information as to the molecular processes involved in this reaction. One possible method of gaining such information is offered by quantitative IR spectroscopy. Investigations of the anion-cation interaction and of hydration in polyelectrolytes [1] and in solutions [2–5] had proved the suitability of this method with regard to the intermolecular interactions and structures in biological systems.

George *et al.* [6] have already pointed out the importance of the hydration and of the intermolecular interaction on ATP hydrolysis. The former suppose that changes to solvation enthalpy are primarily of significance as regards the changes to free enthalpy on ATP hydrolysis.

Theoretical investigations have shown that hydrogen bonds with a double minimum potential well are extremely easily polarizable [7–9]. This leads to these hydrogen bonds interacting strongly with their environment [1, 8, 10, 11]. These interactions cause absorption continua in the IR spectra, which indicate the presence of polarizable hydrogen bonds. Such hydrogen bonds are formed preferably by the excess protons. Hence these hydrogen bonds can be of type $(BH \cdots B)^+$ or, when the acceptors are anions, of type $(BH \cdots B)^-$.

The question now arises as to whether such hydrogen bonds form on ATP hydrolysis, since excess protons come into being here. This also becomes noticeable on investigating biological systems. Herbst and Piontek [12], for instance, showed that on ATP splitting in the muscle the medium briefly becomes more acidic.

A later paper deals with the ATP hydrolysis in aqueous solutions by IR spectroscopy. The following shall provide the basis for interpreting these hydrolysis experiments.

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Results and Discussion

IR spectra of aqueous solutions or of hydrated layers, respectively, were plotted for the substances present on ATP ortho-phosphate hydrolysis. The pure Mg^{2+} complex of ATP could not be investigated, since the initial substance delivered was already 20% hydrolysed (see subsequent paper). The parameter for the series is the mole percent of protons with respect to the phosphates occurring as anions.

Aqueous solution of inorganic phosphate

Fig. 1 shows the IR spectra of $K_{3-n}H_nPO_4$ in aqueous solution, whereby n varies from 0 to 3 in increments of 0.5 ($n = 0$:0%; $n = 3$:300% protona-

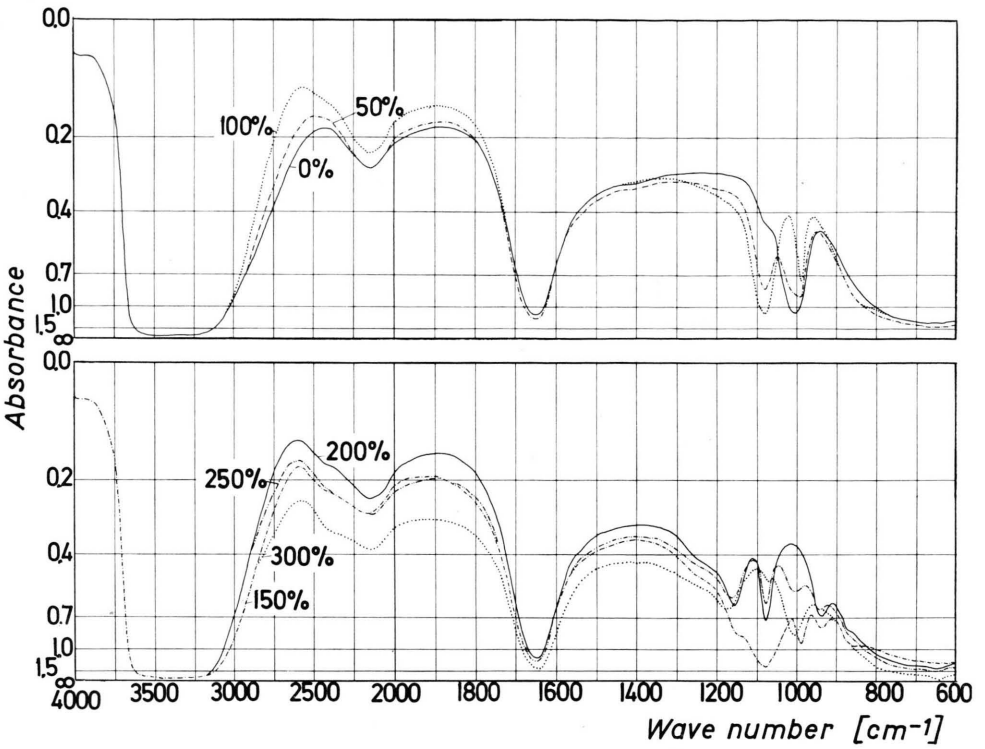


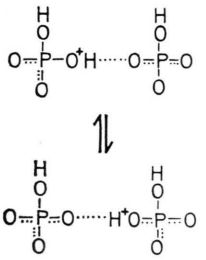
Fig. 1. IR spectra of aqueous solutions of PO_4^{3-} ions dependent on protonation. % figures are mole % H^+ relative to the phosphate ions. Concentration 1 PO_4^{3-}/30 H_2O; sample thickness 13 μm.

tion). The band assignment according to [13, 14] is summarized in Table I. Together with Fig. 1, this table provides information as to the structure in which the phosphate ion is present depending on the respective degree of protonation.

Table I. Assignment of the bands of the phosphate ion.

	Assignment	Band position [cm^{-1}]
K_3PO_4	ν_{as}	1010
	ν_s	942 (after disturbance of the symmetry)
K_2HPO_4	ν_{as} $-P(=O)(-O)_2^{--}$	1083
	ν_s $-P(=O)(-O)_2^{--}$	990
	P−OH	850
KH_2PO_4	$\nu_s > P(=O)(-O)^-$	1160
	$\nu_{as} > P(=O)(-O)^-$	1080
	$\nu_{as} > P(-OH)_2$	942
	$\nu_s > P(-OH)_2$	872

One can see in the range 2700 – 1200 cm^{-1} that the background at 150% protonation is raised in contrast to 100 and 200% protonation. Recently, Schiöberg *et al.* [14a] have shown that this effect don't exist for concentrations less than 1 mole/l (ca. 60 H_2O/PO_4). With increasing concentration the effect becomes more and more pronounced. As, for example, with imidazole [3] this maximum of a continuous absorption indicates that in the range 100 – 150% protonation easily polarizable hydrogen bonds form. This continuum is maximally intense on semiprotonation of the acceptor and then decreases in intensity, since two acceptor groups are no longer available for each proton. The additional proton accordingly forms an easily pola-



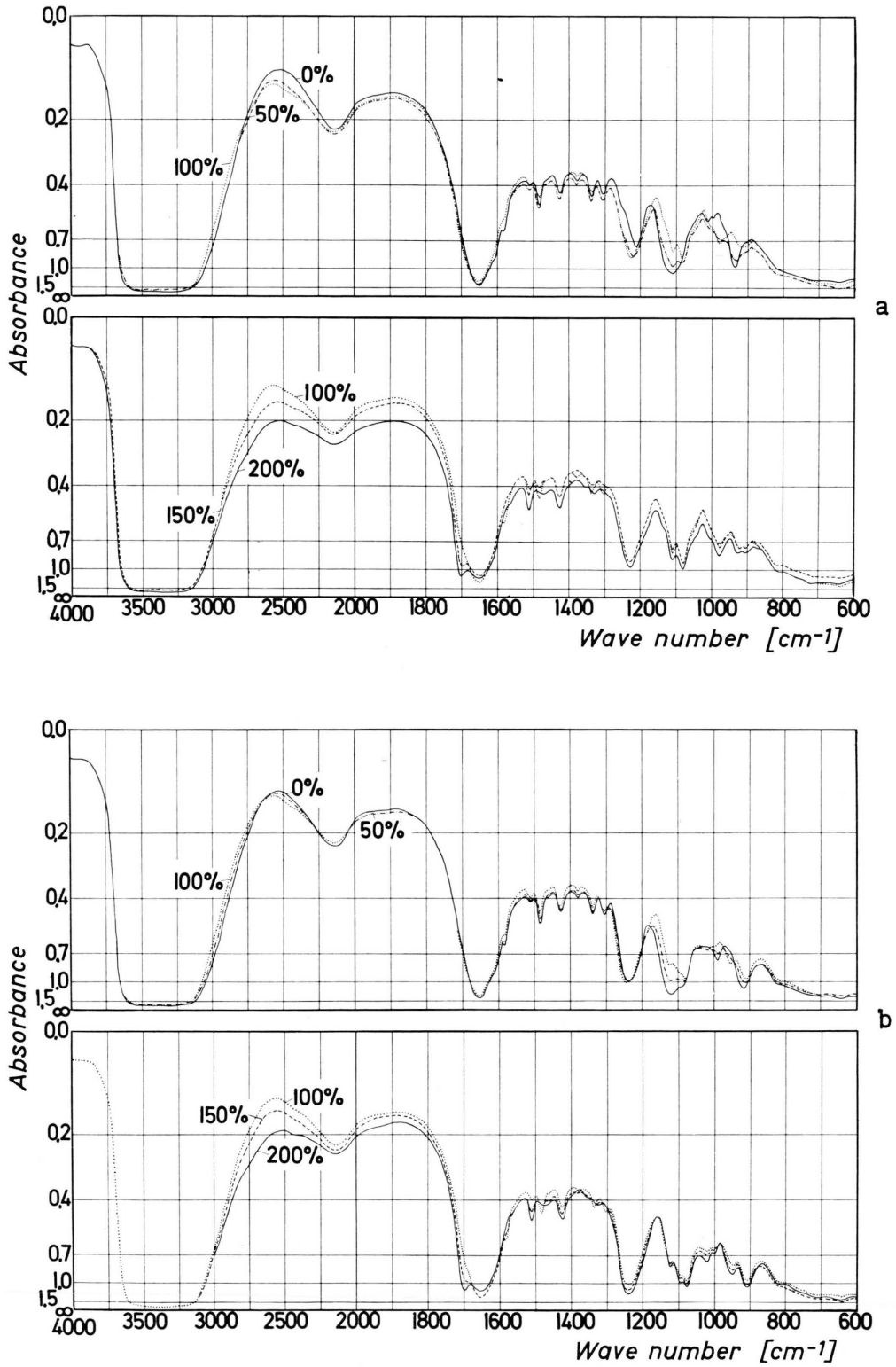


Fig. 2. IR spectra of aqueous solutions of ADP^{3-} or ATP^{4-} Na^+ salts dependent on protonation. % figures are mole % H^+ relative to the solute molecules. Concentration 1 solute molecule/60 H_2O. a. ADP; b. ATP.

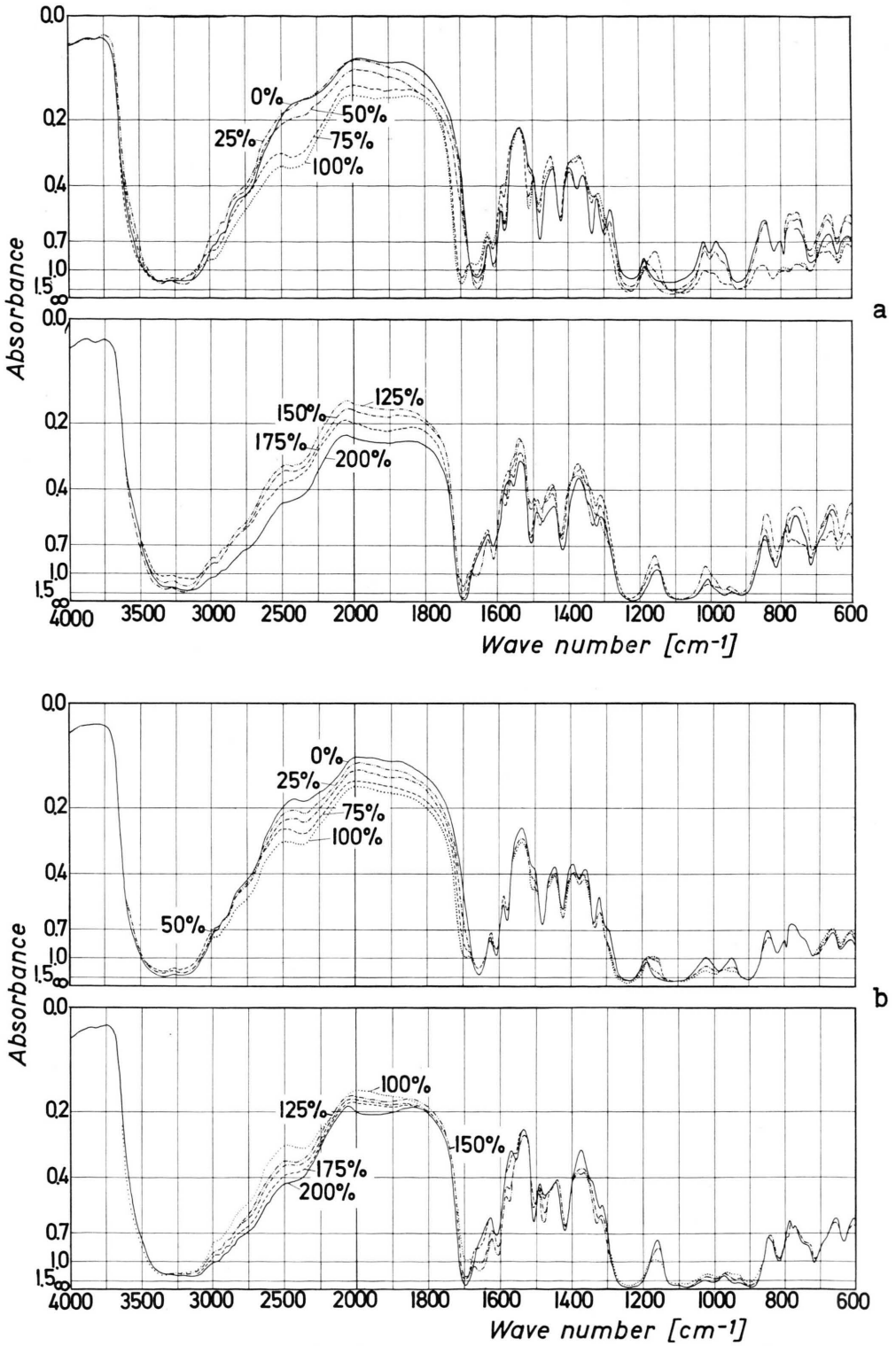


Fig. 3. IR spectra * of films of ADP^{3-} or ATP^{4-} Na^{+} salts dependent on protonation hydrated at 7% relative air humidity at the film. % figures are mole % H^{+} relative to the phosphate molecules. a. ADP; b. ATP.

* The region of the phosphate bands cannot be well evaluated since the absorbance is too strong with the layer thickness choosen. With respect to the reproducibility of the evalution of the continuum, such a relatively large layer thickness was necessary. Therefore spectra of the same samples were plotted with smaller layer thicknesses to control the results with the phosphate bands.

rizable hydrogen bond between hydrogen phosphate ions and is thus to be represented by the two proton boundary structures shown. As far as the phosphate band is concerned, the spectra of the 150% protonated PO_4^{3-} ion cannot be fully explained through mere superposition of the bands of the 100 and 200% protonated ion (*i. e.* 1160 and 1080 cm^{-1}). In fact, the fluctuation of the proton in the polarizable hydrogen bond causes strong broadening of the band of the 150% protonated phosphate ions at 1080 cm^{-1}.

The other protonation ranges shall not be considered here, since they do not occur under physiological conditions with respect to the pH value of these solutions (see ref. 14 a).

Na^+ salts of ATP and ADP in film and solution

pK_a values and assignment

$Na_{1-n}H_nATP$ and $Na_{3-n}H_nADP$ ($n = 0{:}0\%$; $n = 2{:}200\%$ protonation) were, starting from tetra- or tri-Na^+ salt, respectively, protonated in steps of 25% to maximally 200% and investigated in aqueous solutions and as films (Figs 2 and 3). At protonation degrees of up to 200% the acceptors with the lowest and the highest pK_a value of the ATP and ADP play no significant role. The pK_a values 6.5 and 4.0 are assigned spectroscopically [15, 16] to the N(1) at the base residue and to the terminal $-PO_3^{--}$ group.

As will be described in detail (Experimental Procedure), the protonation of the molecule was not carried out by adding acids (titration), since adding other ions (counterions of the acid and the buffer) can make the results of the spectroscopic measurements too complex and thus completely incomprehensible. Therefore we effected protonation by mixing salts and acids.

The assignment of the bands which are relevant for our investigations is summarized – according to [17, 18] – in Table II.

Comparison of ATP and ADP in aqueous solutions

ATP is obtained from ADP by adding a third phosphate group *via* a POP diester bond. As recognized at best with the spectrum of the unprotonated ATP (Fig. 2), the ν_{as} P–O–P band at 940 or 920 cm^{-1}, respectively, is therefore more intense with the ATP than with the ADP (*cf.* Figs 2 a and 2 b).

The protonation occurs similarly with both molecules. The pH range of the protonation of the phosphate group is clearly separated from that of the base residue. The protonation proceeds according to the pK_a values 6.5 or 4.0, respectively, initially at the final phosphate group and then at the N(1) atom of the base. These two positions become occupied in succession by the first and the second proton. On adding the proton at pH 6.5, the PO_3 group with the ATP and ADP becomes rearranged as shown in Table II. Although this rearrangement process is the same with ATP and ADP, different changes occur in the spectrum.

The protonation at the phosphate in the terminal position is indicated in particular by a typical change in the range 1080 – 1120 cm^{-1}. The antisymmetric stretching vibration of the $-PO_3^{--}$

Table II. Band position and assignment for the ATP or ADP in aqueous solutions (60 H_2O/soluted molecule).

Band positions anion		Rearrangement with H^+ addition	Band positions anion + H^+	
ADP	ATP		ADP	ATP
1213	1235	ν_{as} $\left(\begin{matrix}O\\-P-\\O\end{matrix}\right)^-$ ν_{as}	1225	1240
masked	1085	ν_s ν_s	1108 *	1120 *
1112	1120	ν_{as} $\left.\begin{matrix}\\-P=O\\\\\end{matrix}\right\}^{2-}$ → $\left.\begin{matrix}\\-P=O\\OH\end{matrix}\right\}^{-}$ ν_{as}	1080	1095
1020	990	ν_s ν_s		1080
940	920	ν_{as} P–O–P P–O–P–OH	940	905
820	830	ν_s resp. → resp.	820	830
		P–O–P–O–P P–O–P–O–P–OH		

* Eventually C–O–P stretching vibration, then masked withouth protonation; otherwise a ν_s PO_2^- vibration.

group at 1120 cm^{-1} disappears. Thus the symmetrical stretching vibration of the $-PO_2^{--}$ groups which occurs on rearrangement is found at 1080 cm^{-1} (see Table II). With the ATP the band splits at 1080 cm^{-1}, in contrast to the ADP. Moreover, with the ATP $\nu_{as} - PO_2^{--}$ at 1235 cm^{-1} shifts far more weakly toward larger wave numbers than with the ADP and hardly increases in intensity. ATP and ADP can thus be distinguished from one another in the spectra of the protonated substances on the basis of these criteria.

No further significant changes are observed in the range of the PO vibrations on the second protonation step. As protonation increases, the band which occurs when the base residues are protonated (1690 cm^{-1}) becomes visible. In the following, this band indicates the protonation of the base residues. However, in a dilute H_2O solution (Fig. 2) it merely appears as a shoulder at the slope of the H_2O scissor vibration (1640 cm^{-1}). The two bands of base ring vibrations at 1510 and 1480 cm^{-1}, however, change markedly on protonation. With the unprotonated molecule the band at the smaller wave number is always present, and the other band merely as a shoulder. The contrary is the case when the base residues are completely protonated.

The proton added to the phosphate group of the ATP or ADP molecules causes no IR continuum. On protonation of the base residue, in contrast, this effect is marked by the rise of the background as protonation increases, as is shown clearly in the range 3000 – 1700 cm^{-1}. When the dilution is taken into consideration, this continuum is almost as intense as that found by Sessler on protonation of purine [5]. This continuum indicates the formation of easily polarizable $NH^+ \cdots N$ hydrogen bonds between the purine residues. A maximum of the absorbance of the continuum at 150% protonation, as observed with imidazole [3], cannot be expected, since the purine residue possesses not only one acceptor but N(1) and N(3) as acceptors for protons and hydrogen bonds. Furthermore, complex interplay between the formation of the $NH^+ \cdots H$ hydrogen bonds and the addition of water molecules occurs [4].

Comparison with the spectra of the hydrated films

Let us now compare the spectra of the solutions in Fig. 2 with those of the hydrated films in Fig. 3. The spectra of the films exhibit a basic difference from those of the ADP or ATP, respectively, in aqueous solution: the changes to the bands of the phosphate groups discussed as well as those at the ring vibrations are found from 0% upwards as protonation increases. The properties of the proton acceptors evidently change in the layer to such a degree that the phosphate group and base residue protonate simultaneously. This applies equally to ATP and ADP; with ADP, however, the ring protonation is preferred – for it is seen clearly that the band at 1690 cm^{-1}, indicating the ring protonation, emerges far more markedly with ADP at 100% protonation than with ATP. Since the ratio of the bonding constants for the proton at both bonding positions in dilute solutions amounts to $5 \cdot 10^2$, the pK_a values evidently approximate each other as the ADP or ATP concentration, respectively, increases.

Here, too, a continuum occurs which can be observed in the range 3000 – 1700 cm^{-1}. In this case, however, the absorbance of this continuum starts from 0% and increases with protonation. This conforms well with the fact that the proton acceptor strength of the phosphate group and that of the base residue approximate each other. In addition to the easily polarizable $NH^+ \cdots N$ bonds, unsymmetrical hydrogen bonds form in the films as protonation increases. Here the donors are presumably NH^+ groups, which cause a band at 2400 cm^{-1}.

Mg^{2+} complex of ADP in aqueous solutions

The energy-rich phosphates usually occur in nature as Mg^{2+} complexes. The products occurring on orthophosphate hydrolysis of the MgATP complex are the magnesium complex of the ADP and inorganic phosphate.

The complex formation with Mg^{2+} shifts the pK_a value of the protonation of the phosphate residue; on the other hand, the bonding constant for the Mg^{2+} depends on the degree of protonation [23, 25]. This is summarized in Table III.

No final decision can as yet be made concerning the bonding site of the Mg^{2+}. Izatt [24] gives a short

Table III. Mutual dependence of the binding of Mg^{2+} and H^+ with ATP and ADP (taken from ref. 16, 19–25).

log K (Mg^{2+})			log K (H^+) = pK_a		
ATP		ADP	ATP		ADP
4.5	H^+ ↓	3.5	6.5	Mg^{2+} ↓	6.5
2.5		2.0	4.5		5.0

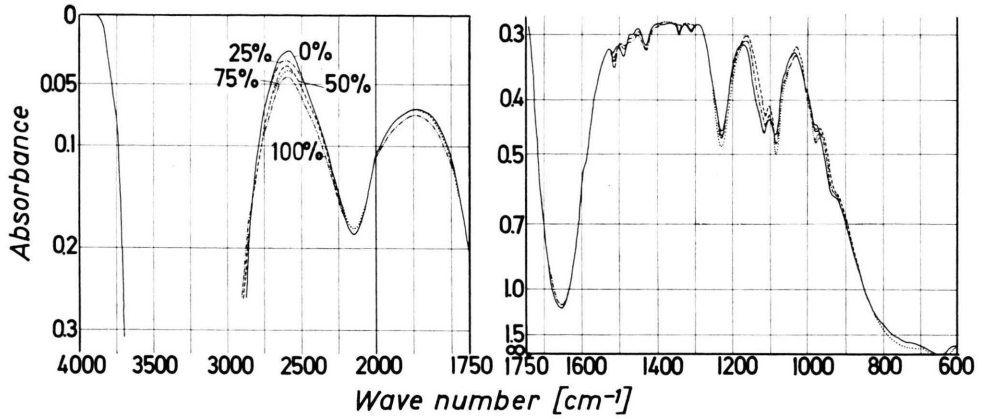


Fig. 4. IR spectra of aqueous solutions of the MgADP complex dependent on protonation. % figures are mole % H^+ relative to the ADP ions. Concentration 1 ADP/170 H_2O,

survey. IR investigations on poly(A) clarified that Mg^{2+} ions cross-link the PO_2^- groups with the base residues [26]. However, no clear evidence that Mg^{2+} ions act directly on the adenine residue could be observed.

Fig. 4 shows the spectra of 0.3 M aqueous $MgK_{1-n}H_nADP$ solutions ($n = 0$:0%; $n = 1$:100% protonation). The protonation was varied in steps of 25% from 0 to 100% protonation.

Comparing the spectra of the Mg^{2+} complex with those of the Na^+ salts (Fig. 2 a) yields the following findings:

1. The vibration ν_{as} of the PO_3 group (1120 cm^{-1}) has already at 0% protonation largely disappeared with the Mg^{2+} complex; that is, the PO_3 group is always rearranged as in the case of protonation (Table II).

2. The position of the ν_{as} of the $-PO_2^{--}$ group with the Mg^{2+} complex does not depend on the protonation. It always lies at 1227 cm^{-1}, which is the position of the vibration at 50% and 100% protonated Na^+ salt of the ADP.

3. With the Mg^{2+} complex, as with the Na^+ salt up to 100%, the vibrations of the base residues undergo no change on protonation.

The findings 1 – 2 reflect first of all the often confirmed fact that the presence of an Mg^{2+} ion has the same effect with regard to the phosphate group as protonation. It may be concluded that the Mg^{2+} ions split H_2O molecules which are linked to the phosphate groups. The locally arising excess protons then lead to the effects observed. Indeed, Brintzinger inferred "local hydrolysis" from IR measurements of the interaction between various divalent ions with the phosphate group of the ATP [27]. Water which is situated between Mg^{2+} ions and phosphate groups is split hydrolytically in unprotonated systems. This hydrolysis is suppressed as protonation increases. Since, due to this buffer effect, the true degree of protonation changes only slightly as protonation decreases, no alterations are observed in the IR spectrum. The background, too, remains almost constant.

Aqueous solutions of the MgADP complex together with phosphate ions

Since the previous sections dealt with systems containing only one anion, measurements shall now be discussed of solutions containing both ADP^{3-} and PO_4^{3-}. Fig. 5 illustrates the spectra of a stepwise protonated 0.3 M aqueous solution of Mg^{2+}:ADP^{3-}:PO_4^{3-} = 1:1:1. The parameter is once again the degree of protonation, K^+ is replaced stepwise by H^+. Of the 6 negative charges available at both anions PO_4^{3-} and ADP^3, two are compensated for by Mg^{2+} with all samples. The protonation which is termed in the following "overall protonation" n is defined as the protonation relative to (ADP^{3-} + PO_4^{3-}) *i. e.*, to both anions. The overall protonation was varied from 50 to 300%.

It can be expected that the PO_4^{3-} ion binds the first proton completely, on account of its high pK_a value of 12. It should be taken into consideration, however, that the local hydrolysis at the MgADP complex also supplies protons. Thus the bands of the 100% protonated PO_4^{3-} ion at 1080 and 990 cm^{-1} already appear at 50% overall protonation

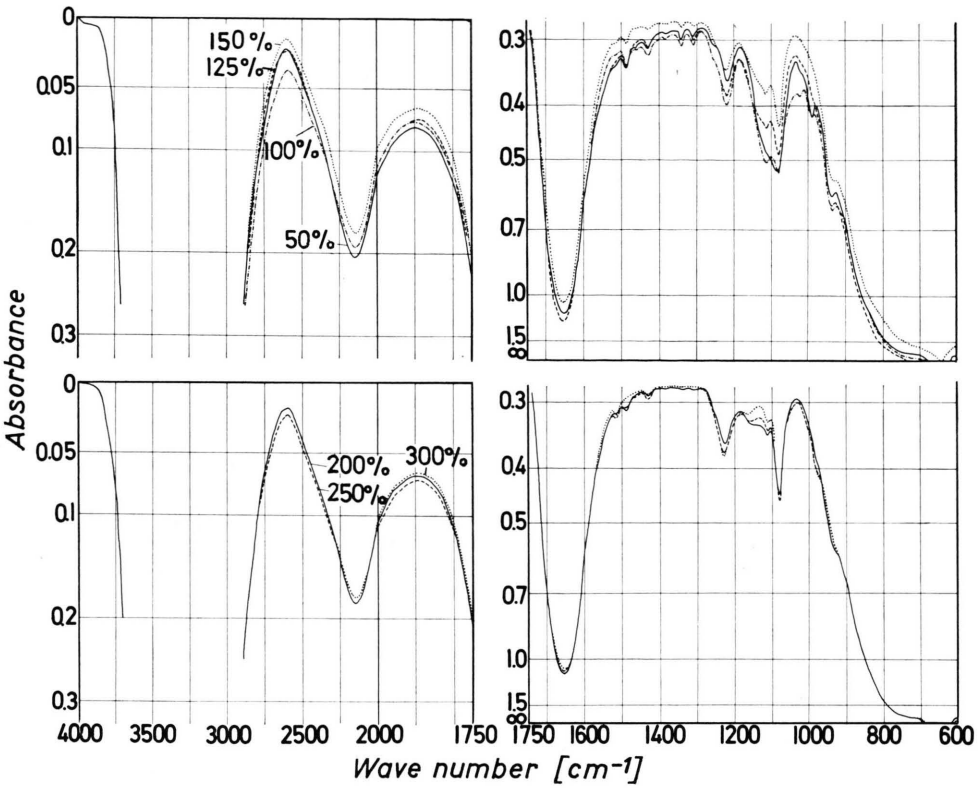


Fig. 5. IR spectra of a 1:1:1 mixture of ADP^{3-} : PO_4^{3-} : Mg^{2+} dependent on protonation. % figures are mole % H^+ relative to (ADP^{3-} or PO_4^{3-}). Concentration 1 ($ADP+PO_4$)/170 H_2O.

instead of the intense antisymmetric stretching vibration of the PO_4^{3-} ion at 1010 cm^{-1} (*cf.* Fig. 1).

The influence exerted by further protonation is particularly clear when overall protonation changes from 150 to 200%. The intense absorption at 1080 cm^{-1}, which overlies the bands of the ADP at 100% protonation, disappears here. As comparison with Fig. 1 shows, the disappearance of this band is the result of transition from the 150 to the 200% protonated PO_4^{3-} ion. The same is indicated by disappearance of the band at 980 cm^{-1}. Thus, on 150% overall protonation, two protons have already been added to most of the PO_4^{3-} ions. The remaining ions are protonated at two sites in the range 150 – 200% overall protonation.

On transition from 200 – 300% overall protonation, the local hydrolysis at the ADP is suppressed and the ADP complex protonated further. This is shown by the decreasing intensity of the band at 1120 cm^{-1}, the increase in intensity of the antisymmetric stretching vibration at 1213 cm^{-1} and the occurrence of the weak shoulder at 900 cm^{-1}. Changes occurring on exceeding 200% protonation of the PO_4^{3-} ion, for instance the occurrence of an intense band at 1005 cm^{-1}, are not observed in the spectra in Fig. 5.

The absorption decrease in the range of the H_2O torsional vibrations, *i. e.*, below 800 cm^{-1} on 150% protonation, deserves attention. This effect is far more marked with the hydrolysing ATP system and is discussed in [28].

Conclusions

Interpreting the hydrolysis experiments [28] described in the following paper is complicated by the fact that, during the hydrolysis, the proportion of the anions and protons present alters as a function of the degree of hydrolysis. The purpose of the present paper was to provide a basis for interpreting these experiments. This was achievied by varying the degree of protonation for given fixed anion concentrations. The changes observed on protonation to the bands of the phosphate groups

and the base ring vibrations clarified the proton distribution over the various acceptors as a function of all the protons present. Further, the occurrence of an IR continuum indicates that under certain conditions $NH^+ \cdots N$ hydrogen bonds form between the adenine residues and that the hydrogen phosphate ions mac be cross-linked *via* $OH^+ \cdots O$ bonds, both of which are easily polarizable. As the continuum indicates, these hydrogen bonds interact strongly with their environment.

Experimental Procedure

The substances were supplied by the following firms: H_4ATP from Fluka AG, Buchs (Switzerland); Na_4ATP from Schuchardt, Munich; H_3ADP from Boehringer, Mannheim; Na_3ADP, K_3PO_4, K_2HPO_4, KH_2PO_4 and H_3PO_4 from Merck AG, Darmstadt.

Mg_3ADP_2 was made from Na_3ADP using an ion exchanger. The subsequent enzyme test (supplied by Boehringer, Mannheim) showed that no significant decomposition had taken place.

The hydration water portion of the initial substances was determined by an ultimate analysis.

All steps were conducted under nitrogen atmosphere in order to prevent the sample solutions from coming into contact with the CO_2 in the atmosphere. The required cation-proton ratios were obtained by mixing the solutions of the initial substances. The ATP or ADP concentrations were determined by means of UV absorption at 260 nm [29]. The K^+ ion content was determined gravimetrically via the complex formation with tetraphenyloborate.

The films of reproducible thickness were made from the solutions on Ge sample carriers with the aid of a centrifugation drying procedure [30]. The water was removed during centrifugation at 7% air humidity (saturated hydrous NaOH solution). The reproducibility of the layer thickness amounted to over 2%.

The films were then investigated in the cells described in [1] at 7% air humidity and 25 °C. The solutions were investigated in the cell shown in Fig. 6 at the same temperature.

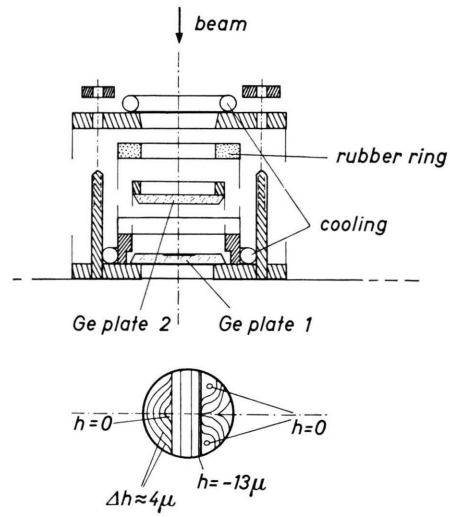


Fig. 6. Liquid cell; longitudinal section. a. Contour lines of Ge plate 1.

The cell is illustrated in Fig. 6. The solution is located in the wedge-shaped space between the two Ge plates. The shape of the surface shown in Fig. 6 a ensures that the two plates touch at three points only. The latter fact together with the even pressure exerted by the rubber ring ensures a layer thickness reproducibility of ± 0.05 μm. At the same time the rubber ring serves as a seal. The average layer thickness amounts to 13 μm. The spectra were plotted with the IR spectro-photometer model 325 supplied by Bodenseewerk Perkin Elmer, Ueberlingen, West Germany (slit program 6.5, response 3, gain 3.0). The ordinate was expanded twice with the spectra shown in Figs 4 and 5.

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