Characterization of the Guanosine 5′-Triphosphate 3′-Diphosphate and Guanosine 5′-Diphosphate 3′-Diphosphate Degradation Reaction Catalyzed by a Specific Pyrophosphorylase from *Escherichia coli*†

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ABSTRACT: Guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp) are specifically degraded by a manganese-dependent pyrophosphorylase present in *spoT*$^+$ but not in *spoT*$^-$ strains of *Escherichia coli*, indicating that the enzyme is the *spoT* gene product. The enzyme catalyzes the release of pyrophosphate from the 3′ position of ppGpp or pppGpp, yielding ppG and pppG, respectively; pppGpp could not be detected as an intermediate in the decay reaction. Degradation of (p)ppGpp is optimal in the presence of 200 to 300 mM potassium or sodium acetate, at a pH of 7.5 to 8 and a temperature of 37 °C. In the present communication in particular, the effect of energy-rich compounds upon the decay reaction has been investigated. This reaction is stimulated in the presence of 4 mM pppA and a nucleoside diphosphate kinase. The primary product of the pppA- and kinase-dependent reaction is pppG, suggesting that the decay reaction is enhanced by phosphorylation of ppG to pppG. Other nucleotides such as pppC or pppU also stimulate the breakdown of ppGpp, whereas p(NH)ppA or p(C)ppA does not. A pyrophosphate–nucleoside triphosphate exchange reaction is observed with pppA or, to a much lesser extent, with pppC and pppI.

It is assumed that guanosine 5′-diphosphate 3′-diphosphate (ppGpp[1]) functions as pleiotropic effector, restricting various metabolic processes in bacteria (Cashel, 1975). This pleiotropic effect is initiated whenever *Escherichia coli* cells are starved for a required amino acid, leading to rapid accumulation of ppGpp and pppGpp. This control, known as the stringent response, is reversed upon readdition of the omitted amino acid, causing the rapid disappearance of ppGpp and pppGpp.

Genetic studies have shown that the genetic locus named *spoT* is involved in this response. *spoT*$^-$ mutant strains fail to synthesize pppGpp, whereas ppGpp is overproduced. When the stringent response is reversed, the rate constant for ppGpp breakdown is significantly lowered in *spoT*$^-$ strains (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974). These data have been interpreted as an indication that the *spoT* gene product functions in the phosphorylation of ppGpp to pppGpp, which in turn is degraded to pppG and ppG (Laffler and Gallant, 1974). This hypothesis has been challenged by a number of groups proposing that degradation starts with ppGpp rather than with pppGpp (de Boer et al., 1976; Chaloner-Larsson and Yamazaki, 1976; Kari et al., 1977; Fiil et al., 1977). No doubt these conflicting models, which suggest different degradation pathways, can only by elucidated by studying the mechanism of this reaction in a cell-free system. Recent reports by two groups (Heinemeyer and Richter, 1977; Sy, 1977) demonstrated that in vitro decay of ppGpp is catalyzed by an enzyme that releases coordinately two phosphates from the 3′ position of the nucleotide, yielding ppG and pyrophosphate (Heinemeyer and Richter, 1978). Although this enzyme, referred to as pyrophosphorylase, is present in a fraction containing ribosomes, the latter are not essential for the decay reaction. This communication describes the further characterization of the ppGpp and pppGpp degradation in an in vitro system from *E. coli*.

Materials and Methods

[α-^{32}P]GTP or [^{3}H]GTP (New England Nuclear, Boston, Mass.) was used as substrate for preparing pp*Gpp or pp[^{3}H]Gpp using a crude stringent-factor system from *E. coli* (Richter et al., 1975); the superscript asterisk to the right side of the "p" indicates the position of the ^{32}P label. ppGpp* was prepared from [γ-^{32}P]ATP, ppGp*p from [β-^{32}P]ATP; the latter was synthesized from [γ-^{32}P]ATP and 5′-AMP by the myokinase reaction (Bergmeyer, 1974). When p*pp[^{3}H]Gpp was prepared, a purified *E. coli* stringent factor (kindly provided by S. Fehr), [γ-^{32}P]GTP (New England Nuclear) and [^{3}H]GTP were used. The specific activities of the labeled guanosine polyphosphates were in the range of 1–15 Ci/mmol. Unlabeled ppGpp, pppGpp, ppApp, pApp, and pppApp were obtained from Sanruku-Ocean Co., Tokyo, Japan. Bovine nucleosidediphosphate kinase (EC 2.7.4.6), pNppA, and pCppA came from Boehringer, Mannheim, Germany. Protein was estimated by the method of Lowry et al. (1951).

Strains, Media, and Growth Conditions. E. coli strains CP78 (*spoT*$^+$, *relA*$^+$, *CCA*$^+$, *his*$^-$, *leu*$^-$, *arg*$^-$, *thr*$^-$, *Bl*$^-$), CP79 (*relA*$^-$, *spoT*$^+$, *CCA*$^+$, *his*$^-$, *arg*$^-$, *thr*$^-$, *Bl*$^-$, *leu*$^-$), or K10 (*relA*$^-$, *spoT*$^-$, *tonA22*) were used in this study. Bacterial cells were grown at 37 °C in medium containing, per liter, 10 g of tryptone, 5 g of yeast extract, 2 g of glucose, and 5 g of NaCl; pH was adjusted to 6.9. Growth was followed by measuring the absorbance at 578 nm; with a 1-cm pathlength cell, 0.5 A_{578} units/mL correspond to 5×10^8 cells/mL. Exponentially growing cells were harvested at a density of 1.0 to 1.5 A_{578} units/mL. The specific activities of the ppGpp-degrading pyrophosphorylase varied with growth conditions and were optimal with cells obtained from the late logarithmic phase. Growth was stopped by adding crushed ice. Cells were collected by centrifugation in a continuous-flow rotor (Sorvall),

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[1] Abbreviations used are: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; pNppA, β,γ-imidoadenosine 5′-triphosphate; pCppA, β,γ-methyleneadenosine 5′-triphosphate; ppApp, adenosine 5′-diphosphate 3′-diphosphate; pppApp, adenosine 5′-triphosphate 3′-diphosphate; pApp, adenosine 5′-monophosphate 3′-diphosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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TABLE I: Subcellular Distribution of the ppGpp Degrading Pyrophosphorylase.[a]

fractions assayed	pyrophosphorylase units/g of cell	yield (%)
crude cell extract	253	(100)
30 000*g* pellet	45.6	18.0
30 000*g* supernat fract.	198.6	78.5
150 000*g* pellet	141.6	55.9
150 000*g* supernat fract.	5.0	0.2
70S ribosomes[b]	11.1	4.4
gradient fraction[b]	45.7	18.1

[a] The various subcellular fractions were isolated and assayed as described under Materials and Methods. [b] Preparation of 70S ribosomes and gradient fraction: 150 000*g* pellet was dissolved in buffer B and 150 A_{260} units was layered on top of a linear sucrose gradient [10–30% sucrose (w/v) in buffer B] and centrifuged at 20 000 rpm for 12 h in a SW27 Beckman rotor. The gradient was fractionated from the top. Fractions containing 70S ribosomes were pooled and concentrated by centrifugation (150 000*g* for 17 h); the top fractions of the gradient (gradient fraction) were pooled and concentrated by $(NH_4)_2SO_4$ precipitation (45 g/100 mL). After dialysis against 20 mM Tris-HCl buffer (pH 7.7), 2 mM $MnCl_2$, 2 mM dithiothreitol, and 5% sucrose (w/v), 70S ribosomes and gradient fractions were assayed for ppGpp degrading activity.

washed once with buffer A [10 mM Tris-HCl (pH 7.7), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, 1 mM $MnCl_2$] and either immediately processed or stored in liquid nitrogen until used.

Preparation of the Fraction Containing the ppGpp-Degrading Pyrophosphorylase. If not otherwise indicated, the ppGpp-degrading enzyme fraction was prepared as reported (Heinemeyer and Richter, 1977); the various steps are summarized as follows. Cells were suspended in 3 volumes of buffer A containing 2 μg/mL DNase and homogenized in a laboratory pressure cell Model 15 M (Gaulin Corp., Everett, Mass.) at 16 000 psi. The homogenate was centrifuged at 8000*g* for 15 min. The supernatant fraction (about 9 mL) was layered on top of a 3-mL sucrose cushion (2 M sucrose, 5 mM magnesium acetate, 2 mM $MnCl_2$, 2 mM dithiothreitol, 0.1 mM ppGpp) and was centrifuged at 200 000*g* for 17 h. The gradient was fractionated as described (Heinemeyer and Richter, 1977); the first 7 to 8 mL from the top was discarded (fractions 1 and 2). Fractions 3 and 4 (containing the majority of ribosomes) were processed by a threefold dilution with 20 mM Tris-HCl buffer (pH 7.7), 2 mM $MnCl_2$, 5 mM magnesium acetate, 1 mM EDTA, 2 mM dithiothreitol, and 5% (w/v) glycerol. A particulate fraction was removed by centrifugation at 60 000*g* for 30 min. The supernatant fraction containing the ppGpp-degrading activity was centrifuged at 150 000*g* for 4.5 h or at 200 000*g* for 12 h. In the latter case, almost all of the ppGpp-degrading activity was found in the pellet which is referred to as the ribosomal fraction. At the shorter centrifugation time, residual activity was also present in the supernatant fraction (S100). The ribosomal pellet was dissolved in 20 mM Tris-HCl (pH 7.7), 2 mM $MnCl_2$, 2 mM dithiothreitol, 1 mM EDTA, and 10% (w/v) glycerol. Aliquots with 16 mg/mL protein were kept frozen in liquid nitrogen until used. The unit of pyrophosphorylase is defined as the amount of enzyme that degrades 1 nmol of ppGpp/min at 37 °C.

Subcellular Distribution of the ppGpp-Degrading Pyrophosphorylase. The method for preparing the pyrophosphorylase suggests that it is associated with ribosomes. Contamination of this fraction by membrane vesicles could not totally be excluded, however, and poses the question of the subcellular distribution of this enzyme. Therefore, preparation and centrifugation of the crude cell extract were modified. Cells were suspended in threefold buffer B [10 mM Tris-HCl (pH 7.7), 60 mM magnesium acetate, 1 mM $MnCl_2$, 6 mM 2-mercaptoethanol, 60 mM NH_4Cl, 5% sucrose, (w/v)] containing 2 μg/mL DNase and homogenized as described above. After removing intact cells at 8000*g* for 15 min, the crude cell extract was centrifuged at 30 000*g* for 30 min and at 150 000*g* for 4.5 h. Table I shows the units of pyrophosphorylase calculated per gram of cells present in the various subcellular fractions. Although the crude extract showed a rather high activity in degrading ppGpp, these figures may not be reliable due to the presence of unspecific ppGpp-degrading activity by pyrophosphatase (Heinemeyer et al., 1978). The particulate fraction (30 000*g* pellet) contains 18% and the 30 000*g* supernatant fraction about 80% of the total activity. A similar distribution was found for the stringent factor (Schmale et al., 1977). Upon further fractionation of the 30 000*g* supernatant fraction by a high-speed centrifugation step, the activity was exclusively found in the ribosomal pellet. When these ribosomes were subjected to sucrose gradient centrifugation, most of the pyrophosphorylase activity remained in the top fractions of the gradient; the remaining activity was present in the 70S ribosomal peak. Ribosomal subunits prepared by dissociation at low Mg concentrations retained very low ppGpp-degrading activity; the latter comigrated with the 30S ribosomal subunit. A corresponding pyrophosphorylase isolated from *Bacillus subtilis* and *Bacillus stearothermophilus* showed similar affinity to ribosomal subunits (Richter and Geis, 1978). It should be noted that pyrophosphorylase activity sometimes also comigrated with the large ribosomal subunit. The implication of this finding is not yet clear. Similar to the stringent factor, the pyrophosphorylase seems to form aggregates in buffers of low ionic strength. These aggregates may reach a size large enough to cosediment with the ribosome and/or ribosomal subunit; they would also explain the wide subcellular distribution of the pyrophosphorylase activity.

Assay for ppGpp Degradation. The 50-μL assay system contained 2.5 μmol of Tris-HCl (pH 7.7), 0.2 μmol of $MnCl_2$, 0.1 μmol of dithiothreitol, 4.5 μmol of sodium formate, 1.25 μmol of ammonium acetate, and 50 to 150 μg of protein of the ppGpp-degrading enzyme fraction. In some experiments sodium formate and ammonium acetate were replaced by 13.5 μmol of potassium acetate. The reaction was started with 0.01 μmol of ^{32}P-labeled or tritiated ppGpp or pppGpp (sp act. 1–2 Ci/mol). Where indicated, pppA or other nucleotides were titrated with the corresponding magnesium concentration. Routinely, the incubation temperature and time were 37 °C and 20 min, respectively. The reaction was stopped with 1 μL of 88% formic acid, a precipitate was removed by centrifugation, and 2- to 6-μL aliquots of the supernatant fraction were spotted together with unlabeled marker nucleotides on polyethylenimine thin-layer plates (Cel 300 PEI, obtained from Macherey and Nagel, Düren, Germany). The degradation products were analyzed by one- or two-dimensional thin-layer chromatography. Chromatograms were developed one dimensionally in 1.5 M KH_2PO_4 (pH 3.5) or two dimensionally as follows: system 1, first dimension, 0.5 M LiCl/4 M sodium formate (pH 3.4); second dimension, 1.5 M KH_2PO_4 (pH 3.5); system 2, 1.5 M LiCl/2 M sodium formate (pH 3.4); second dimension, 1.5 M KH_2PO_4 (pH 3.5); system 3, first dimension, three steps with LiCl; second dimension, three steps with sodium formate (Randerath, 1966); unlabeled nucleotides used as markers were identified under UV light; radioactive spots identified by autoradiography were cut out and counted in Bray's solution.

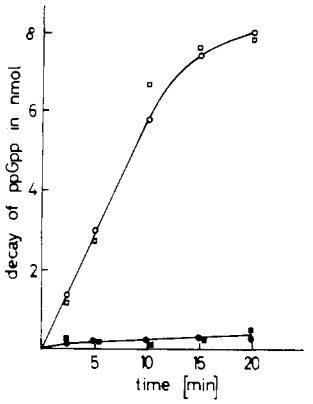


FIGURE 1: Degradation of ppGpp by cell-free extracts prepared from *spoT*$^+$ and *spoT*$^-$ strains. The following isogenic pairs of *E. coli* strains were kindly provided by Dr. N. P. Fiil, Copenhagen through Dr. K. Isono, Berlin: NF859 (*F*$^-$, *metB*$^-$, *argA*$^-$, *relA*$^+$, *spoT*$^+$), NF161 (*F*$^-$, *metB*$^-$, *argA*$^-$, *relA*$^+$, *spoT1*), NF929 (*F*$^-$, *thr*$^-$ *leu*$^-$, *thi*$^-$, *arg*$^-$, *pyr*$^-$, *spoT*$^+$, *relA*$^+$, λ^-, λ^s), and NF930 (*F*$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, *arg*$^-$, *pyr*$^-$, *spoT1*, *relA*$^+$, λ^-, λ^s). Cells were homogenized in buffer B as outlined under Materials and Methods. A particulate fraction was removed by centrifugation at 30 000*g* for 30 min; the supernatant fraction was centrifuged at 150 000*g* for 4.5 h. The pellet was dissolved in 10 mM Tris-HCl buffer (pH 7.7) containing 2 mM $MnCl_2$, 2 mM dithiothreitol, 5% sucrose (w/v); this fraction (100 μg of protein/50 μL of assay) was assayed for decay of ppGpp. At the time indicated, 5-μL aliquots were withdrawn, mixed with 2 μL of 88% formic acid, and analyzed one dimensionally: (○-○) NF859; (●-●) NF161; (□-□) NF929; (■-■) NF930.

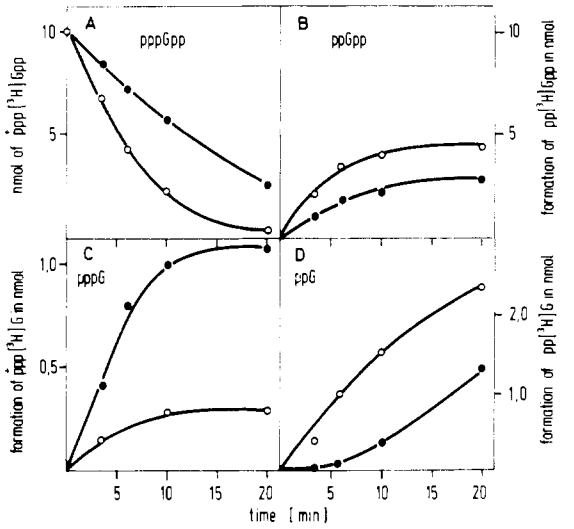


FIGURE 2: Analysis of the products of the p*pp[^{3}H]Gpp degrading reaction. Assay conditions were the same as for the ppGpp-degrading system, except that the decay reaction was started with 0.01 μmol of p*pp[^{3}H]-Gpp. The decay reaction was carried out in the absence (open circles) or in the presence of 1 mM pppG (closed circles). At the time indicated, 5-μL aliquots were withdrawn, mixed with 2 μL of 8.8% formic acid, and applied to polyethylenimine sheets. Analysis of the degradation products were carried out by one-dimensional thin-layer chromatography in 1.5 M KH_2PO_4 (pH 3.5). The following products are expressed in nanomoles at the time indicated: ppGpp (B); pppG (C); ppG (D). In A, the stability of pppGpp is shown at various time points. It should be noted that in A and C the $^3H/^{32}P$ ratio is 1.

Results

In Vitro Conditions for the Degradation of ppGpp. The decay of guanosine polyphosphates was analyzed by using guanosine tetraphosphate (ppGpp) as substrate. The conversion of ppGpp to various degradation products was followed by one- or two-dimensional thin-layer chromatography on polyethylenimine coated sheets. Initially, guanosine pentaphosphate (pppGpp) was not assayed because of its rapid conversion to ppGpp by unspecific GTPases. As was recently reported, the decay of ppGpp is catalyzed by an enzyme associated with the ribosomal fraction that releases pyrophosphate from the 3′ position of the nucleotide (Heinemeyer and Richter, 1977; Heinemeyer and Richter, 1978). The decay reaction is dependent upon 2 to 4 mM $MnCl_2$ but not upon Mg^{2+} (Heinemeyer et al., 1978). In vitro degradation of ppGpp was greatly enhanced by monovalent cations. In the presence of 200 to 300 mM potassium or sodium acetate, the reaction was stimulated fourfold compared to assay conditions where monovalent cations had been omitted; at these concentrations, ammonium acetate stimulated the reaction twofold, whereas LiCl was inhibitory (R. Harder, in preparation) The pH optimum of the decay reaction is between 7.5 and 8, while optimal incubation temperature is at 37 °C. Kinetic studies have revealed a K_m value of 0.8 mM for ppGpp. Neither pApp, ppApp, nor pppApp significantly inhibited the ppGpp degradation reaction at concentrations fivefold in excess over ppGpp. The ppGpp-degrading pyrophosphorylase has been found in extracts prepared from stringent or relaxed strains, although the extract of the latter strain is about one-fifth as active as the wild-type strain. Whether the reduced activity of the ppGpp-degrading enzyme obtained from the relaxed strain is due to a greater lability of the enzyme or whether it is less strongly expressed is not yet known. When compared with the ppGpp-degrading pyrophosphorylase from isogenic *spoT*$^+$ strains, little or no ppGpp-degrading activity was found in *spoT*$^-$ strains, strongly suggesting that the pyrophosphorylase is the *spoT* gene product (Figure 1).

Degradation Does Not Proceed Via Phosphorylation of ppGpp to pppGpp. The finding that certain *E. coli* strains (*spoT*$^-$) degrade ppGpp in vivo at a significantly lower rate and contain little or no pppGpp led to the hypothesis that the initial step in the decay reaction proceeds via phosphorylation of ppGpp to pppGpp (Laffler and Gallant, 1974). Although the intermediate product pppGpp could not be found in the in vitro system, we could not exclude the possibility that pppGpp may immediately be converted to pppG and ppG and, hence, not be detectable. To test whether pppGpp was stable in our assay system, double-labeled p*pp[^{3}H]Gpp was used as substrate. Figures 2A,B show that p*pp[^{3}H]Gpp was immediately converted into pp[^{3}H]Gpp and P_i, indicating that the ppGpp-degrading enzyme fraction was contaminated by various GTPases. This unspecific hydrolysis of pppGpp was reduced when an excess of pppG was added to the assay mixture (Figure 2A). It is evident that p*pp[^{3}H]Gpp was degraded to p*pp[^{3}H]G in the presence of cold pppG but to pp[^{3}H]G when pppG was omitted (Figures 2C and D). It is apparent that the pyrophosphorylase converts pppGpp directly to pppG and pyrophosphate under conditions where unspecific GTPases were saturated with higher concentrations of pppG. Therefore, any pppGpp formed during incubation should be detectable under these conditions. As pointed out, the only degradation products we found were pp*G and pyrophosphate but no pppGpp, excluding the possibility that the latter is an intermediate in the ppGpp decay reaction. That degradation of ppGpp via pppGpp may depend on pppA as phosphate donor can also be excluded, since no pppGpp was detectable in the presence of pppA. It should be noted that with a ppGpp-degrading enzyme fraction prepared from *spoT*$^-$ strain K10 little degradation of ppGpp and pppGpp was observed, indicating that, although the activity is very low, the pyrophosphorylase degrades both ppGpp and pppGpp (data not shown). Although our data are consistent with the in vivo finding that the rate of

TABLE II: Stimulation of the ppGpp Degrading Reaction by Various Nucleotides.[a]

assay conditions	compds formed (nmol) pppG	ppG
control	0	2.8
+ pppA	1.6	3.1
+ pppI	0.5	1.4
+ pppU	2.0	2.4
+ pppC	1.6	2.0

[a] Where indicated the concentration of the nucleoside triphosphate was 4 mM. Incubation was carried out at 37 °C for 5 min in the presence of 160 μg of pyrophosphorylase.

TABLE III: $P^{32}P_i$-Nucleoside Triphosphate Exchange Reaction.[a]

nucleotides assayed	^{32}P-labeled material released from ppGp*p & determined in % as $P\sim{}^{32}P_i$	nucleoside triphosphate
control	61.4	
+pppA	36.3	38.6 (pppA)
+pppG	62.1	0 (pppG)
+pppC	65.6	3.2 (pppC)
+pppU	77.1	0 (pppU)
+pppI	48.4	1.3 (pppI)

[a] The nucleoside triphosphate concentrations used were 4 mM. The products were analyzed by one- or two-dimensional thin-layer chromatography (system 3). In the control assay no nucleoside triphosphates were added.

degradation of ppGpp is significantly lower in *spoT*$^-$ than in *spoT*$^+$ strains, the failure to detect pppGpp in vivo in *spoT*$^-$ strains remains obscure.

Decay of ppGpp in the Presence of Various Nucleotides. The first suggestion that in vitro decay of ppGpp may be effected by energy-rich precursors came from experiments where degradation of ppGpp was stimulated by pppA (Heinemeyer and Richter, 1977). As shown in Table II, pppA enhances the decay reaction about twofold; a similar activation was observed with pppC and pppU. Analysis of the degradation products revealed that, in the presence of the nucleotides assayed, predominantly pppG was formed and upon longer incubation ppG and pG were observed. Apparently, a nucleoside diphosphate kinase contaminating the ppGpp-degrading enzyme fraction accelerates the decay reaction by shifting the equilibrium toward the formation of pppG (Heinemeyer et al., 1978). The possibility that ppGpp is first phosphorylated to pppGpp and then degraded to pppG can be excluded, since no pppGpp was found as the intermediate product. Optimal degradation was obtained with 4 mM pppA or higher, and little or no stimulation was found with pppA analogues such as pNppA or pCppA (data not shown).

It is not yet clear whether the observed pppA-dependent stimulation can be correlated with the in vivo experiments wherein decay of ppGpp was reduced by an order of magnitude when formation of energy-rich compounds was impaired (Gallant and Margason, 1972; Raué and Cashel, 1975; Chaloner-Larsson and Yamazaki, 1976; de Boer et al., 1976). Although with some ppGpp-degrading enzyme preparations up to a fivefold stimulation of the decay reaction was observed with pppA and a nucleoside diphosphate kinase, this stimulatory effect may only be secondary in nature.

We have recently shown that two phosphates are released

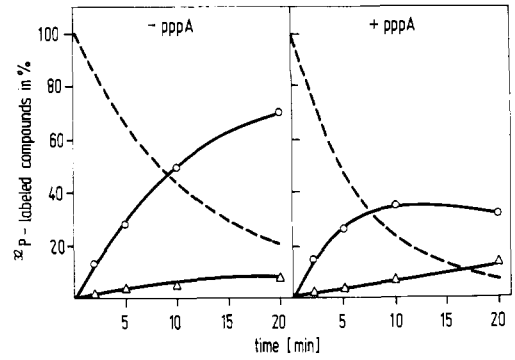


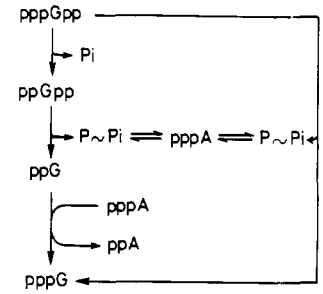
FIGURE 3: Release of pyrophosphate from the 3′ position of pp[^{3}H]Gp*p in the presence or absence of pppA. Where indicated, the concentration of pppA was 4 mM. The dotted line represents the overall decay of ppGpp; (O–O) P ~ P_i; (Δ–Δ) P_i. At the time indicated, 5-μL aliquots were withdrawn from the assay mixture and stopped with 2 μL of 8.8% formic acid. Aliquots were chromatographed on polyethylenimine sheets as outlined under Materials and Methods (system 1 or 2).

in a one-step reaction from the 3′ position of ppGpp, yielding pyrophosphate (Heinemeyer and Richter, 1978). When the decay of radioactively labeled ppGp*p or ppGpp* was assayed in the presence of pppA, we noticed a significant reduction of pyrophosphate formation while the overall decay reaction was stimulated (Figure 3). Since the formation of inorganic phosphate did not significantly increase, it was assumed that pyrophosphate either was transferred to an appropriate acceptor or was exchanged with pppA. The former assumption could not be put to a decisive test, since experiments with tritiated pA and ppGpp* as substrate did not result in a significant formation of pppA. The alternative assumption predicts that a pppA–P ~ P_i exchange reaction exists. Table III shows that with ppGpp* (or ppGp*p) as substrate the ^{32}P label appeared in the pppA spot. This pyrophosphate exchange reaction predominantly occurred with pppA, whereas little or no effect was observed with other nucleotides. Again this pppA-mediated exchange reaction may not be directly coupled to the ppGpp decay reaction and may merely reflect the activity of an aminoacyl-tRNA synthetase present in the ppGpp-degrading enzyme preparation. On the other hand, aminoacyl-tRNA synthetase assayed under these conditions shows little or no activity in the pyrophosphate exchange reaction. It is evident that highly purified enzyme preparations of the ppGpp degradation reaction should solve this problem.

Discussion

The ppGpp and pppGpp degradation reactions may be summarized in Scheme I. With pppGpp as substrate, it is either

SCHEME I: ppGpp and pppGpp Degradation Reactions.



degraded directly to pppG and pyrophosphate or converted to ppGpp by hydrolysis (Cashel, 1975). The ppGpp degradation reaction which yields ppG and pyrophosphate is stimulated by pppA; in this reaction, ppG is phosphorylated to pppG by a

nucleoside diphosphate kinase. Apparently, the pppA-mediated effect is indirectly correlated to the decay reaction. A more direct role for pppA in the decay reaction may be implied by our finding with more purified enzyme preparations: the conversion of ppGpp to ppG is enhanced by pppA, whereas no pppG is formed (unpublished data). That breakdown of ppGpp is accompanied by a pppA-P $\sim$ P_i exchange reaction may also indicate that pppA has a central function in the decay reaction.

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Phosphate Content of *Escherichia coli* Alkaline Phosphatase Isozymes. The Effect of Phosphate and Zinc on the Separation of Isozymes†

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ABSTRACT: Alkaline phosphatase from *Escherichia coli* was isolated as two major isoenzyme forms that were separated by DEAE-cellulose chromatography. Each form contained 2 equiv of endogenous phosphate. The endogenous phosphate, although difficult to remove, readily exchanges with phosphate. The forms also were separable by polyacrylamide gel electrophoresis. Apoenzyme prepared from native enzyme by the removal of zinc (and phosphate) also contains electrophoretically distinct enzyme forms which are indistinguishable from the native forms on gel electrophoresis. The isozymes were also found to have similar affinities for inorganic phosphate and susceptibilities to inactivation by EDTA. These results are not consistent with the notion that the formation or separation of isoenzyme forms is dependent upon different amounts of bound phosphate. They are consistent with the suggestion that a difference in amino acid composition is the basis for the occurrence and separation of these isoenzymes.

Alkaline phosphatase from *Escherichia coli* is composed mainly of three isoenzyme forms which are separable by ion-exchange chromatography or electrophoresis (Lazdunski & Lazdunski, 1967; Levinthal et al., 1962). It is concluded that the isozymes are the result of epigenetic modifications since a single cistron codes for both subunits of the enzyme (Singer, 1961).

The nature of the differences between isozymes of alkaline phosphatase has been investigated in a number of laboratories (Lazdunski & Lazdunski, 1967; Schlesinger & Anderson, 1968; Bosron & Vallee, 1975). In general there has been a lack of agreement about the basis of the isozyme differences. On one hand, evidence has been presented which supports differences in the N terminus of the isozymes as the basis for the isozymes (Kelley et al., 1973; Schlesinger et al., 1975). On the other hand, there is evidence that suggests that differences in the amount of inorganic phosphate bound to the isozymes is the basis for the isozymes (Bosron & Vallee, 1975). Neither of these interpretations excludes the other, as it is possible that differences in the N terminus of the two subunits could be re-

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