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

I. Hydrolysis Reactions of ATP[1] with Some Continuous-Chain Polyamines

SHINNICHIRO SUZUKI, TADAYOSHI HIGASHIYAMA, AND AKITSUGU NAKAHARA

Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka, Japan

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The nonenzymatic, polyamine catalyzed hydrolysis of adenosine 5′-triphosphate has been investigated in order to shed light on ATPase function.

It has been discovered that pentaethylenehexamine(pentaen) and tetraethylenepentamine(tetraen) enhance the hydrolysis of ATP by considerable factors, whereas small amines such as ethylenediamine(en) show virtually no effect on the hydrolysis of ATP. It has also been found that the enhancing effect on the hydrolysis of ATP increases with the increasing number of the imino nitrogen atoms in the polyamines, although the spacing in the carbon chain of those polyamines cannot be ignored.

In all cases the products of the hydrolysis reactions have been ADP and inorganic orthophosphate(P_i), unaccompanied by either AMP and pyrophosphate(PP_i). During the course of the hydrolysis reactions, the formations of one-to-one complexes of ATP and the polyamines have been considered as intermediates, in which several hydrogen (or electrostatic) bonds between the positively charged amino or imino nitrogen atoms of the polyamines and the adenine, ribose and triphosphate moieties of ATP are formed, activating the phosphate group so that the hydrolytic attack of water becomes easier.

INTRODUCTION

Adenosine triphosphate plays an essential role in the metabolism of living organisms. It is the substance of special importance in the supplementation, transportation and preservation of energy in many biological systems, and participates in numerous enzymatic reactions. Two of the high energy phosphate bonds of ATP are extremely labile, releasing on hydrolysis about 7–9 kcal/mole. The mechanism whereby this free energy is utilized has been under study for the more complex processes such as muscle contraction and ion transport. Since the mechanism of hydrolysis of the phosphate bond by enzymes such as myosine ATPase in muscle contraction has not been clarified, it is worthwhile to study ATPase function on the basis of the results of nonenzymatic hydrolysis of ATP.

When ATP is hydrolyzed by phosphatase systems, magnesium or calcium ion is believed to play an important role. From this point of view, the interaction of metal ions

[1] The abbreviations used are as follows: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate.

with ATP and the influence of metal ions on the kinetics of nonenzymatic hydrolysis reactions of ATP have already been studied in considerable detail (*1–6*).

The present paper describes the results of investigations on ATP hydrolysis in the presence of various polyamines in order to examine their effect on hydrolysis reactions, as a preliminary study on systems containing metal ions. This is the first case dealing with the hydrolyses of ATP in the presence of organic compounds of low molecular weight.

EXPERIMENTAL SECTION

Materials. Disodium salts of ATP and 2′-deoxy-ATP were purchased from the Sigma Chemical Company. The amount of inorganic orthophosphate (P_i), which was present as an impurity was determined by the method of Martin and Doty (*7*). All the commercial polyamines except spermine and spermidine were converted into the hydrochlorides, which were recrystallized repeatedly from water–alcohol mixtures. As to the pentaethylenehexamine, the above treatment was carried out after the base had been distilled *in vacuo* (188–193°C/0.5 mm Hg). Commercial spermine- and spermidine-hydrochloride and *NN′*-(di-2-hydroxyethyl)-ethylenediamine were used without further purification. The synthesis of 1,5,8,11,15-pentaazapentadecane (papd) was performed according to the following scheme:

$$HN(CH_2CH_2OH)_2 \xrightarrow[HBr,\ PBr_3]{} HBr{\cdot}HN(CH_2CH_2Br)_2$$

$$\xrightarrow[\text{excess of } NH_2CH_2CH_2CH_2NH_2]{} \text{papd.}$$

The final purities of the polyamine hydrochlorides were determined by elemental analysis, and by the use of an amino acid analyzer. All the polyamines and their related compounds used in this study are tabulated in Table 1.

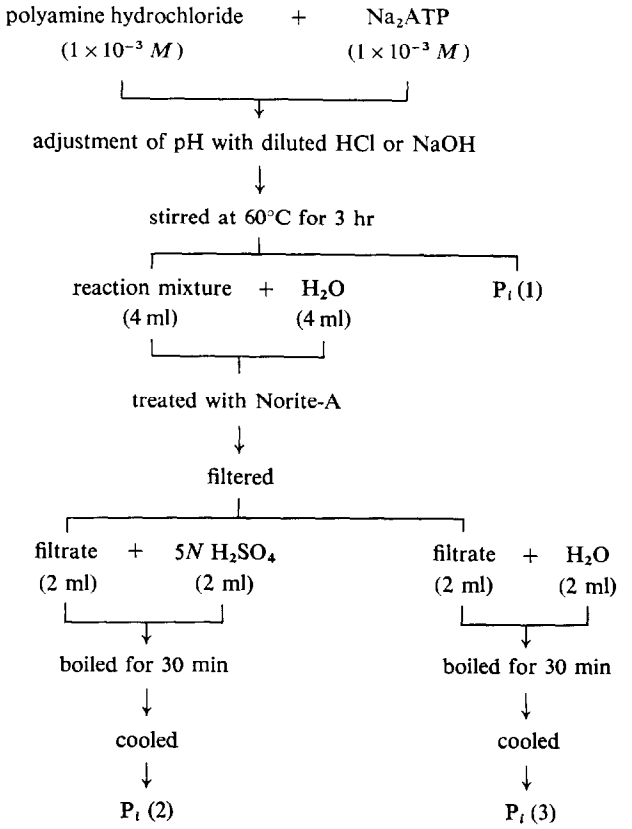
TABLE 1

CLASSIFICATION OF POLYAMINES AND ALCOHOL DERIVATIVE USED IN HYDROLYSIS REACTIONS

Number of nitrogen atoms	Name	Abbreviation	Formula
N_6	pentaethylenehexamine	(pentaen)	N2N2N2N2N2N[a]
N_5	tetraethylenepentamine	(tetraen)	N2N2N2N2N
	1,5,8,11,15-pentaaza pentadecane	(papd)	N2N2N2N2N
N_4	triethylenetetramine	(trien)	N2N2N2N
	spermine	(spmn)	N3N4N3N
N_3	diethylenetriamine	(dien)	N2N2N
	dipropylenetriamine	(ditn)	N3N3N
	spermidine	(spmd)	N3N4N
N_2	ethylenediamine	(en)	N2N
	1,10-decamethylenediamine	(dmen)	N10N
N_2O_2	*NN′*-(di-2-hydroxyethyl) ethylenediamine	(dheen)	O2N2N2O

[a] The numerals such as 2, 3, 4 and 10 symbolize the numbers of methylene groups between the two amino or imino nitrogen atoms.

Hydrolysis of ATP with various polyamines. Aqueous solutions of ATP (1×10^{-3} *M*) and polyamine hydrochloride (1×10^{-3} *M*) were adjusted to desired pH-values with small quantities of dilute hydrochloric acid or sodium hydroxide solution, and were heated at 60°C with constant shaking in a water bath for specific periods. After being cooled to room temperature, 0.5 or 1 ml portions of the reaction mixtures were taken out from each solution, and were used as samples to determine the liberated P_i. The method of Martin and Doty (*7*), employed in this study to determine the amount of P_i,



The amount of PP_i was determined by the following calculation: $PP_i = (P_i(2) - P_i(3))/2$.

FIG. 1. The scheme of the experimental procedure for the determination of values of P_i and PP_i.

utilizes colorimetry of the molybdenum-blue produced by reduction of ammonium phosphomolybdate(VI), which is extracted by a 1 : 1 mixture of benzene and isobutanol from the original aqueous layer. The determination of pyrophosphate (PP_i) liberated in the hydrolysis reaction of ATP into AMP and PP_i was carried out by using 4 ml portions of reaction mixtures according to the direction of Tetas and Lowenstein (*3*). The outline of the whole procedure for the evaluation of P_i and PP_i is schematically shown in Fig. 1.

The reproducibility of the values of P_i and PP_i was confirmed by repeating the experiment; although experiments were always accompanied by inevitable errors,

which were estimated to be less than $\pm 3\%$ for the values of P_i and $\pm 5\%$ for those of PP_i.

For some particularly important data concerning the values of P_i and PP_i, the results were reexamined on the basis of the amount of nucleotides such as ATP, ADP and AMP existing in solutions which had been separately prepared under the same reaction conditions. Those experiments, carried out according to the directions of Cohn and Carter (*8*), always showed satisfactory agreement with the results from P_i- and PP_i-determination.

Measurements. The ultraviolet absorption spectra were recorded at room temperature with a Shimadzu Multipurpose Recording Spectrophotometer MPS-50L. The difference spectra were obtained by the use of a reference cell containing ATP and a sample cell containing ATP mixed with the polyamines, since it had been ascertained in advance that the polyamine itself has no absorption in the region of 220–300 nm.

The colorimetry for the P_i-determination by Martin–Doty method was made by use of the wavelength of 720 nm with a Spectronic 20 of Shimadzu–Bausch and Lomb.

A Hitachi-Horiba M-5 pH meter equipped with a 1026-05T Horiba glass electrode and a 2010-05T reference electrode was used after standardization with a Nakarai standard buffer solution (pH 4.01 and 6.86 at 25°C).

The pmr spectra in D_2O were obtained by the use of a Japan Electron Optics JNM-C-60HL Spectrometer using the sodium salt of 3-(trimethylsilyl)-propanesulfonic acid as a internal standard.

RESULTS AND DISCUSSION

In order to reveal further aspects of the hydrolysis reactions of ATP in the presence of various polyamines, a brief kinetic study was carried out, the results of which are illustrated in Fig. 2. The two curves in Fig. 2 indicate that the effect of polyamines on

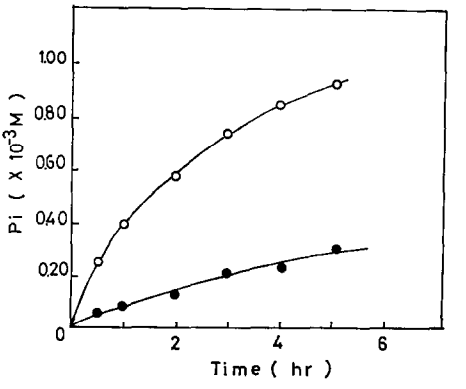


FIG. 2. The values of inorganic phosphate as the function of reaction time. (○: pentaen, ●: trien). Reaction conditions: ATP (1×10^{-3} *M*), amine (1×10^{-3} *M*), temp. 60°C, pH 3.5.

the hydrolysis of ATP can roughly be evaluated by using only values of P_i liberated in 3 hr reaction at 60°C. We have, therefore, chosen 3 hr as the period of time for hydrolysis, and compared the effect of various continuous-chain polyamines on the hydrolysis of

ATP. Figure 3 represents the results of this examination, in which the values of P_i liberated are plotted against the pH-variance. As is seen in Fig. 3, polyamines of $NH_2—CH_2—CH_2—(NH—CH_2—CH_2—)_nNH_2$ type enhance the hydrolysis of ATP, and the effect of enhancement increases with the increasing number of the imino nitrogen atoms. Of interest is the fact that there is a great difference between the effect of tetraen and trien on the hydrolysis of ATP. Inspection of Fig. 3 also reveals that the values of PP_i liberated through the hydrolysis reaction of ATP are negligibly small in all cases. This finding suggests that the hydrolysis reactions of ATP with these polyamines proceed preferentially according to the reaction route ATP → ADP + P_i. It has also been found that the hydrolysis reactions of ADP in the presence of pentaen and tetraen essentially do not occur, suggesting that a side reaction ADP → AMP + P_i is negligible.

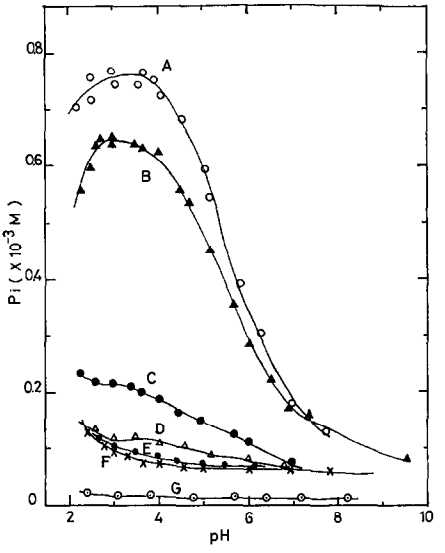


FIG. 3. The results of hydrolysis reactions of ATP with polyamines. (After the reactions using pentaen and tetraen as the catalyst, the initial pH values of the reaction mixture were slightly varied as follows: pentaen, 2.98 (initial value) → 2.99 (final value); 3.60 → 3.55; 4.01 → 3.91; tetraen, 3.02 → 3.01; 3.55 → 3.50; 4.12 → 4.00.) Reaction conditions: ATP (1×10^{-3} *M*), amine (1×10^{-3} *M*), temp. 60°C, time 3 hr. A: pentaen, B: tetraen, C: trien, D: dien, E: en, F: in the absence of amine, G: PP_i liberated as the result of the side reaction: ATP → AMP + PP_i (negligibly small amount for all the reactions A–E).

The effect of concentration variance of pentaen on the value of P_i liberated in the hydrolysis of ATP is indicated in Fig. 4. Apparently the curve in Fig. 4 tends to level off at around the region corresponding to the equimolar ratio of pentaen and ATP, suggesting that the hydrolysis reaction proceeds through the formation of a 1:1 pentaen:ATP intermediate complex. In addition, the extent of complex formation of the pentaen-ATP is considered to be so large that the most of all ATP-species are complexed with pentaen when the reaction system contains pentaen and ATP in an

equimolar ratio, since the value of P_i never increases in spite of the further increases in value of pentaen/ATP at 60°C.

In order to confirm complex formation of polyamine and ATP, pmr and uv studies were carried out, the results of which are illustrated in Figs. 5 and 6, respectively.

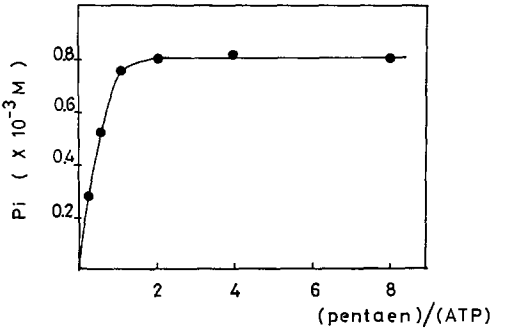


FIG. 4. The reaction activities against the ratio of pentaen/ATP. Reaction conditions: ATP (1×10^{-3} *M*), temp. 60°C, time 3 hr, pH 3.5.

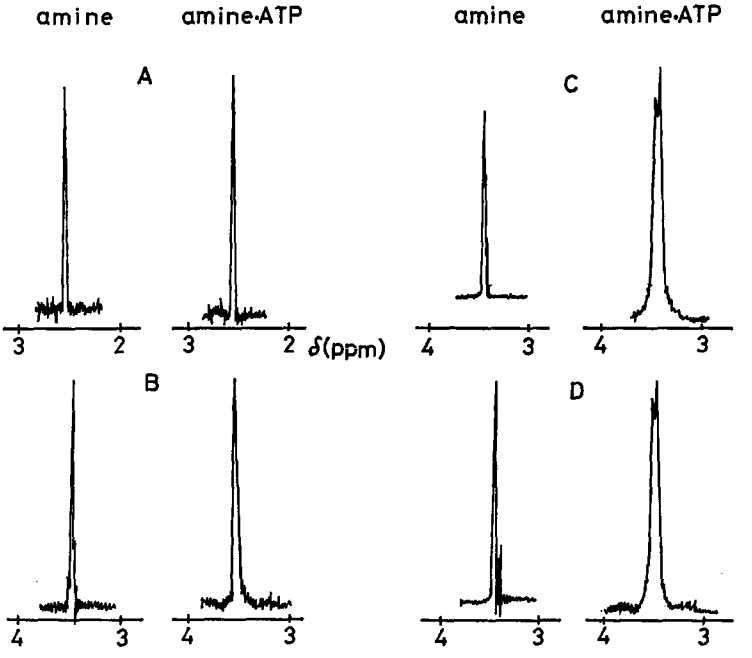


FIG. 5. The pmr signals for the methyl- or methylene- protons of amines in the absence and presence of ATP in D_2O (ATP 0.1 *M*, amine 0.1 *M*, pD 3.5). A: methyl amine, B: dien, C: tetrane, D: pentaen.

Figure 5 represents the comparison of pmr signals arising from the methyl or methylene protons of the various amine molecules in the absence and presence of ATP. As for the signal for methylamine (N_1), no change is observed with addition of ATP; whereas in the cases of dien (N_3), tetraen (N_5) and pentaen (N_6), broadening or even splitting (only

in cases of tetraen and pentaen) of the signals are distinctly observed upon the addition of ATP. The results can be understood in terms of complex formation between ATP and the various polyamines, including the presumption that longer polyamines such as tetraen and pentaen can form far more stable complexes with ATP than smaller amines such as methylamine or ethylenediamine. The above presumption is thought to be quite reasonable, since there are many binding sites for the formation of hydrogen bonds or electrostatic interaction both in the ATP and the longer polyamines, but only few binding sites in the smaller amines. Further, it is expected that exchange between bound and free polyamines in the presence of ATP is quite slow in the cases of the longer polyamines,

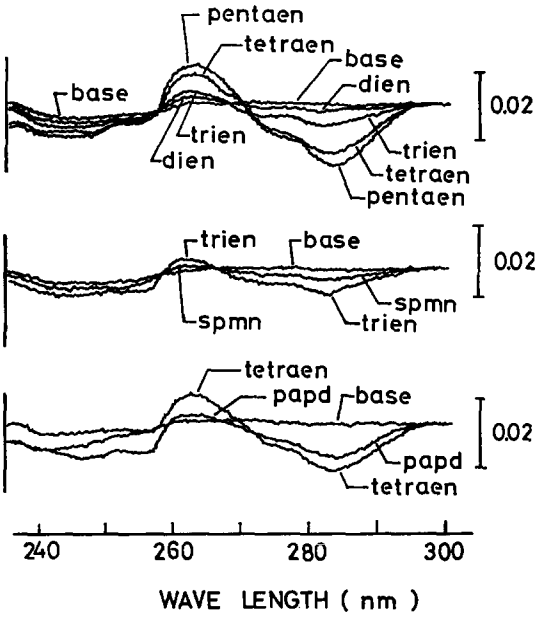


FIG. 6. The ultraviolet absorption difference spectra of amine-ATP mixture (ATP 5×10^{-3} *M*, amine 5×10^{-3} *M*, I = 0.01 (KCl), pH 3.3).

but comparatively rapid in the cases of the smaller amines. The broadening or splitting of the signal for methylene protons, which were observed only in the cases of longer polyamines mixed with ATP, are thus interpreted in Fig. 5.

Since it is well known that ATP exhibits the absorption maximum at 260 nm due to the adenine ring, we have examined whether some variations of this absorption band are observed in the presence of the polyamines (Fig. 6). Inspection of Fig. 6 reveals that the influence of those polyamines on the adenine ring of ATP tends to decrease in the order: pentaen > tetraen > trien > dien, and this order is exactly in accord with the decrease in activities in the hydrolysis reactions as illustrated in Fig. 3. Though the reason why there is an increase and a decrease in absorption at 260 and 285 nm, respectively, is at this moment unexplainable, the extent of influence on the absorption should be proportional to the extent of perturbation occurring in the adenine moiety. The pentaen or tetraen may be long enough to reach either terminus of the ATP, and thus to affect the electronic state of adenine moiety as well as that of phosphate group.

The influence of polyamines on the adenine moiety was further investigated by employing structurally different polyamines such as spmn and papd. Though the number of amino and imino nitrogen atoms in spmn or papd are the same as those in trien or tetraen, respectively, the variation of the absorption band at 260 nm are always observed greater in the cases of polyethylenepolyamine type (trien > spmn; tetraen > papd). This finding is again compatible with the order of activities in the hydrolysis reaction (Table 2). As is apparent from these experiments, the hydrolysis reactions of ATP are closely related to the interaction between polyamines and the adenine moiety of ATP, though the interaction between the phosphate or ribose moiety of ATP and the imino or amino nitrogen atoms of polyamine is also important.

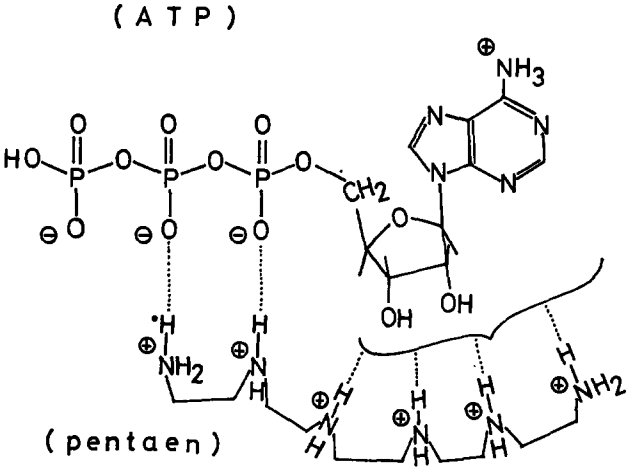


FIG. 7. Proposed structure of 1:1 ATP:pentaen complex. Though it is obvious from the spectral study that there must be some interaction between the adenine moiety of ATP and the polyamine molecule, it cannot be decided which nitrogen atoms of the adenine moiety are hydrogen bonded with the polyamine molecule.

Thus we propose the structure depicted in Fig. 7 as the intermediate complex of the hydrolysis reaction of ATP. In this structure the amino and imino groups of polyamine molecule link to oxygen atoms of the phosphate and ribose group, and nitrogen atoms of the adenine group of ATP through hydrogen bonds or electrostatic linkages. Under the optimal pH (3–4) of the hydrolysis reactions, it may be considered that the continuous chain of the polyamine molecule elongates appreciably, since there must exist repulsive forces between the protonated nitrogen atoms in the molecule. In the case of tetraen the most abundant tetraen-species are computed[2] as H_4L^{4+} (68%), in which L denotes the neutral tetraen molecule, H_5L^{5+} (30%) and H_3L^{3+} (2%) at pH 3.5; and in the same way, the most abundant ATP-species are evaluated as H_2ATP^{2-} (78.5%) and $HATP^{3-}$ (21.5%) at the same pH. The folded structure of ATP may also, more or less, be elongated upon the formation of ATP-polyamine complex on account of the above

[2] The abundance percentages of the various tetraen species were computed by making use of their stability constants cited in L. G. Sillén and A. E. Martell, "Stability Constants of Metal-Ion Complexes," Special Publication No. 17, The Chemical Society, London (1964).

described elongated polyamine molecule. According to a recent X-ray study (*9*), the ATP molecule possesses a bent structure, in which the adenine moiety makes a right angle with the ribose moiety, and the triphosphate group is folded. In the proposed structure the ATP molecule may, therefore, have strain to some extent, and suffer a decrease of electron density at the phosphorous atom as the result of hydrogen bonds or the proximity effect of the positively charged amino or imino nitrogen atoms of the polyamine molecule. The variations occurring in the ATP molecule may promote the nucleophilic attack of water molecule on the phosphate group of ATP, and then the terminal phosphate group will be cleaved.

Of special interest is the fact that those polyamines having lengths of carbon chain different from that of the polyethylenepolyamine type do not enhance the hydrolysis of ATP so effectively as pentaen or tetraen. Although the tetraen and papd have the same number of amino or imino nitrogen atoms, the activity of the latter in the hydrolysis reaction of ATP is not to be compared with that of the former, as is seen from Table 2.[3]

TABLE 2

A COMPARISON OF ACTIVITIES OF VARIOUS POLYAMINES IN ATP-HYDROLYSIS REACTION[a]

	Amine	P_i ($\times 10^{-3}$ *M*) pH 3	4	5
N_2	en	0.16	0.12	0.13
	dmen	0.24	0.11	0.11
N_3	dien	0.22	0.25	0.22
	spmd	0.13	0.11	0.11
	ditn	0.13	0.11	0.11
N_4	trien	0.38	0.34	0.30
	spmn	0.10	0.11	0.11
	dheen	0.16	0.14	0.13
N_5	tetraen[b]	0.64	0.61	0.48
	papd[b]	0.19	0.17	0.13

[a] ATP (1×10^{-3} *M*), amine or alcohol (1×10^{-3} *M*), temp. 60°C, time 3 hr 40% dioxane–60% water.
[b] 100% water.

This suggests that the periodically suitable spacing of amino or imino nitrogen atoms of polyamines seem to be necessary in order to have the effective interaction with ATP. For instance, the skeletal structure of polyamine such as —N—C—C—N— is considered to fit nearly exactly the interval of oxygen atoms of triphosphate chain, as shown in Fig. 7.

As to the bonding site of the ribose moiety with the polyamines, we prefer the hydroxy group at 3′ position of ribose, since 2′-deoxy-ATP was hydrolyzed as easily as ordinary

[3] As far as the experiments on N_2-, N_3-, and N_4-amine are concerned, the solvents used were dioxane–water mixture, for the convenience of comparing those hydrolysis activities.

ATP with pentaen under the same conditions. In any case, the effective interaction between ATP and the polyamines (especially pentaen or tetraen) seems to require an extraordinarily highly steric restriction, as in the case of an enzyme reaction. In the ATP-polyamine complex, the hydrogen (or electrostatic) bonds between the phosphate residue and polyamine may be of direct importance for the hydrolysis reaction, whereas those between the adenine or ribose moieties and polyamine are probably of significance in helping formation of the stable intermediate complex.

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