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# Nonenzymatic hydrolysis of adenosinetriphosphate (ATP) at high temperatures and high pressures

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**Abstract**

Marine organisms living in the deep sea near to hot wells show a fascinating tolerance to extremely high temperatures and pressures. Under the given conditions the synthesis and stability of biomolecules seem to be limiting facts for the basis of life. We studied the influence of high pressures and high temperatures on the hydrolysis of ATP, a universal component for the storage of energy in all known organisms and therefore an extremely interesting and important molecule.

The hydrolysis of ATP, ADP and AMP was studied in unbuffered solutions at temperatures between 353 and 369 K at pH values between 3.4 and 10.0. The pressure dependence was determined to $p_{max} = 220$ MPa also at pH 5.

All data can be explained by a proton catalyzed mechanism that removes in consecutive steps the final phosphate group. In none of the experiments could pyrophosphate be detected. The influence of phosphate and magnesium ions on the hydrolysis is discussed.

*Keywords:* ATP; High pressure; Kinetics; Hydrolysis

## 1. Introduction

All presently known forms of life rely on the availability and timely release of the energy stored in the anhydride bonds of the common nucleoside triphosphates. In the absence of functional phosphatases and/or intact cells these compounds are relatively stable at ambient pressure and temperature in the common biological range of pH. In the last decades many forms of life have been found that thrive in hot aquatic environments at temperatures around and well above 100°C [1,2]. These exotic temperatures are often found in the mid-oceanic rift zones where they are combined with high-hydrostatic pressures [3] or near to hot wells at ambient pressures.

A description of the hydrolysis constants for ATP determined around room temperature and at ambient pressure by a single Arrhenian yields an increase of the rate of hydrolysis by approximately three orders of magnitude, when the temperature is increased from 298 to 373 K. It is thus obvious, that a further

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significant increase of $T$ must lead to a situation where the nucleosidetriphosphates (NTP) in aqueous solution become very instable and hydrolyze completely within minutes.

Our studies, presented below, were carried out in order to characterize the limiting temperatures for life more precisely. The main emphasis was to determine the influence of hydrostatic pressure upon the hydrolysis of the NTP. The inclusion of pressure as an additional variable in the analysis of the kinetics permits the activation volume $\Delta V^*$ to be determined and thus gives more information about the mechanism for this process. From a more biological point of view it also provides information about the effect pressure has upon the limits of life. In previous studies it has been shown that high pressures and high temperatures destabilize proteins and other cellular components [5] and cause cell lysis of thermophilic archebacteria [6].

Because of the central importance that adenosinetriphosphate (ATP) has in cell metabolism, its hydrolysis has been studied for a very long time. The early results have been summarized [4,7,8]. The influence of metal ions on hydrolysis rates was recognized very early and has been explored in many publications [7,9,10]. The mechanism of ATP hydrolysis in acidic solution has been described as occurring via a five-coordinated oxyphosphorane intermediate due to an addition–elimination reaction [8]. For alkaline solutions the metaphosphate ion has been postulated as the first product of hydrolysis [8,11].

In view of the marked influence that metal ions have on hydrolysis rates and since the emphasis in this work was more on the characterization of the limiting temperatures for the practical stability of ATP, we studied the reactions in unbuffered solutions. For the high concentrations of ATP used for the NMR measurements and under the given high pressures and temperatures no good buffering systems exist [16]. Additionally the introduction of buffers or salts with high-ionic strength leads to complications in the analysis. These effect could for example be demonstrated by the influence of magnesium ions on the hydrolysis rate of several compounds. Application of $^{31}P$ NMR permits the unambiguous assignment of all phosphorous acid-containing substances in the reaction paths and was thus chosen as the analytical tool.

## 2. Materials and methods

The adenosine phosphates in the form of their disodium salts, tetrasodium diphosphate decahydrate, pentasodiumtriphosphate, magnesium chloride hexahydrate p.A. and sodium deuteroxide (37%) were purchased from E. Merck (Darmstadt, Germany). They were dissolved in $D_2O$ 99.9% deuterated from MSD (IC Chemikalien, München, Germany).

The starting pH was adjusted by titrating the solutions with the 37% sodium deuteroxide in $D_2O$. The pH was determined with a glass electrode. The deuterium ion concentration pD [12] was also determined from the instrument (pD = pH + 0.35).

In order to minimize systematic errors caused by differences in heat transfer to the solutions during temperature equilibration, all experiments including the one at ambient pressure were carried out in a high-pressure autoclave. A home-made steel autoclave with a bore of 20 mm diameter was used. Pressure generation was achieved with commercial 400 MPa equipment (HIP, Erie, Pennsylvania, USA). The pressures were measured to $\pm 5$ MPa by a Bourdon gauge (Heise, Newtown, Connecticut, USA). The autoclave was immersed in an oil bath and thermostated to $\pm 0.3$ K.

Approximately 5 $cm^3$ of the solutions were filled into silicone tubings (i.d. 10 mm, o.d. 12 mm) sealed at either end by glass stoppers and thermostated in the autoclave that had been heated to the desired temperature before-hand. Ethylene glycol was used as the pressure transmitting medium because its low compressibility minimizes possible overheating effects caused by the rapid compression of the liquid in the high-pressure experiments. The temperature inside the tubing was monitored by a metal-sheathed chromel/alumel thermocouple. Under all experimental conditions it took approximately 30 min to reach a constant final temperature. The rather long time spans needed to attain thermal equilibrium restricted the kinetic studies to temperatures at which the hydrolysis occurred with half-life times longer than 3 h. For these rather slow reactions the temperature uncertainty during the initial period was eliminated by numerical methods.

After the planned period of incubation the samples were removed from the autoclave, thrown into an ice bath, neutralized to pH 7 after cooling, and

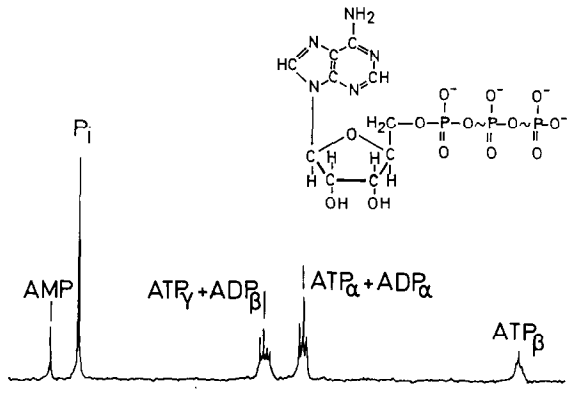



Fig. 1. 121.5 MHz $^{31}$P NMR spectrum of the products of ATP hydrolysis. (Conditions for hydrolysis: 9 h at pH 5.0, 351 K, 0.1 MPa.) The spectrum was obtained at room temperature at pH: 7.0.

stored in a refrigerator until the final relative concentrations of the phosphate containing compounds could be determined by $^{31}$P NMR. No further hydrolysis took place during the storage.

For the determination of the phosphate concentrations $^{31}$P NMR was chosen, since this method permits the simultaneous quantitative identification of all phosphate compounds in one experiment, although this experiment is fairly time consuming. The pH 7 solutions were analyzed in a Bruker MSL 300 spectrometer operating for phosphorous at a frequency of 121.5 MHz in 10 mm tubes.

Fig. 1 gives a typical spectrum. In order to avoid magnetic saturation effects that could distort the relative intensities, a pulse delay of 75 s had to be applied. The relative ATP, AMP and inorganic phosphate $P_i$ concentrations were determined directly from the integration of the well separated signals. The signal for $P_\beta$ of ADP overlaps with $P_\gamma$ of ATP and the two $P_\alpha$ signals also do the same. The ADP concentration is thus obtained by subtracting the ATP concentration as determined for the $P_\beta$ peak of ATP from the total intensity observed in the range of chemical shift where the ATP $\alpha$ and $\gamma$ signals overlap with the two ADP resonances.

## 3. Results and discussion

In all spectra obtained only $P_i$, AMP, ADP and ATP $^{31}$P signals were observed. No signal assignable to pyrophosphate (PP) was found. (Given the quality of the spectra concentrations $c \geq 2\%$ of the total phosphate should have been detectable). This is in accordance with the results obtained by Ramirez et al. [7] and Mayerson et al. [8] but disagrees with the data published by Hutchings et al. [4] that have recently been reanalyzed [13]. In these experiments significant PP concentrations were observed. These results were obtained in highly concentrated perchloric acid solutions and the PP concentrations were determined by an enzymatic assay. The authors observed a significant PP concentration formed by the hydrolysis of ATP to AMP and PP.

Our data were collected in the pH range $3.4 \leq$ pH $\leq 10.0$. They can be quantitatively described by the sequential removal of the terminal phosphate group:

$$\text{(I)}\quad \mathrm{ATP} + \mathrm{H_2O} \underset{k_{ATP}}{\rightarrow} \mathrm{ADP} + \mathrm{P_i} + \mathrm{H_2O} \underset{k_{ADP}}{\rightarrow} \mathrm{AMP} + 2\mathrm{P_i} + \mathrm{H_2O} \underset{k_{AMP}}{\rightarrow} \mathrm{A} + 3\mathrm{P_i}$$

In order to determine $k_{ADP}$ and $k_{AMP}$ more precisely and to minimize the errors of the data analysis of the hydrolysis experiments for ATP, several experiments were performed with ADP and AMP as the starting materials.

After the initial period needed for establishing the final temperature in the sample, the logarithms of the concentrations $\ln([x]_t/[x]_0)$ of all starting compounds decreased linearly with time showing that the reaction was pseudo first order. From the slope of this plot the hydrolysis constants were calculated. Because of the influence of the heating period, this straight line does not pass at $t = 0$ through the point $\ln([x]_t/[x]_0) = 0$. In the subsequent determination of the hydrolysis constants for the products of hydrolysis (ADP, AMP) this effect was taken care of by shifting the time axis of the experiment in such a way that at $t_0$ (corr) the condition $\ln([x]_t/[x]_0) = 0$ was fulfilled. In a typical experiment this demanded a shift of the time scale by approximately 10 min. Assuming constant temperature, pressure and pH, the hydrolysis is a pseudo first-order reaction. The concentrations of all four phosphate groups containing compounds as function of time are given by a set of coupled linear differential equations. Solving these equations yields for the concentrations of the three

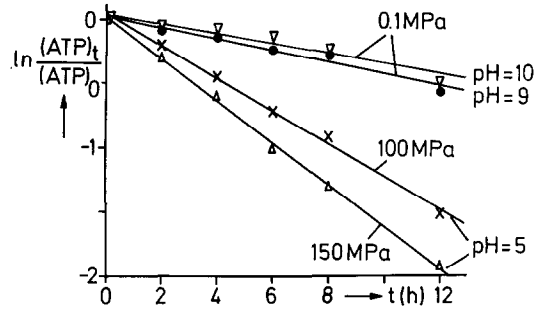



Fig. 2. $\ln([ATP]_t/[ATP]_0)$ as a function of time at two pressures ($T = 353$ K, pH = 5.0), $[ATP]_0 = 10$ mM, and as a function of time at two values of pH ($T = 353$ K, $p = 0.1$ MPa). The large scatter of the experimental concentrations results from the low reaction rates and from the rather large drop of pH during hydrolysis in these slightly alkaline conditions.

nucleoside phosphates as a function of time the following results

$$[ATP]_t = [ATP]_0 \cdot e^{-k_{ATP}t} \quad (1)$$

With the condition $[ADP]_0 = 0$ and $[AMP]_0 = 0$ the corresponding results for these two compounds are:

$$[ADP]_t = \frac{k_{ATP}[ATP]_0}{k_{ADP} - k_{ATP}}\left(e^{-k_{ATP}t} - e^{-k_{ADP}t}\right) \quad (2)$$

$$[AMP]_t = \frac{k_{ATP}k_{ADP}[ATP]_0}{k_{ADP} - k_{ATP}}\left(\frac{e^{-k_{ATP}t} - e^{-k_{AMP}t}}{k_{AMP} - k_{ATP}} + \frac{e^{-k_{AMP}t} - e^{-k_{ADP}t}}{k_{AMP} - k_{ADP}}\right) \quad (3)$$

For all conditions studied the first estimates of $k_{ATP}$ ($T$, $p$, pH) were derived from plots of the type shown in Fig. 2. The full data analysis for the derivation of the three kinetic constants of Scheme I was carried out by an iterative computer parameter fitting program. The iteration was stopped, when the deviations between the experimental and the calculated concentrations became smaller than 1%.

Fig. 3 shows some typical results. The lines drawn through the experimental points result from the computer fits. Fig. 4 gives the ambient pressure hydrolysis constants $k_{ATP}$ and $k_{ADP}$ as function of the initial pH. It appears obvious that in the pH range studied here the hydroxyl ion- or base-catalyzed mechanisms do not contribute significantly to the decomposition

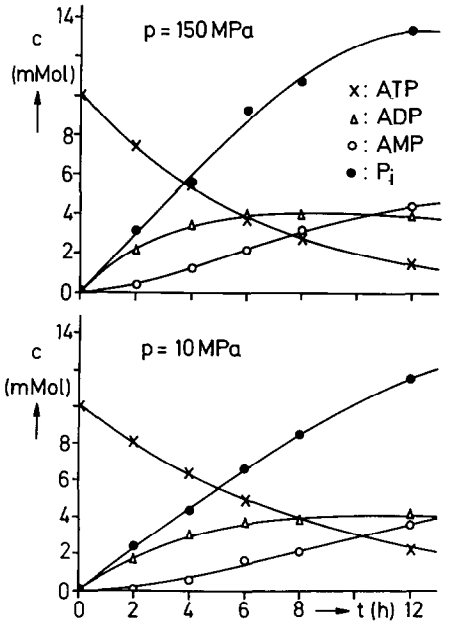



Fig. 3. Concentration of the four phosphate compounds as a function of time at two pressures. The lines drawn through the experimental points are the result of the computer fits to Eqs. (1–3). ($T = 353$ K, pH = 5.0.)

of the anhydrides. The insert in Fig. 4 gives the logarithm of the quotient of the kinetic constants $k_x(pH)/k_x(pH = 3.38)$. The data are described by

$$k_x \sim e^{-c_x pH} \quad (x = ATP \text{ or } ADP) \quad (4)$$

with $c_{ATP} = 0.22 \pm 0.01$ and $c_{ADP} = 0.21 \pm 0.02$ and yield further evidence that only one mechanism, the

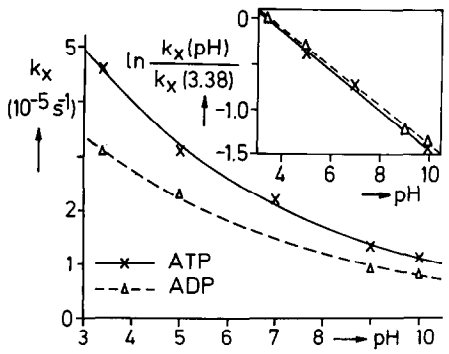



Fig. 4. $k_{ATP}$ and $k_{ADP}$ as a function of starting pH. $T = 353$ K, $p = 0.1$ MPa. Insert $\ln(k_x(pH)/(k_x(pH = 3.4))$ as function of pH.

Table 1
Influence of magnesium ions, inorganic orthophosphate and the adenosine moiety on the rate constants for the hydrolysis of ATP, ADP and AMP at pH 5 and 353 K. Starting concentrations 10 mM of adenosine phosphates. Phosphate additions — ATP, $[P_i] = 0$ mM; ADP, $[P_i] = 10$ mM; AMP, $[P_i] = 20$ mM. This set of experiments was aimed at studying the hydrolysis at constant overall $P_i$ concentration. Magnesium additions — ATP and ADP: $[Mg^{2+}] = 10$ mM

| | Neat | $+Mg^{2+}$ | $+P_i$ |
|---|---|---|---|
| X | $k_x(10^{-5}\ s^{-1})$ | $k_x(10^{-5}\ s^{-1})$ | $k_x(10^{-5}\ s^{-1})$ |
| ATP | $3.1 \pm 0.2$ | $2.0 \pm 0.2$ | – |
| ADP | $2.6 \pm 0.2$ | $1.8 \pm 0.2$ | $2.5 \pm 0.2$ |
| AMP | $0.2 \pm 0.02$ | – | $0.6 \pm 0.03$ |
| PPP | $5.4 \pm 0.3$ | – | – |
| PP | $2.7 \pm 0.2$ | – | – |

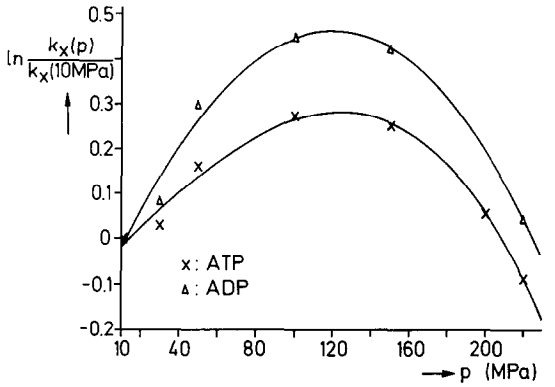



Fig. 5. Logarithm of the normalized rate constants for the hydrolysis of ATP and ADP at 353 K vs. pressure. (Starting pH = 5.0.)

proton catalyzed hydrolysis, is observed in the range of pH studied.

In order to have an independent control of $k_{ADP}$, several hydrolysis experiments were run with AMP and also ADP as starting material. In addition, the influence of magnesium ions and of inorganic phosphate on the hydrolysis rates were studied.

Table 1 compiles the data for the influence of $Mg^{2+}$ and $P_i$ upon the hydrolysis. The addition of the magnesium ions reduces the hydrolysis rates of ATP and ADP by ca. 30%. Since in the living cell most ATP is thought to be complexed to $Mg^{2+}$, this complexing obviously results in a slight stabilisation of the triphosphate corresponding to a drop in temperature by ca. 2 K. Inorganic phosphate does not seem to have any influence upon ADP hydrolysis. However, the ester hydrolysis of AMP is enhanced by a factor of 3. While the stabilizing influence of $Mg^{2+}$ is easily rationalized by complex formation between the metal ion and the phosphate moieties, the reason for the catalytic effect $P_i$ has upon the ester hydrolysis remains obscure.

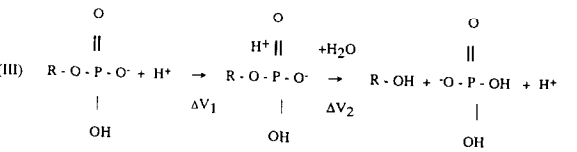
The discrepancy between our hydrolysis Scheme I where no phosphate is formed and the mechanism proposed by Hutchings et al. [4] could also result from the rapid decomposition of the pyrophosphate under the conditions of our experiments. If this is the case, the PP formed by the reaction

(II) $ATP + H_2O \rightarrow AMP + PP$

would be removed rapidly from the reaction mixture and never be present in measurable quantities. In order to exclude this possibility the hydrolysis constants for triphosphate (PPP) and PP were determined. They are also included in Table 1. $k_{PP}$ is within experimental error identical to the constants found for ATP and ADP. The fact that PPP decomposes with twice the rate of the three other compounds is readily explained by the two identical sites for hydrolysis in each PPP molecule. The presence of the adenosine moiety does thus only direct the hydrolysis towards the terminal anhydride bond, but does not influence the actual reaction rate.

In Fig. 5 the pressure dependence of the two hydrolysis constants $k_{ATP}$ and $k_{ADP}$ are given. The pressure dependence of rate processes is usually described by the activation volume $\Delta V^*$.

The initial slope yields for both curves $-15 \pm 4$ $cm^3\ mol^{-1}$. However, at pressures around 120 MPa both curves flatten and pass through a maximum. Further increase of pressure leads to a sharp drop of the hydrolysis constants. The initial negative activation volume is best explained by the most common mechanism of anhydride and ester hydrolysis [14] the $A_{Ac}$ 2-mechanism.




This mechanism is typically accompanied by a negative $\Delta V^*$ of the order of $-8$ to $-15$ $cm^3$

mole$^{-1}$. Tentatively a consistent description of the pressure dependence of the hydrolysis reactions can be given: at ambient pressure, the second reaction of Scheme III is rate limiting. Its rate increases rapidly with pressure ($\Delta V_2$ negative) such that at high pressures step one becomes the limiting reaction, which in the case of the nucleoside phosphates is accompanied by at least a partial neutralisation of the ionic charges and thus should be accompanied by a positive $\Delta V_1$.

## 4. Concluding remarks

All data collected here can be described quantitatively by the consecutive hydrolysis mechanism [7,8] given in Scheme I. Pyrophosphate was not observed in any of the spectra. Also the influence of additions of $Mg^{2+}$ and $P_i$ can be fully described with the reactions of Scheme I. The influence of $P_i$ on the ester hydrolysis remains obscure and needs further investigations. The most important result of the study in the alkaline solution is, that hydroxyl ion catalyzed hydrolysis, that is very efficient in ester hydrolysis, is at least an order of magnitude slower than the proton catalyzed splitting of the anhydride bonds.

Pressures in the range of those encountered in the accessible biosphere ($p \leq 100$ MPa) enhances the rate of all steps of ATP hydrolysis. The inversion of the pressure dependence of $k_{ATP}$ and $k_{ADP}$ at $p > 120$ MPa yields interesting mechanistic information for these reactions. Obviously the rate limiting step of the hydrolysis is at higher pressures different from the ambient pressure conditions.

Extrapolating the ambient pressure data to higher temperatures yields the result that around 400 K and at slightly acidic pH the half-life time of ATP is in the range of a few minutes. Organisms thriving under the most extreme temperature encountered hitherto (383 K) [3] must thus have mechanisms for protecting their ATP from hydrolysis or resynthesize this compound permanently at a very high rate. At first glance a reduction of the water activity in the cytosol or at least in the regions of the cell interior where the triphosphates are accumulated could provide a partial protection. However, kinetic studies of the ATP hydrolysis in media where the water activity was reduced by up to 90% by the addition of dimethylsulphoxide or ethylene glycol [15], showed quite unexpectedly a 4 to 6 fold increase of the hydrolysis rates.

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