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Effect of Salts and Polyamines on T_4 Polynucleotide Kinase†

Johan R. Lillehaug and Kjell Kleppe*

ABSTRACT: The activity of T_4 polynucleotide kinase (EC 2.7.1.78) was found to be greatly stimulated by salts, such as NaCl and KCl, and polyamines such as spermine and spermidine. Up to a sixfold increase in initial rates was observed with a variety of different single-stranded DNAs and mono- and oligonucleotides. The optimal concentrations of salts were 0.125 *M*, corresponding to a total ionic strength of $\mu = 0.19$. For polyamines the optimal concentrations were found to be at approximately 2 m*M*. With low enzyme concentration and in the absence of activators complete phosphorylation was not achieved for a number of substrates. In the presence of salts or polyamines or high concentration of enzyme the phosphorylation proceeded to completion. Addition of salt led to an increase in both the apparent V_{max} and the Michaelis constant for the DNA substrate whereas the Michaelis constant of ATP remained unchanged. Polyamines had a similar influence on the kinetic constants for the DNA substrate whereas a decrease was found for the apparent Michaelis constant for ATP. The overall mechanism in the presence of activators was found to be sequential but probably of a rapid equilibrium random type. Of the inorganic anions tested both P_i and PP_i inhibited the enzyme in a competitive manner with both substrates.

Polynucleotide kinase (T_4) (EC 2.7.1.78) catalyzes the phosphorylation of 5′-hydroxyl termini of nucleic acids and oligo- and mononucleotides using ATP as a phosphate donor (Richardson, 1965). The enzyme has recently been purified to homogeneity and some of its properties studied (Panet et al., 1973). Despite its wide use today in structural work on nucleic acids little is known about the function of this enzyme in vivo.

A number of factors are likely to influence the activity of T_4 polynucleotide kinase. With regard to practical application, function, and regulation in vivo, information about these aspects would seem to be of importance. In the present work we describe the effect of some activators and inhibitors of T_4 polynucleotide kinase employing mono- and

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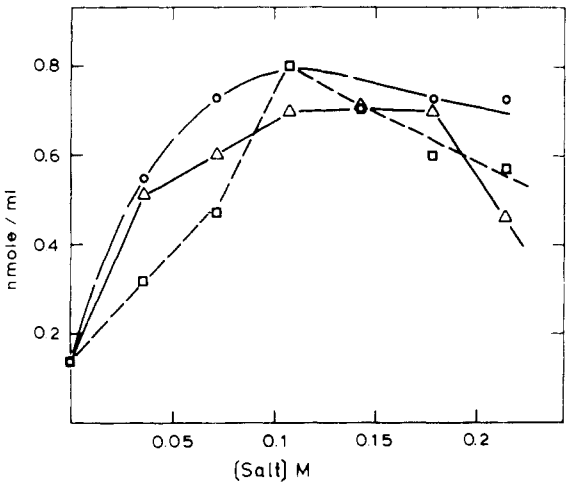

FIGURE 1: Effect of different monovalent cations on phosphorylation of micrococcal nuclease treated calf-thymus DNA by T_4 polynucleotide kinase. Standard assay conditions were used, except that the salt concentration was varied as indicated, each experimental point being the average of two parallel experiments: (□) NaCl; (○) KCl; (△) CsCl.

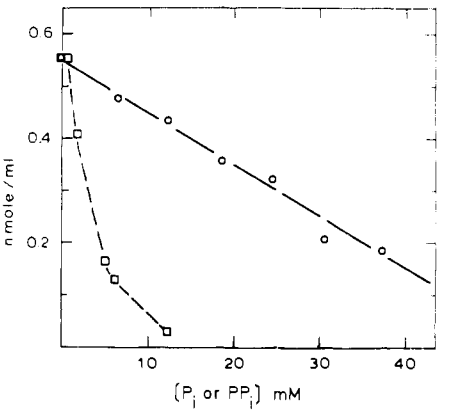

FIGURE 2: Inhibition of T_4 polynucleotide kinase by inorganic phosphate (P_i) and pyrophosphate (PP_i). Standard assay conditions with micrococcal nuclease treated calf-thymus DNA were employed except that P_i and PP_i were added to the concentrations indicated. Sodium salts of P_i and PP_i were used: (○) P_i; (□) PP_i.

Table I: Activation of T_4 Polynucleotide Kinase by Inorganic Anions.[a]

Salt	nmol/ml	% Act.
None	0.19	100
NaCl	0.94	595
NaBr	0.94	595
NaF	0.83	437
$NaNO_3$	0.70	370
$NaHCO_3$	0.70	370
$NaHSO_4$	0.72	380

[a] Assay conditions were as described under Materials and Methods. The anions were present at a concentration of 0.125 *M*.

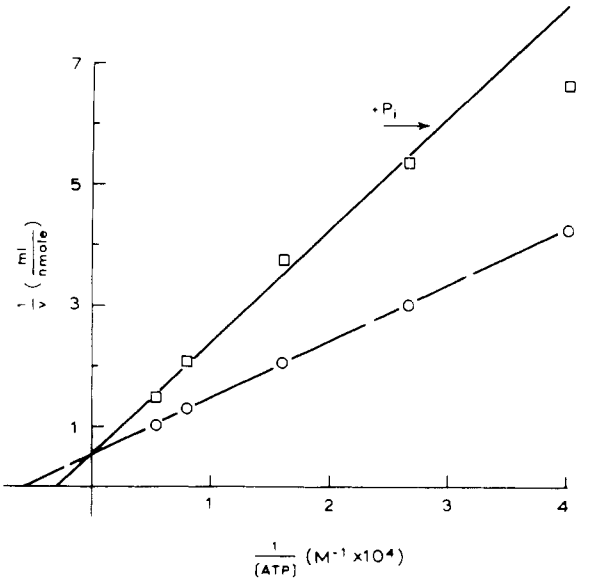

FIGURE 3: Effect of concentration of ATP on the P_i inhibition plotted according to Lineweaver and Burk (1934). Standard conditions with micrococcal nuclease treated calf-thymus DNA were employed, except that the concentration of ATP varied. The concentration of sodium phosphate was 25 m*M*.

oligonucleotides and single-stranded DNAs as substrates. Studies on the kinetics of the enzyme are reported in the preceding paper (Lillehaug and Kleppe, 1975).

Materials and Methods

The materials used were as described in the preceding paper (Lillehaug and Kleppe, 1975). The standard assay mixture contained 60 m*M* Tris (pH 8.0), 9 m*M* $MgCl_2$, 15 m*M* β-mercaptoethanol, 0.066 m*M* [γ-^{32}P]ATP, 0.23 m*M* micrococcal nuclease treated calf-thymus DNA, and approximately 1–1.7 units/ml of T_4 polynucleotide kinase. The normal incubation time was 30 min at 37° if not otherwise stated. The other methods used are described in the preceding paper (Lillehaug and Kleppe, 1975).

Results

Effect of Monovalent Cations. Figure 1 shows the influence of different concentrations of NaCl, KCl, and CsCl on the rate of phosphorylation of single-stranded micrococcal nuclease treated calf-thymus DNA. All three salts greatly stimulated the activity; up to a fivefold increase in activity was observed. The maximum rate of phosphorylation was found at approximately 0.125 *M*, corresponding to a total ionic strength of $\mu = 0.19$. At higher concentrations of salts, the activity decreased slightly. With LiCl and NH_4Cl, not shown, similar results were obtained. The same stimulation with the salt was also observed with double- and single-stranded T_7 DNA, oligonucleotides, and mononucleotides.

Effect of Inorganic Anions. The results above suggested that the concentration of salt may be of great importance when assaying T_4 polynucleotide kinase. These experiments were, however, only carried out with Cl^- as anion. The effects of other anions using Na^+ as a cation are shown in Table I. Br^- was as effective as Cl^- whereas F^-, NO_3^-, HCO_3^-, and SO_4^{2-} showed less stimulation at the same concentrations. P_i and PP_i at 0.125 *M* concentration gave almost 100% inhibition. The inhibitory action of these ions has also been described by others (Novogrodsky et al., 1966; Richardson, 1965). Both P_i and PP_i might be considered to be substrate analogs for the enzyme. Their effects were therefore investigated in more detail and the results are shown in Figure 2. It is evident that PP_i is a much stronger inhibitor than P_i. Above a concentration of approximately 6 m*M* a Mg^{2+}-PP_i precipitate was formed and thus Mg^{2+} became the rate-limiting factor. For both com-

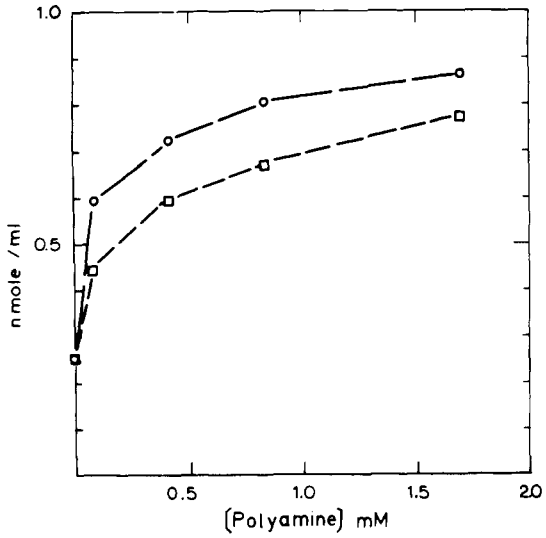

FIGURE 4: Effect of spermine (○) and spermidine (□) on the rate of phosphorylation of micrococcal nuclease treated calf-thymus DNA by T_4 polynucleotide kinase. Standard assay conditions were employed.

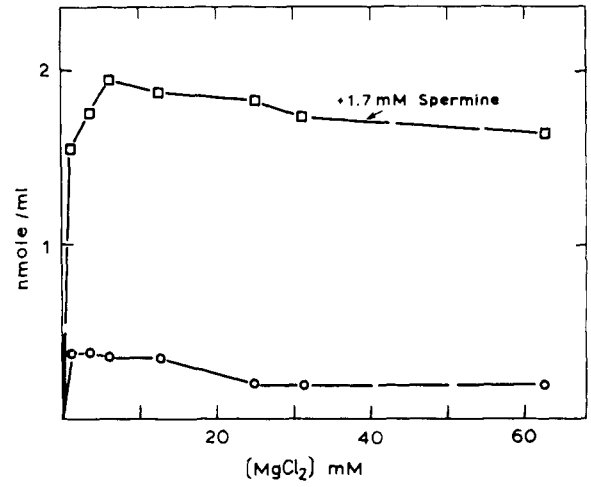

FIGURE 5: Influence of $MgCl_2$ concentration on the spermine stimulation. Standard assay conditions with micrococcal nuclease treated calf-thymus DNA were employed, except that the concentration of $MgCl_2$ varied as indicated. Spermine concentration was 1.7 m*M*.

pounds the kinetic data, using several types of DNA, were plotted according to Lineweaver and Burk (1934). An example, P_i, with varying ATP concentrations, is shown in Figure 3. The data indicate competitive inhibition. Similar results were also obtained with PP_i. From such plots the K_i for P_i and PP_i was estimated to be 29 and 2 m*M*, respectively. The data for P_i were also plotted according to Hill (1910) (not shown) and the Hill coefficient, n_H, was found to be 1.6, thus indicating a minimum of two binding sites per catalytic unit. Assuming that there is a binding site both for ATP and DNA in each active site it is possible that P_i binds to both of these sites. Experiments were therefore carried out to test whether P_i also was a competitive inhibitor with the DNA substrate and the results (not shown) indicate that this indeed is the case.

With PP_i similar results were obtained both with regard to the Hill plot and inhibition studies.

Influence of Polyamines. Previously it has been established that both T_2 and T_4 polynucleotide kinase are completely dependent on the presence of a divalent metal ion such as Mg^{2+} (Richardson, 1965; Novogrodsky and Hurwitz, 1966). Other divalent metal ions give less activity. Polyamines are present in large amounts in both procaryotic and eucaryotic cells and bacteriophages. Hence they might also affect the activity of phage-induced enzymes. The results shown in Figure 4 suggest that this is the case with T_4 polynucleotide kinase. Increasing concentrations of spermine greatly stimulated the activity of this enzyme. Thus, at a concentration of 1.7 m*M* approximately threefold stimulation in activity was seen. Similar results were also obtained with other DNAs, oligonucleotides, and mononucleotides. The effects of other polyamines were also tested and for all of them the increase in activity observed was less than that observed for spermine. The following order of effectiveness was obtained: spermine > spermidine > cadaverine > putrescine.

Both Mg^{2+} and polyamines are positively charged ions at the pH employed. They might therefore be expected to have similar effects. However, this does not appear to be the case as shown in Figure 5. In the absence of Mg^{2+} no enzymatic activity was detected. Maximal activity was observed at a concentration of approximately 10 m*M* Mg^{2+} and the stimulation by spermine was not dependent on the concentration of Mg^{2+}. It is therefore unlikely that spermine and Mg^{2+} exert their effect at the same sites on the enzyme or on the same stages in the reaction sequence.

Table II: Activation of T_4 Polynucleotide Kinase by KCl and Spermine.[a]

Substrate	Act. (nmol/ml) Control	0.1 *M* KCl	1.5 m*M* Spermine	0.1 *M* KCl + 1.5 m*M* Spermine
Micrococcal nuclease treated calf thymus	1.1	2.4	3.2	3.1
$dT(pT)_{14}$	34.1	58.2	76.5	80.8
$dC(pC)_9$	27.5	53.9	64.4	70.1

[a] Assay conditions were as described under Materials and Methods. Oligonucleotide concentrations were: 13 μM $dT(pT)_{14}$ and 7 μM $dC(pC)_9$. The activity values given are based on initial velocities.

Several experiments were also carried out to test whether or not the effects of salt and polyamines were additive. The results shown in Table II suggest that their effects are not, or only partially, additive.

Time Course of Phosphorylation and Effect of Enzyme Concentration. The time course of phosphorylation of a number of different substrates in the absence and presence of salt or polyamines was investigated. This aspect is particularly relevant with regard to practical applications of this enzyme. A typical example, the phosphorylation of 3′-rUp, is shown in Figure 6. In the presence of 1.5 m*M* spermine or 0.1 *M* KCl (not shown) the phosphorylation reached a plateau level corresponding to 100% phosphorylation after approximately 120 min. In the absence of spermine or KCl the initial rate of phosphorylation was approximately two- to threefold less than that obtained in the presence of the activators, and after 90 min a plateau was reached corresponding to approximately 40% phosphorylation. When spermine or KCl was added after 30 min the rate of phosphorylation immediately increased to the same rate as that obtained

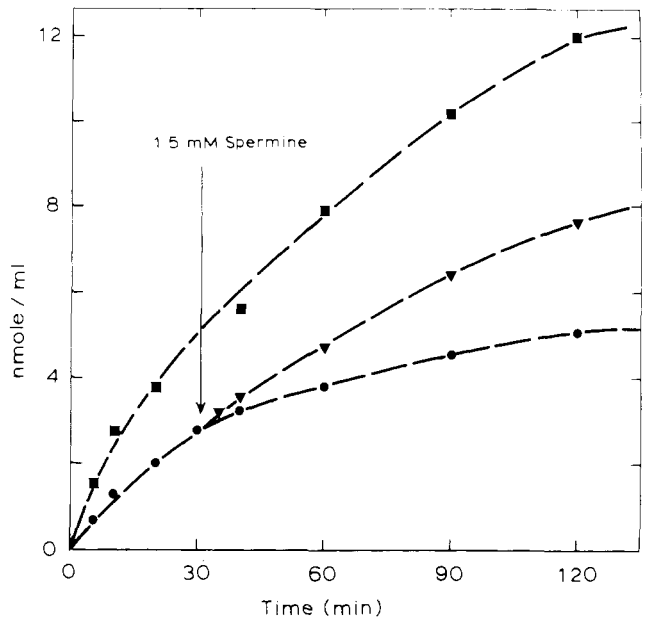

FIGURE 6: Time course of phosphorylation of 3′-rUp. Standard assay conditions were employed: (●) control without spermine; (■) control with 1.5 m*M* spermine; (▲) 1.5 m*M* spermine added at 30 min.

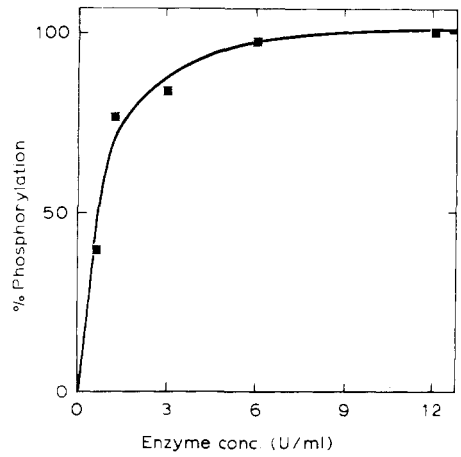

FIGURE 7: Influence of enzyme concentration on level of phosphorylation. Standard assay conditions were employed, 3′-rUp (20 μM) being the phosphate acceptor. The incubation time was 90 min and the enzyme concentration was varied as indicated.

when the activators were added initially. Similar incomplete phosphorylation in the absence of activators was seen with a number of other substrates as well. The results above clearly show that the low phosphorylation obtained in the absence of activators is not due to heat inactivation of the enzyme. Addition of an equal amount of substrate to the reaction mixture after plateau level had been established caused an increase in the amount phosphorylated.

The level of phosphorylation in the absence of activators was found to depend markedly on the concentration of enzyme as illustrated with 3′-rUp in Figure 7. At a concentration of 9 units/ml complete phosphorylation was achieved even in the absence of activators. It should be noted that the deoxyoligonucleotides required considerably higher enzyme concentrations for complete phosphorylation than do the mononucleotides.

Effect of Salts and Polyamines on Kinetics and Kinetic Parameters. As expected both salts and polyamines influenced the kinetic parameters of T_4 polynucleotide kinase.

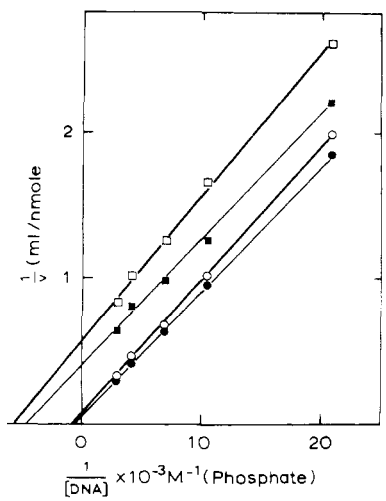

FIGURE 8: Effect of spermine on kinetic pattern. Standard assay conditions were employed using micrococcal nuclease treated calf-thymus DNA as the substrate varied. Incubation time was 15 min: (□) 94 μM ATP; (■) 156 μM ATP; (○) 94 μM ATP with 1.5 m*M* spermine present; (●) 156 μM ATP with 1.5 m*M* spermine present.

The overall kinetics was still sequential in the presence of these activators (Lillehaug and Kleppe, 1975), i.e. double reciprocal plots still gave nonparallel lines having a common crossover point as illustrated for spermine in Figure 8. Furthermore, as was the case in the absence of activators (Lillehaug and Kleppe, 1975), P-DNA was a competitive inhibitor of HO-DNA.[1] ADP, in this case, however, was found to be a competitive inhibitor of ATP (data not shown). These data therefore suggest that in the presence of activators the mechanism is of a rapid equilibrium random type (Cleland, 1970). Thus, the mechanism differs somewhat from that postulated in the absence of activators. Addition of polyamines resulted in a large increase both in $K_{iHO\text{-}DNA}$ and in apparent V_{max} and Michaelis constant for HO-DNA. Thus, in the presence of 1.5 m*M* spermine the $K_{iHO\text{-}DNA}$ increased from 2.6 to 5.5 μM (5′-hydroxyl). Furthermore, spermine caused a decrease in the apparent Michaelis constant for ATP from 0.14 to 0.09 m*M* (not shown). Similar results were obtained with other substrates as well. In the presence of salt, KCl, a similar increase in apparent V_{max} and Michaelis constant for HO-DNA was found. In this case, however, the apparent Michaelis constant for ATP remained unchanged. The effect of spermine concentration on apparent V_{max}, Figure 9, offers further evidence that the reaction may proceed through alternate pathways in the presence of activators (Cleland, 1970). Addition of salt resulted in a similar influence on the apparent V_{max}.

The fact that both types of activators caused an increase in apparent V_{max} and decreased the affinity for substrate ($1/K_{iHO\text{-}DNA}$) might suggest that the rate limiting step in the reaction sequence is the dissociation of the product. Such an explanation is also in agreement with the observation that in general substrates having the highest apparent Michaelis constants also gave the highest apparent V_{max} (Lillehaug and Kleppe, 1975).

Discussion

The present work concerns the effect of some activators

[1] Abbrevations used are: HO-DNA, a DNA carrying a free 5′-hydroxyl group; P-DNA, a DNA carrying a 5′-phosphoryl group.

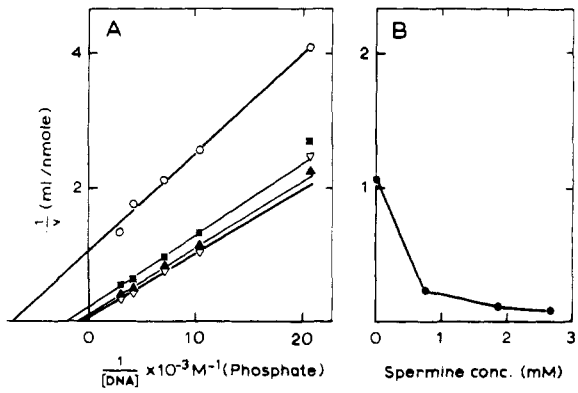

FIGURE 9: (A) Influence of spermine on apparent V_{max} and Michaelis constant. The ATP concentration was 66 μM and the spermine concentrations were: (○) control without spermine; (■) 0.75 mM spermine; (▲) 1.8 mM spermine; (▽) 2.7 mM spermine. Other conditions were as in Figure 8. (B) A replot of apparent V_{max} vs. spermine concentration.

and inhibitors of T_4 polynucleotide kinase. The mechanism of action of two types of activators, namely polyamines and salts, was investigated in detail. Both compounds increased the rate of phosphorylation up to five- to sixfold for a number of different DNAs and oligo- and mononucleotides. Since the effects of the activators were not additive they may act on similar sites on the enzyme–substrate or on the same step in the reaction sequence. Previously it has been reported that T_4 polynucleotide kinase is inhibited by agar, dextran sulfate, and other polysaccharide sulfates and further this inhibition could be overcome by addition of spermine (Wu, 1971). It could therefore be argued that the enzyme used in the present work actually contains an inhibitor whose effect can be counteracted by salts and polyamines. It is, however, unlikely that this is the real explanation since a homogeneous enzyme was used having the expected specific activity and furthermore the enzyme had been exposed to high salt concentration during the purification procedure.

A more likely explanation involves the idea that both salts and polyamines affect subunit interaction as well as binding of substrates and products to T_4 polynucleotide kinase. The marked effect of enzyme concentration on the plateau level of phosphorylation may suggest that several interconvertible forms of the enzyme are present in the reaction mixture. We propose that the enzyme exists in two forms A and B which are in equilibrium with each other: A $\rightleftarrows$ B. Form A could be subunits of the enzyme, either dimers or monomers. Form B would be the more active catalytic species having a lower affinity for the substrates than form A. Both salts and polyamines and perhaps also substrates would shift the equilibrium in favor of form B. The products, on the other hand, we suggest favor form A. At a high concentration of the enzyme form B would be the predominating species. Dilution would lead to an increase in A. Physiochemical studies on T_4 polynucleotide kinase currently in progress should provide more evidence concerning such a mechanism. There are many examples in the literature of similar interconversions, the equilibrium being affected by a number of different agents. In the case of polyamines the most well-documented studies are perhaps those on glycogen phosphorylase b (Wang et al., 1968) and UDP-galactose-4-epimerase (Darrow and Rodstrom, 1966). In both cases polyamines caused aggregation of subunits similar to the mechanism postulated above. The concentrations employed were approximately the same as in the present study.

The micrococcal nuclease treated calf-thymus DNA substrate used in the present study was of small molecular weight and largely single stranded. With regard to double-stranded DNAs both polyamines and salt are known to stabilize such molecules. These agents might therefore also be expected to influence the rate of phosphorylation of bihelical DNAs, particularly those having protruding 3′-hydroxyl ends. Indeed, preliminary studies have shown that polyamines actually inhibit the phosphorylation of the latter DNA types, probably due to stabilization of the bihelical structure. It should also be pointed out that native large molecular weight DNAs such as T_7 DNA tend to aggregate in the presence of a high concentration of the polyamines, in particular spermine, and thus the concentration of the substrate becomes rate limiting.

Acknowledgment

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