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Nonenzymatic Hydrolysis Reactions of Adenosine 5′-Triphosphate and Related Compounds

II. Kinetic Studies of the Hydrolysis of ATP[1] and Related Compounds with Various Polyamines

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Various structural features of polyamines which are responsible for the acceleration of the hydrolysis of ATP to ADP and P_i at pH 3–4 were surveyed by means of kinetic studies, leading to the following conclusions: 1) The ethyleneimine chain of the polyamines should be as long as possible; 2) the number of methylene carbon atoms between the two adjacent nitrogen atoms of the polyamine has to be two; 3) the terminal groups of the ethyleneimine chain should be primary amino groups.

The rate of ATP hydrolysis in the presence of pentaethylenehexamine (pentaen), which possesses the above properties, was found to be 15 times as high as that of hydrolysis at pH 3.5 in the absence of amines. The kinetic data support the previous assumption that there is formation of an ATP–pentaen complex in the hydrolysis reaction. The formation constant of the complex has been calculated to be $K = 1.9 \times 10^4\ M^{-1}$ at pH 3.5 and 50°C from the kinetic data. From the temperature dependence of the rates for pentaen or tetraethylenepentamine, the thermodynamic data of these reactions have been obtained.

On the other hand, it has been found that pentaen enhances the hydrolysis of GTP and UTP as well as ATP. No phosphate ester bonds of AMP, *p*-nitrophenylphosphate and α-D-glucose-1-phosphate were hydrolyzed. Therefore, it may be concluded that hydrolysis of the phosphate ester bond with the polyamine is characteristic of ATP, GTP and UTP.

INTRODUCTION

We reported in a previous paper (*1*) hydrolysis reactions of ATP with continuous-chain polyamines, observations believed to shed light on ATPase function. The amounts of inorganic orthophosphate liberated from ATP by those polyamines were found to decrease, in the pH range 2–7, in the order: Pentaethylenehexamine (pentaen, N2N2N2N2N2N)[3] > tetraethylenepentamine (tetraen, N2N2N2N2N) > triethylenetetramine (trien, N2N2N2N) > diethylenetriamine (dien, N2N2N) > ethylenediamine

[1] The abbreviations used are: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; UTP, uridine 5′-triphosphate; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate.

[2] Taken in part from the Ph.D. thesis of S. Suzuki, Osaka University, 1974.

[3] The N and 2 denote the nitrogen atom and the number of methylene groups between two nitrogen atoms, respectively.

(en, N2N). Tetraen was distinctly more active than 4,7,10-triazatridecane-1, 13-diamine (N3N2N2N3N). These findings were explained in terms of an increasing catalytic effect that increases with the numbers of the imino nitrogen atoms in the molecules, although the spacing in the carbon chain of the amines was of importance.

The present paper describes in detail the results of kinetic studies of those hydrolysis reactions. Thermodynamic data of ATP hydrolysis with pentaen and tetraen, hydrolysis of GTP and UTP with pentaen, and results of the ATP hydrolysis in the presence of 2,14-dimethyl-2,5,8,11,14-pentaazapentadecane and 1,9-bis(2′-pyridyl)-2,5,8-triazanonane are also reported.

EXPERIMENTAL SECTION

Materials. The disodium salt of ATP, trisodium salt of GTP, and tetrasodium salt of UTP were purchased from the Sigma Chemical Company. These nucleotides were employed without further purification, and the amounts of P_i as contaminants in the samples were determined in advance by the method of Martin and Doty (*2*). The slight amount of P_i present did not actually affect the kinetic measurements. All amines used in this study are tabulated in Table 1. The preparations of the hydrochlorides of pentaen, tetraen, trien and tatd have already been described (*1*). Papd was synthesized by a condensation reaction between a large excess of *N,N*-dimethylethylenediamine and 1,5-dibromo-3-monoazapentane hydrobromide (refer to the experimental section of (*1*)). Pynonane was obtained by reduction of the Schiff base produced from diethylenetriamine and pyridine-2-aldehyde (*3*).

The final purity of all polyamine hydrochlorides was confirmed by elemental analyses.

Kinetic measurements. The reaction vessel used for kinetic measurements was a jacketed glass cell of 50-ml capacity, covered with a rubber stopper having several openings. The cell is equipped, through the openings of the stopper, with a combined electrode (Radiometer GK 2332C) for pH measurement, an induction tip of a Radiometer ABU12 autoburette and a thermometer.

The initial concentration of substrate of polyamine was adjusted to 1.00×10^{-3} *M*, and the ionic strength was maintained at 0.10 with sodium chloride. Fifty milliliters of the mixture where placed in a thermostated cell.[4] After the desired temperature had been attained, a sample (about 1.3 ml) was taken out with a syringe from the reaction mixture at regular intervals and placed in a test tube that was then quickly cooled in an ice bath to stop the reaction. The samples were kept in a refrigerator before analysis of inorganic phosphate.

The pH value of a solution during the course of the reaction was maintained at pH 3.50 ± 0.02 by adding very small amounts of 0.03–0.05 *N* sodium hydroxide from the autoburette which was connected with a Radiometer TTT1C titrator.

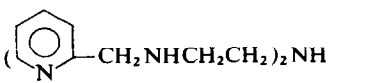
The reaction rate was determined by measuring the amounts of P_i liberated in series of 0.50- or 1.0-ml portions of the reaction mixture, according to the method of Martin and Doty (*2*). The determination of the amounts of PP_i have been described (*1*).

Spectral measurements. The ultraviolet absorption spectra were recorded at room temperature with a Union Giken spectrophotometer SM 401.

[4] In the experiment involving the addition of the metal ion, 50 ml of the solution containing ATP (1.00×10^{-3} *M*), pentaen (1.00×10^{-3} *M*) and metal chloride (1.00×10^{-3} *M*) were heated in a cell.

TABLE 1

CLASSIFICATION OF POLYAMINES USED IN THE HYDROLYSIS REACTIONS

Number of nitrogen atoms	Name	Abbreviation	Formula
6	[Pentaethylenehexamine	Pentaen	$NH_2CH_2CH_2(NHCH_2CH_2)_4NH_2$
5	Tetraethylenepentamine	Tetraen	$NH_2CH_2CH_2(NHCH_2CH_2)_3NH_2$
	4,7,10-Triazatridecane-1,13-diamine	Tatd[a]	$(NH_2CH_2CH_2CH_2NHCH_2CH_2)_2NH$
	2,14-Dimethyl-2,5,8,11,14-pentaazapentadecane	Papd	$((CH_3)_2NCH_2CH_2NHCH_2CH_2)_2NH$
	1,9-bis(2′Pyridyl)-2,5,8-triazanonane	Pynonane	
4	[Triethylenetetramine	Trien	$NH_2CH_2CH_2(NHCH_2CH_2)_2NH_2$

[a] In the previous paper (*1*), 1,5,8,11,15-pentaazapentadecane(papd) in Table 1 should be 4,7,10-triazatridecane-1,13-diamine(tatd).

The difference spectra were obtained at pH 3.50 by the use of a reference cell containing GTP or UTP (2.00×10^{-4} *M*) and a sample cell containing GTP or UTP (2.00×10^{-4} *M*) mixed with pentaen (2.00×10^{-4} *M*),[5] since pentaen itself shows no light absorption in the region of the spectrum concerned.

RESULTS AND DISCUSSION

1. Rates of ATP Hydrolysis in the Presence of Various Polyamines

In the previous study (*1*), we investigated the catalytic effects of various polyamines on the hydrolysis of ATP by estimation of the amounts of P_i liberated in the reaction ATP → ADP + P_i. The P_i determination at pH 2–7 revealed that the increasing order of catalytic activity is: En < dien < trien < tetraen < pentaen. The side reactions, ATP → AMP + PP_i and ADP → AMP + P_i, were negligible.

Now the initial rates of hydrolysis of ATP with these polyamines have been quantitatively obtained by kinetic means. The experiments were carried out at an optimum pH of 3.5. The rates of hydrolsyis with pentaen, tetraen and trien are 15, 10 and 2.6 times higher, respectively, than those of hydrolysis in the absence of the amines (Table 2). These findings support the proposal that the polyamine with longer ethyleneimine chain more easily forms the reactive ATP–polyamine complex, as was already discussed (*1*).

TABLE 2

INITIAL RATES OF ATP-HYDROLYSIS WITH VARIOUS POLYAMINES AND WITH PENTAEN AND METAL ION[a]

Catalyst	Initial rate ($V_0 \times 10^6$ ($M \cdot min^{-1}$))	Relative rate (ratio)
Polyamine only		
None	0.109	1
Trien	0.281	2.6
Tetraen	1.13	10
Pentaen	1.67	15
Tatd	0.298	2.7
Papd	0.327	3.0
Pentaen and metal ion		
Ca(II)	1.67	15
Mg(II)	1.66	15

[a] ATP, 1.00×10^{-3} *M*; polyamine, 1.00×10^{-3} *M*; metal chloride, 2.00×10^{-3} *M*; temperature, 50.0 ± 0.1°C; pH 3.50 ± 0.02; $I = 0.10$ (NaCl).

In order to obtain further information on the relationship between the catalytic activity and the structure of the polyamines, hydrolyses of ATP were carried out by using several amine derivatives containing five nitrogen atoms, such as tetraen, papd, tatd and pynonane. The amounts of P_i produced in runs of 3 hr at 50°C are shown in

[5] In the previous paper (1), the concentrations (5×10^{-3} *M*) of ATP and various polyamines in the difference spectra should have appeared as 5×10^{-5} *M*.

Fig. 1. The order of the catalytic activity was found to increase: Pynonane < tatd < papd < tetraen. The initial hydrolysis rates with papd and tatd at pH 3.5 are also given in Table 2. The rate for tatraen is three to four times as high as the rates for tatd and papd.

The results in Table 2 and Fig. 1 enable us to discuss the structural features of the amine which are responsible for the marked catalytic effect in the hydrolysis reaction.

First, the number of methylene groups between two nitrogen atoms of the polyamine has to be two, as visualized in the structural formula –N–C–C–N–C–C–N–. In the previous paper (*1*), a brief comparison was made between the catalytic activity of tatd

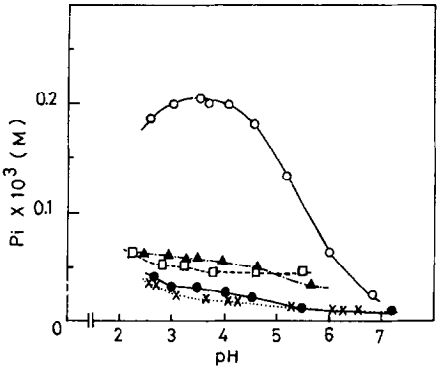


FIG. 1. ATP hydrolysis with various amines containing five nitrogen atoms. Reaction conditions: ATP, 1×10^{-3} *M*; amine, 1×10^{-3} *M*; temperature 50.0°C; time, 3.0 hr; $I = 0.10$ (NaCl). ×, No amine; ●, pynonane; □, tatd; ▲, papd; o, tetraen.

and that of tetraen. It was suggested that the skeletal structure of tatd does not exactly match the interval of oxygen atoms of the triphosphate chain (ATP). This surmise has now been confirmed with the observation that hydrolysis with tatd proceeds only slowly compared with that in the presence of tetraen (Table 2).

Second, the terminal functional group of the polyamine should be a primary amino group. Papd, which has tertiary amino groups at the termini of the molecule, exhibits, by far, less catalytic activity. Pynonane, containing pyridine rings as the terminal groups, also exerts only a slight catalytic effect. The formation of the ATP–papd complex might be difficult on account of the steric repulsion between the ATP molecule and two bulky methyl groups on the terminal nitrogen atoms of papd. Likewise, the formation of the ATP–pynonane complex may be difficult because of the lack of effective hydrogen bonding between the phosphate oxygen atom of ATP and the pyridinium nitrogen atom of pynonane.[6]

The binding of ATP with pynonane presumably is not due to electrostatic interaction between the negative charge of the phosphate moiety and the positive charge of the pyridinium group. If this were so, the catalytic activity of pynonane would be of the same order of magnitude as that of tetraen, contrary to the experimental results shown in Table 2. Accordingly, the linking between the phosphate moiety of ATP and the

[6] The pyridine nitrogen atoms in pynonane are almost protonated at pH 3–4, since the pK_a value for the pyridine group of α-picoline, being analogous to pynonane, is 6.20 (L. G. Sillen and A. E. Martell, "Stability Constants of Metal Ion Complexes," Special Publication No. 17, The Chemical Society, London, 1964).

polyamine (e.g., pentaen and tetraen) is considered to be due to directional hydrogen bonding (rigidly held hydrogen bond) (*4*).

The effect of magnesium(II) or calcium(II) ion on ATP hydrolysis with the polyamine was also examined in order to assess the role of these metal ions in ATPase function (*5*). However, these metal ions did not accelerate the hydrolysis of ATP with pentaen as is clear from Table 2. This finding leads us to conclude that complex formation between ATP and pentaen is not affected by Mg(II) or Ca(II) ion. Thus the polyamine-catalyzed systems in aqueous solution are not suitable for the study of the cooperative effect of Mg(II) or Ca(II) ion.[7]

2. Kinetic Measurements of ATP Hydrolysis with Pentaen

Figure 2 represents the relationship between the initial rates of hydrolysis and the initial concentrations of ATP. The initial rates (V_0) increased in proportion to the substrate concentrations ($[ATP]_0$) until the concentrations of ATP and pentaen became approximately same, and then a saturation phenomenon was observed at higher sub-

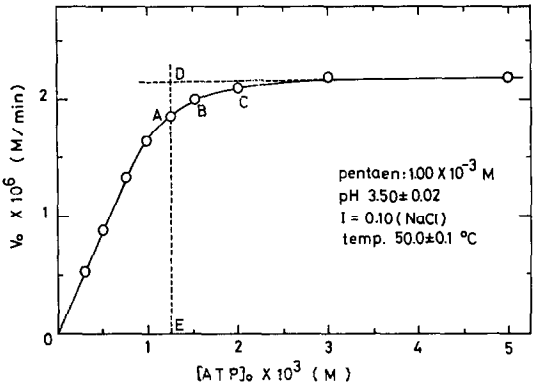


FIG. 2. Initial rates of ATP hydrolysis in the presence of pentaen. Refer to the text for description of *A*, *B* and *C*.

strate concentrations. This kinetic behavior is similar to that of enzymatic reactions. From this result, the reaction of the hydrolysis with pentaen is proposed to be: ATP + pentaen $\overset{K}{\rightleftharpoons}$ ATP–pentaen $\rightarrow$ ADP + P_i + pentaen. The Michaelis constant for this reaction was unobtainable, since the Lineweaver–Burk plot (*6*) of the data in Fig. 2 showed a nonlinear relationship. This is because of the very large formation constant of the ATP–pentaen complex. The formation constant ($K = [ATP\text{–pentaen}]/[ATP][pentaen]$) was determined from the three data, *A*, *B* and *C*, in Fig. 2 ($K = 1.9 \times 10^4$ M^{-1}, at pH 3.5 and 50°C).[8] The large K value reflects the ease of formation of the ATP–pentaen complex, believed to occur between H_2ATP^{2-} and protonated pentaen (*1*).[9]

[7] A brief account of ATP hydrolysis with various cobalt(III) complexes was given previously (S. Suzuki, S. Kimura, T. Higashiyama, and A. Nakahara, *Bioinorg. Chem.*, **3**, 183 (1974).

[8] See Appendix for the calculation of the formation constant.

[9] The proposed structure of 1:1 ATP:pentaen complex appears in a previous paper (1). However, according to another recent paper (R. M. Izatt, J. J. Christensen, and J. H. Rytting, *Chem. Rev.* **71**, 439 (1971)), protonation of the adenine ring should take place at the N(1) nitrogen atom instead of the amino group.

3. *Evaluation of Thermodynamic Data of Activation*

The thermodynamic data of activation in the hydrolysis of ATP with pentaen or tetraen were compared with those in the noncatalytic hydrolysis. The Arrhenius plots of these reactions are illustrated in Fig. 3. In the cases of the catalytic hydrolysis with pentaen and tetraen, the values of k_1, indicated on the ordinate, were calculated from

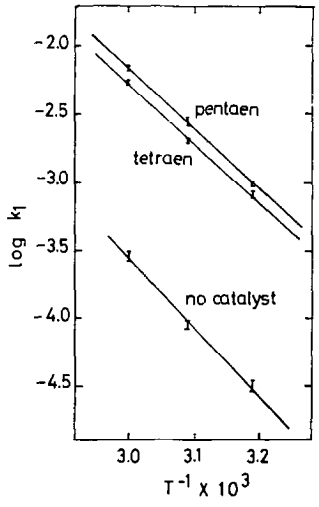


FIG. 3. Arrhenius plots of ATP hydrolysis with no catalyst and with tetraen and pentaen. Reaction conditions: ATP, 1.00×10^{-2} *M*; catalyst, 1.00×10^{-3} *M*; pH 3.50 ± 0.02; $I = 0.10$ (NaCl). For the noncatalytic hydrolysis, the logarithms of the first-order rate constant (k_1') obtained from the equation $-d[\text{ATP}]/dt = k_1'[\text{ATP}]$ were plotted on the ordinate.

the relationship, $V_0 = k_1[\text{polyamine}]_0$, where k_1 represents the rate constant, and $[\text{polyamine}]_0$, the initial concentration of polyamine. The reactions were carried out in the presence of a large excess of ATP (ATP, 1.00×10^{-2} *M*; polyamine, 1.00×10^{-3} *M*) at pH 3.5. Therefore, the k_1 values at various temperatures (40, 50 and 60°C) give the activation parameters of the hydrolysis of the ATP–polyamine complex itself. The results obtained from Fig. 3 are summarized in Table 3. The activation enthalpy (ΔH^*) shown in Table 3 reveals that the transition state in the hydrolysis of the ATP–polyamine complex is more advantageous than that in the noncatalytic hydrolysis of

TABLE 3

THERMODYNAMIC DATA OF ACTIVATION[a]

Catalyst	ΔH^* (kcal/mol)	ΔS^* (cal/mol·degree)
Pentaen	19.6 ± 0.3	-10 ± 1
Tetraen	19.7 ± 0.7	-10 ± 2
None	23.4 ± 0.7	-5 ± 2

[a] ATP, 1.00×10^{-2} *M*; catalyst, 1.00×10^{-3} *M*; temperature, 60.0°C; pH 3.50; $I = 0.10$ (NaCl).

ATP. The decrease of the activation enthalpy results in a facilitation of the hydrolysis with the polyamine.

4. Hydrolysis of GTP or UTP in the Presence of Pentaen

The hydrolysis of GTP or UTP was also carried out in order to investigate the extent of selectivity of a substrate. Both the reactions were accelerated by pentaen and gave the corresponding nucleoside diphosphates (GDP or UDP) and P_i (Table 4). The GTP- and UTP-pentaen complexes are considered to exist during the course of the reactions, as in the hydrolysis of ATP.

TABLE 4

THE HYDROLYSIS OF GTP AND UTP IN THE PRESENCE AND ABSENCE OF PENTAEN[a]

Substrate	Catalyst	$V_0 \times 10^6$ ($M \cdot min^{-1}$)	Relative rate (ratio)
GTP	None	0.0637	1
	Pentaen	1.47	23
UTP	None	0.0788	1
	Pentaen	1.50	19

[a] Substrate, 1.00×10^{-3} M; pentaen, 1.00×10^{-3} M; temperature, 50.0 ± 0.1°C; pH 3.50 ± 0.02; $I = 0.10$ (NaCl).

Since both GTP and UTP provide absorption maxima in the ultraviolet region, due to their base moieties, we have inquired whether variations of these absorption bands are observed in the presence of pentaen. As is seen in Fig. 4, it is clear that the ethyleneimine chain of pentaen perturbs, to some extent, the electronic states of the base moieties of GTP and UTP, although the details are not obvious. These findings suggest that

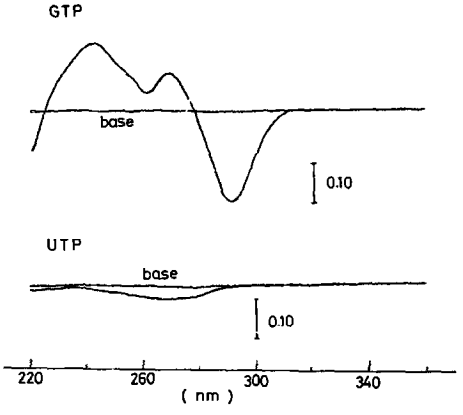


FIG. 4. Ultraviolet absorption difference spectra of GTP- and UTP-pentaen mixture (GTP or UTP, 2×10^{-4} M; pentaen, 2×10^{-4} M, $I = 2 \times 10^{-3}$ ($NaClO_4$); pH 3.5). The baseline was obtained by the use of a reference and a sample cell containing equal amounts of GTP or UTP.

binding of the nucleoside triphosphates with pentaen is actually independent of the structure of their base moieties.

On the other hand, no catalytic function of pentaen has been observed in the hydrolysis of AMP, *p*-nitrophenylphosphate and α-D-glucose-1-phosphate. The initial rate of ADP hydrolysis (ADP → AMP + P_i) with pentaen has also been determined as less than one-tenth of that for ATP hydrolysis with pentaen. Of interest is the fact that the hydrolysis of the phosphate ester bonds with the polyamine is specific for ATP, GTP and UTP.

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APPENDIX

The formation constant (K) was calculated by the method described below. Complex formation between ATP and pentaen is assumed to be 100%, broken line (the saturated rates) in Fig. 2. On the other hand, the initial concentration of ATP ($[ATP]_0$) is higher than that of pentaen ($[pentaen]_0$) at the points A, B and C. Therefore, we can represent the concentration of the complex ([ATP–pentaen]) at the point A as

$$[\text{ATP–pentaen}] = [\text{pentaen}]_0 \times (AE/DE). \quad [1]$$

Then the formation constant can be written as

$$K = [\text{ATP–pentaen}]/[\text{ATP}]\,[\text{pentaen}],$$

where free ATP is given by

$$[\text{ATP}] = [\text{ATP}]_0 - [\text{ATP–pentaen}] \quad [2]$$

and free pentaen by

$$[\text{pentaen}] = [\text{pentaen}]_0 - [\text{ATP–pentaen}]. \quad [3]$$

Thus, we can now compute K from Eqs. [1]–[3] at the point A. The formation constant ($K = 1.9 \times 10^4$) was taken as the average of the constants calculated from the three data at A, B and C.

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