

Monitoring Hydrothermal Reactions on the Millisecond Time Scale Using a Micro-Tube Flow Reactor and Kinetics of ATP Hydrolysis for the RNA World Hypothesis

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A new method for monitoring hydrothermal reactions on the millisecond time scale has been developed using a micro-tube flow reactor. The system was designed based on a high-pressure pump, a sample-loop injector, a narrow-bore size capillary, a cooling bath, back-pressure tubing, and a sampling port. Samples were analyzed using high-performance liquid chromatography. Kinetic analyses of the hydrolyses of adenosine 5'-triphosphate (ATP) and adenosine 5'-monophosphate (AMP) on the millisecond time range at 250—315 °C were demonstrated so as to evaluate the performance of the method. In conclusion, monitoring was successful for the hydrolysis of ATP and AMP in 2—50 ms using fused-silica capillary tubing with 0.015 and 0.025 mm inner diameter (ID). The method enables real-time monitoring of hydrothermal reactions in about 100-times shorter time range than other techniques. This paper proposes apparent rate constants and activation energy for the consecutive hydrolysis of ATP to adenine at high temperatures that were difficult to determine by a conventional batch method. On the basis of the rate constants, kinetics and mechanistic analyses of the step-wise decomposition of ATP were investigated from the viewpoint of the RNA world hypothesis under hydrothermal environments.

The discovery of ribozymes has suggested that the first genetic information emerged on ribonucleic acids (RNA) or RNA-like molecules on the primitive earth.^{1–4} To evaluate this hypothesis, a number of successful studies have been carried out on the spontaneous formation of RNA.^{5–8} On the other hand, it is widely believed that hydrothermal environments played an important role in the chemical evolution of biomolecules.^{9–12} For example, this hypothesis is supported by phylogenetic analyses which suggests that the last common ancestor of all present organisms had the nature of hyperthermophiles.^{13,14} Thus, the less stability of RNA is a major stumbling block for the RNA world hypothesis. However, it seems to be surprising that less quantitative analysis has been carried out concerning the formation and decomposition of RNA under hydrothermal environments.^{15–18}

There has been a limited number of investigations on the chemical evolution of RNA at high temperatures, because of the difficulty to monitor rapid reactions under hydrothermal conditions. Thus, the reaction rates under hydrothermal conditions were occasionally estimated by extrapolating the temperature dependence of the rate constants at low temperatures. Besides, the kinetics of hydrothermal reactions are important in both fundamental and practical areas.¹⁹ Recently, in situ spectral measurements of hydrothermal reactions have been successfully developed using Raman spectroscopy²⁰ and FT-IR.^{21–23} Although the usefulness of these systems has been demonstrated for the in situ monitoring of the decomposition of organic compounds, it is difficult if the reactants and products do not have some spectral difference. Further, monitoring of the time range in

these systems was limited in the sub-second to second time range.

Thus, we have attempted to develop a monitoring method of hydrothermal reactions in the millisecond time range using micro-flow reactors.^{24,25} In the present system, after the reaction samples are taken through a sampling port of the flow system, those containing reactants and products are analyzed by a conventional analytical technique, such as for high-performance liquid chromatography (HPLC). Moreover, the system is easy to set up, since the flow system easily generates high pressure in the flow-tube reactor by back pressure. In this study, the details and scope of the present system were investigated, in which monitoring in millisecond time range at 250—315 °C was successful. The results of these studies on the step-wise hydrolysis of ATP led to proposals concerning the RNA world hypothesis under hydrothermal environments.

Experimental

Flow System and Apparatus. The flow system of hydrothermal reactions is illustrated in Fig. 1. The system consists of a 1 L (1 L = 1 dm³) H₂O reservoir, a high-pressure HPLC pump (model PU-980, JASCO Corporation, Japan), a sample injector (Rheodyne, with 100 µL sample loop), a hydrothermal reactor, a cooling bath, and a back-pressure tubing and sampling port, as well as a temperature controller. A 2 µm stainless-steel line filter was placed before the hydrothermal reactor to remove any particles. The system was connected with transfer lines (0.1 or 0.25 mm inner diameter (ID) of stainless steel (SUS) tubing), and double-distilled water, which was filtered with a 0.2 µm membrane filter, was pumped from the reservoir against the back pressure on the system. The two types

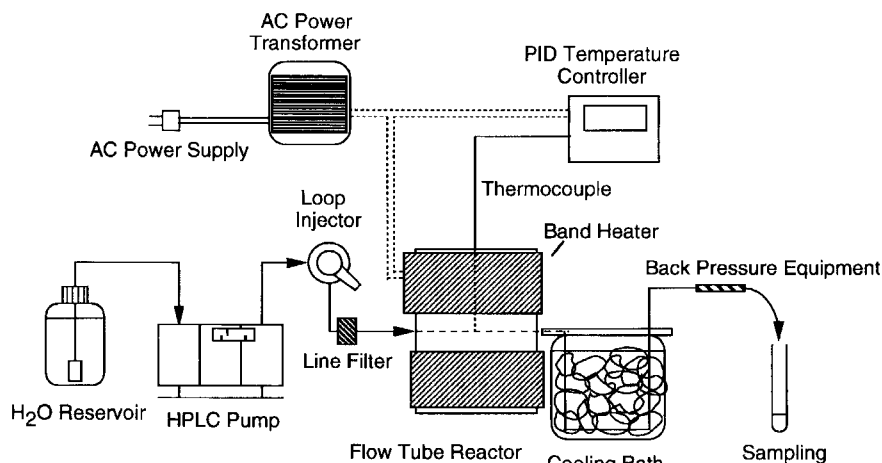


Fig. 1. Hydrothermal system. The system consists of a 1 L H₂O reservoir, a high pressure HPLC pump, a sample injector, a hydrothermal reactor, a cooling bath, a back pressure equipment and sampling port, and a temperature controller.

of flow reactors (reactor I and II in Fig. 2) consisted of a capillary and a heating block, of which the temperature was controlled by using a Proportional-Integral-Derivative (PID) controller (Shimaden, Tokyo, Japan) at $\pm 1\%$. The pressure in the reactor could be regulated over the vapor pressure of water using back-pressure tubing. A 100 μ L reaction sample was injected and heated up at the reaction temperature, and then quenched immediately in a cooling bath. The sample exposed to high temperature was withdrawn from the sampling port after an appropriate time period from injection, which is known from either a calculation based on the flow rate and the inner volume of the system or an experimental calibration using a marker solution, such as diluted HCl and pH test paper. The residence time in capillary was controlled by changing the flow rate of the pump and/or the capillary with different sizes. The inner volume of 0.1 or 0.25 mm ID tubing was determined by weighing with and without filling water at 25 $^{\circ}$ C, and that for tubing smaller than 0.1 mm ID was determined by a calculation based on the ID and length of the

tubing.

Reactor I was used for tubing longer than 10 cm, which was coiled between the main part (A) and the cover (B). For reactor II, 5 or 10 cm fused-silica capillary tubing was placed between two aluminum blocks (Fig. 2). To connect the capillaries, a capillary mini union (22 mm $\times$ 1/16 inch) with graphite ferrules (GL Science Co., Tokyo, Japan), which is commercially available for gas chromatography, was used, and was effective up to 30 MPa. SUS and fused-silica capillary tubing were joined using polyetheretherketon (PEEK) and/or a stainless-steel union (GL Science Co., Tokyo, Japan), with a PEEK connector of fused-silica capillary tubing for capillary electrophoresis (JASCO, Tokyo, Japan), which was tested to bear up to 30 MPa.

The back-pressure was regulated using back-pressure tubing, which was made using a 0.375 mm OD of capillary tubing that was fixed with epoxy resin inside of SUS tubing (0.5 mm ID). The back-pressure tubing was efficient to control the pressure up to 30 MPa

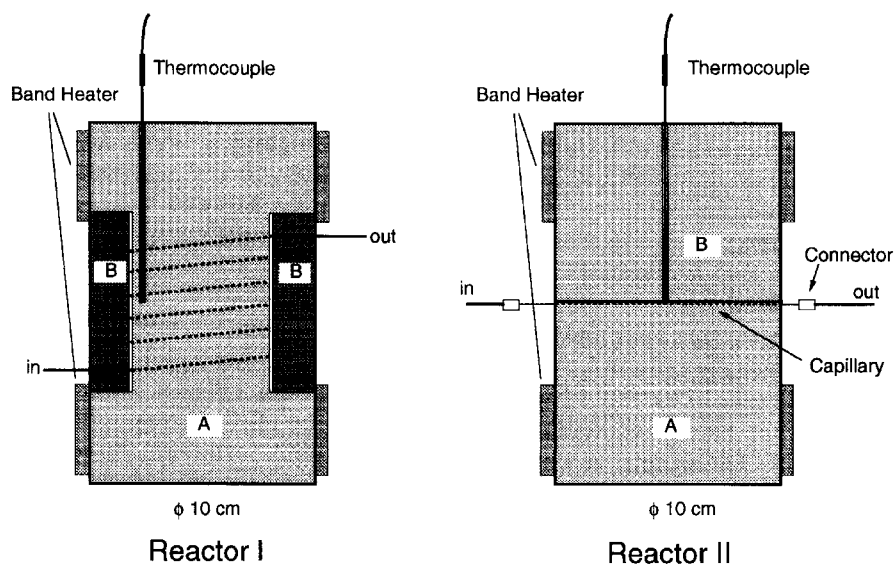


Fig. 2. Cross section view of the hydrothermal reactors. Reactor I: The reactor is made of aluminum block and consists of main part (A) and cover (B). Tubing or capillary longer than 10 cm is coiled between the main part and cover. The reactor is covered by band heater. Reactor II: Capillary of 5 and 10 cm in length is placed between 2 aluminum blocks (A and B). The capillary tubing is located in a groove 0.4 mm depth of the upper surface of the part A. Thermocouple in both reactors is at 3–4 mm from the tubing.

with the acquisition of $\pm 10\%$ by using an appropriate length of back-pressure tubing.

Chemicals and Reaction Procedure. All of the reagents used were of analytical grade. To evaluate the flow system, the hydrolytic reaction of ATP was monitored at 250 °C and that of AMP was monitored at 270–315 °C. The reaction was carried out in a buffer solution containing 0.005 M (1 M = 1 mol dm⁻³) of ATP or 0.001 M AMP, and 0.1 M NaCl, 0.05 M MgCl₂, 0.05 M imidazole (initial pH at 25 °C is 7.0). The samples were analyzed by HPLC (Shimadzu HPLC LC-10A) with a ODS-2 column (GL Science Co., Tokyo, Japan) using a gradient of 0.02 M NaH₂PO₄ with 0.005 M tetrabutylammonium bromide (TBABr) in aqueous solution at pH 3.5 mixed with 0.02 M NaH₂PO₄ with 0.005 M TBABr in 60% CH₃OH at pH 3.5. The rate constants were calculated using a kinetic program, SIMFIT.²⁶

Results and Discussion

Pressure Drop and Heat-Up Time. Rapid monitoring by the flow system can be achieved with a small residence time and quick heating up of the sample solutions. A small residence time can be attained by either increasing the flow rate or reducing the capillary ID and length. Here, the pressure drop in capillary restricts the minimum residence time, because the pressure drop rises with decreasing the capillary ID. The pressure drop (F) is given by

$$F = (32 \cdot l \cdot \mu \cdot v) / (g_c \cdot d^2 \cdot \rho) \quad (1)$$

for laminar flow, where l is the length of tubing, μ is viscosity of the fluid, v is the flow rate in length base (m s⁻¹), g_c is the gravitational conversion coefficient, d is the inner diameter of the tubing, and ρ is the density of the fluid.²⁷ In the present system, the Reynolds number is in the range of 100–2000. Additionally, the pressure drop is proportional to the flow rate for laminar flow, which was actually observed in this system, while the pressure drop for turbulent flow is proportional to the square of v . Thus, it is considered that the flow in the present system was laminar flow. According to Eq. 1, the pressure drop under a constant volumetric flow rate (L s⁻¹) is inversely proportional to the fourth power of d , since v is inversely proportional to the square of d . This is a critical problem when a very narrow bore size capillary is used, and it is obvious that the pressure drop limits the maximum value of the flow rate. In the present system, because the maximum pressure allowed for the pump was 50 MPa, the limits of the flow rate and size of the capillary were justified on the basis of the maximum value of the pressure.

Besides, because the pressure drop in tube reactor is proportional to the viscosity of the fluid (μ), the pressure drop decreases with increasing the temperature, since the viscosity of water decreases with temperature. This effect is fairly important to reduce the pressure drop at high temperatures, for example, μ at 250 °C is approximately one tenth of that at room temperature.²⁸ As a result, it is recommended that the flow rate is initially regulated at a low value i.e. 0.02–0.05 mL min⁻¹ and gradually rises up with increasing the temperature. This procedure prevents any over pressurizing at lower temperatures using a narrow bore size smaller than 0.025 mm ID. In the present system, the ranges of the

flow rates that could be used were 0.06–0.17 mL for 0.015 mm ID $\times$ 5 cm and 0.06–0.35 mL min⁻¹ for 0.025 mm ID $\times$ 10 cm. For a larger ID of the tubing, the pressure drop in the capillary was not significant.

On the other hand, quick heating up of the sample is necessary for rapid monitoring. The heat-transfer rate increases with decreasing the capillary ID. The time required for heating up the sample solution (the heat-up time) was evaluated on the basis of an analysis of ATP hydrolysis. The induction period, where the reaction does not proceed, was observed, and the heat-up time was determined as the interval period of the reaction curves.²⁵ The heat-up time determined using several sizes of capillary were 20–40 ms (0.1 and 0.05 mm ID), 4 ms (0.025 mm ID), and 2 ms (0.015 mm ID). The heat-up time decreased with decreasing the capillary ID, and was almost independent of the temperature at 250–315 °C. In conclusion, the heat-up time was notably reduced with decreasing the ID, even though the pressure drop also increased with decreasing the ID. Thus, the problem of a significant pressure drop in a narrow capillary can be avoided by shortening the capillary length.

In addition, a preheater, which was designed to be the same as reactor I, was tested to reduce the heat-up time. The reaction curves are shown in Fig. 3 for ATP hydrolysis using SUS tubing (0.1 mm ID $\times$ 38 cm) at which the preheater was controlled at 200 °C using SUS tubing (0.1 mm ID $\times$ 38 cm). The reaction curves with and without a preheater were in good agreement, except that the induction period with preheater was shorter than that without a preheater. Consequently, because the use of a longer capillary is more efficient to reduce the heat-up time, rather than using a preheater, a preheater is not necessary in the present system.

Hydrolysis of ATP and AMP. The performance of the system was evaluated by monitoring the reactions of hy-

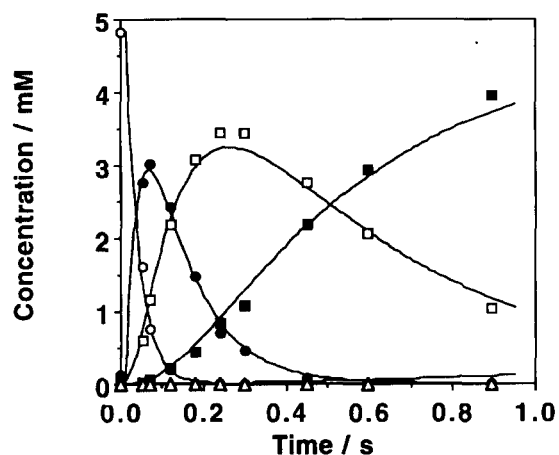
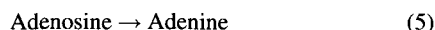


Fig. 3. Reaction curves for ATP hydrolysis using preheater. The reaction was performed in aqueous solution containing 0.005 M ATP, 0.1 M NaCl, 0.05 M MgCl₂, 0.05 M imidazole (initial pH at 25 °C = 7.0) with 38 cm tubing in main heater and 38 cm tubing in preheater which is heated at 200 °C, 14 MPa. ○, ATP; ●, ADP; □, AMP; ■, adenosine; △, adenine. The line drawn through the experimental points were fitted by SIMFIT.

drolyses of ATP and AMP. The reaction curves of ATP hydrolysis were successfully obtained in the time range of 50 ms using 0.025 mm ID $\times$ 10 cm tubing in reactor II, as shown in Fig. 4.²⁵ In the reaction, ATP is consecutively hydrolyzed to adenine, as shown in Eqs. 2, 3, 4, and 5:



The disappearance of ATP could be clearly observed at 250 °C (Fig. 4), while it was not clear using 0.1 mm ID SUS tubing (Fig. 3). First-order rate plots using 0.015 mm ID $\times$ 5 cm fused-silica capillary at 250 °C have been evaluated,²⁵ in which the plots indicate that the heat-up time is 2.1 ms. The apparent rate constant of the disappearance of ATP determined at 250 °C was in good agreement with that estimated from an extrapolation of the rate constants determined at 125–225 °C.²⁴

In order to evaluate the performance of the system at higher temperatures, the hydrolysis of AMP was monitored

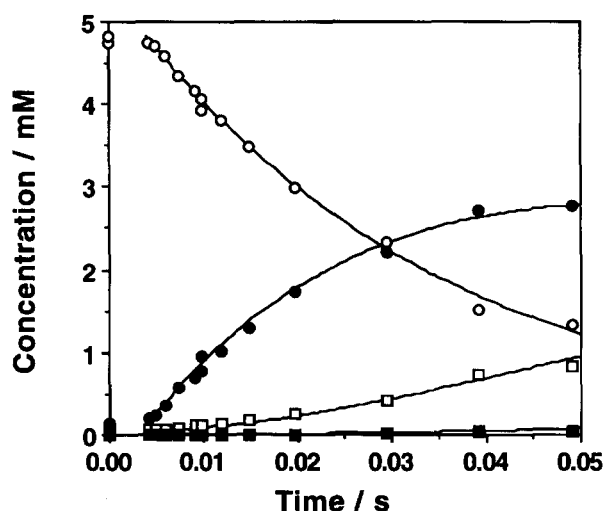


Fig. 4. Reaction curves for hydrolysis of ATP at 250 °C using 0.025 mm $\times$ 10 cm fused-silica capillary tubing. The reaction was performed in aqueous solution containing 0.005 M ATP, 0.1 M NaCl, 0.05 M MgCl_2 , 0.05 M imidazole (initial pH at 25 °C = 7.0), 21 MPa. $\circ$, ATP; $\bullet$, ADP; $\square$, AMP; $\blacksquare$, adenosine. The line drawn through the experimental points were fitted by SIMFIT.

at 270–315 °C, because the hydrolysis of ATP is too rapid (Figs. 5a, 5b, 5c, and 5d). The results demonstrate that the present system is capable of being applied at over 300 °C. First-order rate plots were determined for 270–300 °C, as shown in Fig. 6, and show that the present system has sufficient accuracy. Because monitoring at 315 °C is somewhat less accurate, a further improvement is necessary for monitoring at higher temperature. For a further analysis, the reaction curves shown in Fig. 4 and Figs. 5a, 5b, 5c, and 5d were fitted by SIMFIT to determine the rate constants for each step (Table 1). The reaction curves shown as solid lines were nicely fitted with the experimental points. This fact indicates that the present system provides good quality of data to produce rate constants which are not able to be directly determined using any conventional method.

To evaluate the method, the rate constants were compared with those in previous studies. The rate constants determined in both this and previous studies^{17,24} are plotted as Arrhenius plots (Fig. 7); the result shows that the rate constants in the present study are very consistent with those of previous studies. The apparent activation energy was determined from the slope of the Arrhenius plots, and those for ATP and ADP are in good agreement with a previous study (121–133 kJ mol⁻¹).²⁹ The apparent activation energy for the hydrolysis of AMP and adenosine were obtained for the first time in this study Table 2. Consequently, these results indicate the following. First, the inner wall is inert for the reaction of the consecutive hydrolysis of ATP. Second, the pressure dependence of the rate constants considered in this study is not significant; this fact is consistent with a previous study of ATP hydrolysis at low temperatures.³⁰ Third, the influence by dilution in the capillary was not a significant problem concerning the reaction.

Stability of ATP and the RNA World Hypothesis. The present study has proposed rate constants for a wide range temperatures, as shown in Fig. 7,^{17,24,29,30} which could not be determined in real time by conventional techniques. The de-

Table 2. Apparent Activation Energy for Consecutive Hydrolysis of ATP to adenine

Step	Activation energy/kJ mol ⁻¹
ATP $\rightarrow$ ADP	120
ADP $\rightarrow$ AMP	123
AMP $\rightarrow$ Adenosine	135
Adenosine $\rightarrow$ Adenine	144

Table 1. Rate Constants for Hydrolysis at 250–315 °C/s⁻¹

T/ °C	ATP	ADP	AMP	Adenosine
250	$(3.00 \pm 0.03) \times 10^1$	$(1.08 \pm 0.04) \times 10^1$	$(3.83 \pm 0.17) \times 10^0$	ND
270	ND	ND	$(8.00 \pm 0.11) \times 10^0$	$(1.29 \pm 0.64) \times 10^0$
285	ND	ND	$(1.27 \pm 0.07) \times 10^1$	$(2.95 \pm 0.28) \times 10^0$
300	ND	ND	$(2.87 \pm 0.04) \times 10^1$	$(4.33 \pm 0.59) \times 10^0$
315	ND	ND	$(7.71 \pm 0.26) \times 10^1$	$(1.43 \pm 0.14) \times 10^1$

Reactions in 0.1 M NaCl, 0.05 M MgCl_2 , 0.05 M imidazole, initial pH = 7.0 (25 °C). The rate constants were determined by SIMFIT. ND: not determined.

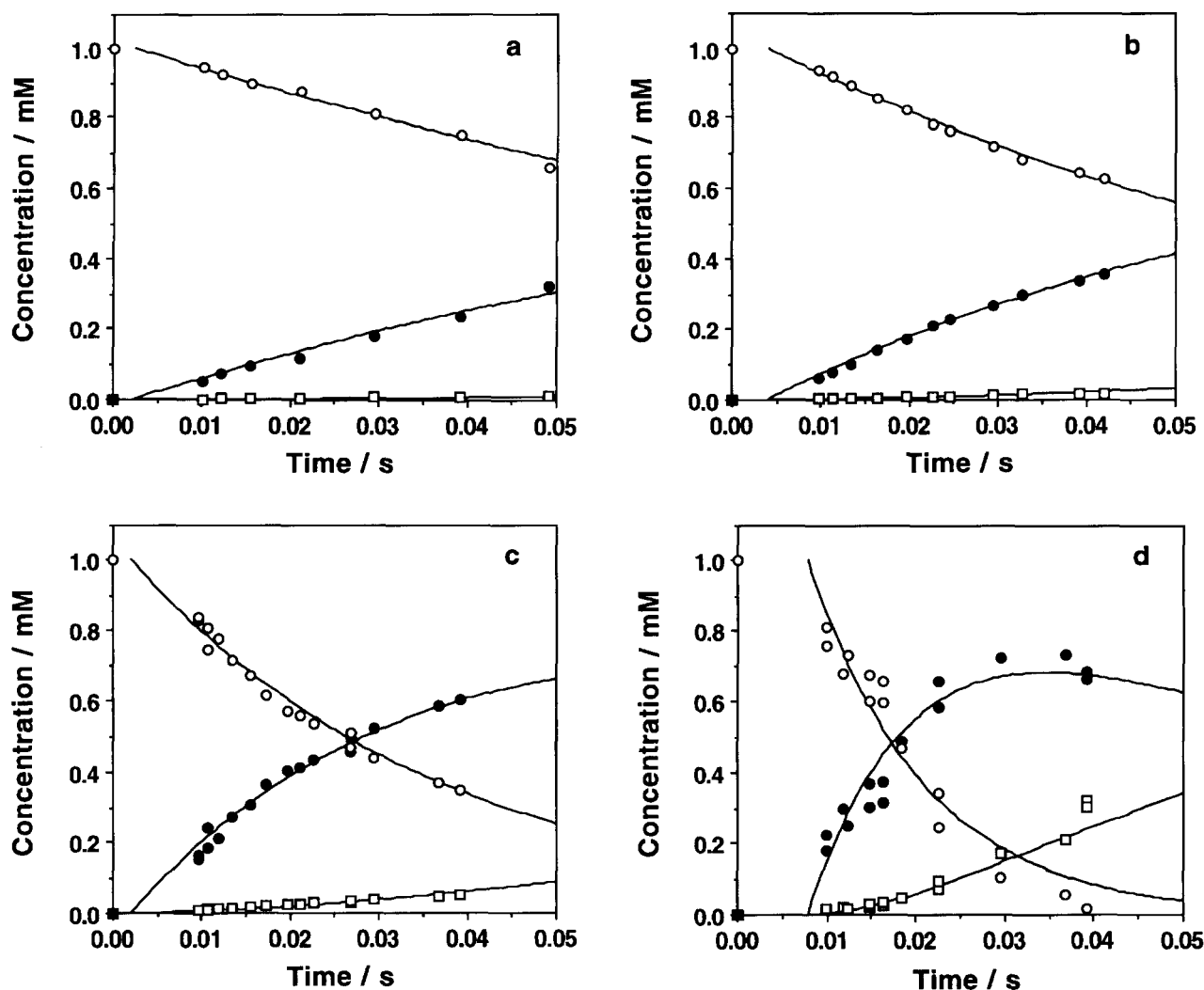


Fig. 5. Reaction curves for the hydrolysis of AMP using 10 cm fused-silica capillary tubing. (a): 270 °C, (b): 285 °C, (c): 300 °C, (d): 315 °C. $\circ$, AMP; $\bullet$, adenosine; $\square$, adenine. The reaction is performed in aqueous solution containing 0.001 M AMP, 0.1 M NaCl, 0.05 M MgCl_2 , 0.05 M Imidazole (initial pH at 25 °C = 7.0), 21 MPa. The line drawn through the experimental points were fitted by SIMFIT.

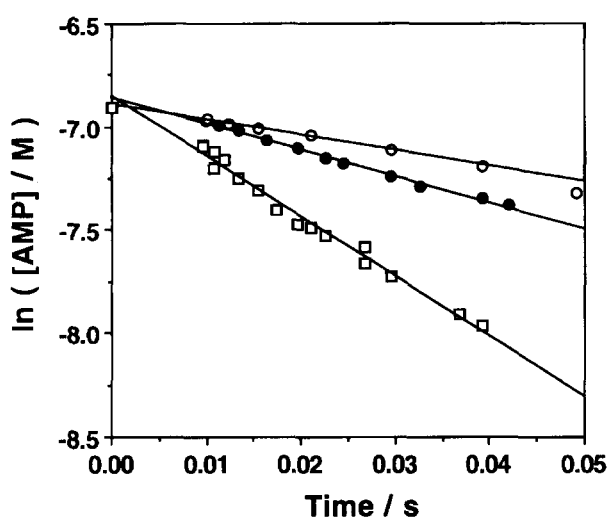


Fig. 6. First-order rate plots for the disappearance of AMP. $\circ$, 270 °C; $\bullet$, 285 °C; $\square$, 300 °C.

pendence of the rate constants over a wide range temperature is valuable for understanding the chemistry of nucleotide stability at high temperatures, and to evaluate the stability of adenosine nucleotides as a monomer unit of RNA from the viewpoint of the RNA world hypothesis under hydrothermal conditions.

The results give the first demonstration that the rate constants decrease in the order $\text{ATP} > \text{ADP} > \text{AMP} > \text{adenosine} > \text{adenine}$ at room temperature to 315 °C, while an erroneous estimation from the rate constants, which were determined at lower temperatures using batch method in our previous study, suggested that the order of the magnitude of the rate constants would change at high temperatures.¹⁷ Moreover, the results show that the apparent activation energy remains constant for the step-wise hydrolysis of ATP at 100–315 °C. In other words, the fact is proof that the Arrhenius plot can be applied for aqueous solution reactions over a wide range of temperatures, which is normally impossible to be confirmed by the batch method and

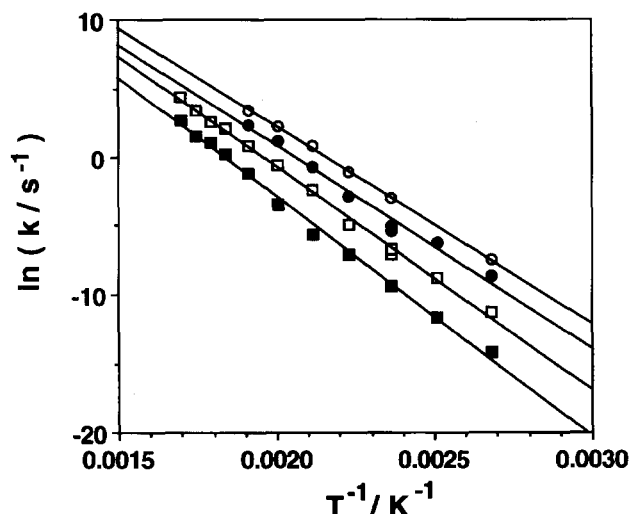


Fig. 7. Comparison of the rate constants on the Arrhenius plots for the hydrolysis of ATP to adenine by the present method and previous studies. 100–150 °C (ATP, ADP, AMP, adenosine, adenine): conventional batch method,¹⁷ 150–225 °C (ATP and ADP) and 150–250 °C (AMP and adenosine): flow reactor in previous study,²⁴ 250 °C (ATP and AMP) and 250–300 °C (AMP and adenosine): this study.

other techniques.^{20–24} The trend concerning the rate constants shows that the mechanism of hydrolysis is basically the same as that at lower temperatures, in which the hydrolysis of ATP occurs consecutively. However, it is also important that the ratio of the rate constants decreases with increasing the temperature. For instance, the ratios of the rate constants of ATP to AMP hydrolysis are 54 at 150 °C²⁴ and 7.8 at 250 °C, and those for AMP and adenosine are 8.1 at 250 °C²⁴ and 5.4 at 315 °C. This fact indicates that the difference in the relative strength among the phosphoester and *N*-glycoside bonding for ATP hydrolysis becomes small with increasing the temperature.

ATP is important in the biosphere, because it acts as a monomer unit of RNA and energy-rich phosphate. Besides, the decomposition of ATP to adenosine is very rapid and the half-life of ATP is 23 ms at 250 °C and that of AMP is 9 ms at 315 °C.²⁴ This fact indicates that the RNA world was difficult to survive during primitive earth if the RNA world had been exposed to hydrothermal systems, such as hydrothermal vents in the deep ocean. Although this speculation may be correct, it is important to take into account that the accumulation of RNA was determined by both the rates of the formation and the decomposition of RNA. That is to say, if the RNA world had mechanisms to increase the stability of ATP and/or accelerate to form RNA, the RNA world could survive under hydrothermal environments. For example, additives, such as protein-like compounds and mineral surfaces, could stabilize and/or catalyze the formation of adenosine nucleotides and RNA.⁸ To evaluate this postulate, the proposed method will be absolutely useful. Besides, the half-lives for ATP and AMP are regarded as being comparable to those in the presence of the corresponding enzymes

at low temperature, since the enzymatic reactions normally proceed in the millisecond time range.^{17,18} In other words, it is impressive that the hydrolysis rates with modern enzymes are as great as those at high temperatures without any enzymes. If primitive enzymes for the hydrolysis of ATP and AMP existed in ancient hydrothermal systems, the hydrolytic reactions of ATP and AMP in the presence of the primitive enzymes should be much faster than those without enzymes. This speculation indicates that the emergence of enzymes under hydrothermal systems was also difficult, since primitive enzymatic reactions require a catalytic ability at high temperatures.

Conclusions. The present system is superior to previous methods^{20–24} regarding a few points. First, this system enables the monitoring of hydrothermal reactions in a much shorter time range (more than 100 times) than other techniques.^{20–24} Second, the system is easy to set up with low cost, and many types of conventional analytical techniques other than HPLC can be used for samples withdrawn from the flow system. Thus, it is obviously useful for practical applications, such as hydrothermal decomposition of chemical waste. Third, monitoring at high temperatures is safely performed, since reactions at high temperatures are carried out in an nL size reactor. Further, it is expected that this system can be applied for in situ detection using spectroscopic techniques.

In conclusion, the present system was well demonstrated as a monitoring method of hydrothermal reactions in the millisecond time range. The method enables real-time monitoring (not in situ) of the hydrolysis of ATP and AMP at 250–315 °C within 50 ms. The usefulness of the method is pronounced as a new approach for studying the chemical evolution of RNA under hydrothermal environments. Moreover, in general, the present method is expected to be applied in several fields of relevance with hydrothermal reactions from practical and fundamental aspects.

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