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THE EXTRACTION OF GUANOSINE 5'-DIPHOSPHATE, 3'-DIPHOSPHATE (ppGpp) FROM ESCHERICHIA COLI USING LOW pH REAGENTS: A REEVALUATION

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Summary

It has been found that the most widely used method for the extraction of guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) from E. coli (1 M formic acid at 0°) results in its in vitro degradation to ppGp and GDP. A comparison with several other extraction procedures indicated that this breakdown is due to the low pH of the reagents used during extraction. This degradation can largely be prevented by using a new extraction technique which involves freezing and thawing of the cells in the presence of lysozyme at a neutral pH followed by treatment with deoxycholate. With this method it is possible to recover from three to five times as much ppGpp from both unstarved and amino acid starved stringent strains of E. coli as compared with the most widely used formic acid procedure. Consequently, it will be necessary to reevaluate the ppGpp values obtained from cells when formic acid or other low pH reagents were used during extraction.

Introduction

Guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) has been directly implicated in the control of RNA synthesis in prokaryotes (1-4). Studies conducted with mutants of E. coli which are defective in ppGpp catabolism (spoT) indicated that this control is correlated with the intracellular level of ppGpp (5,6). The concentration of ppGpp within the cell, therefore, plays a vital role in the control of cell growth.

Our search for the in vivo degradation product of ppGpp resulted in the unexpected discovery that extensive in vitro degradation of this compound occurs when it is extracted by 1 M formic acid at 0°. With the advent of studies regarding ppGpp, especially the kinetics of its synthesis (7-11) and degradation (9-14), these findings are of considerable significance because cold formic acid has always been the most widely used reagent for ppGpp extraction (15). A comparison of those procedures which have been used to extract ppGpp from E. coli

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was therefore made in an attempt to find one which would prevent this breakdown. It was found that the level of ppGpp varied with the extraction procedure used and was always accompanied by degradation to ppGp and GDP. The use of an enzymatic technique at a neutral pH, however, proved to be a very effective method of extracting ppGpp and at the same time greatly minimizing its in vitro degradation.

Materials and Methods

Bacterial cultures were grown with shaking at 37° in Hershey's Tris minimal medium (16) with 0.33 mM phosphate, glucose (0.3%), and thiamine (2 μg/ml). Cultures of E. coli JC-355 (metB1, leu-6, his-1, argG6, and relA$^+$, spoT$^+$) (17), NF-161 (metB1, argA52, spoT1, and relA$^+$) (5,6), and PC-0207 (thi-1, his-68, trp-45, tyrA2, purH57, guaC23, and relA$^+$) (18) were each supplemented with their required amino acids at 14 μg/ml. Cultures of PC-0207 were also supplied with adenosine (24 μg/ml) and guanosine (4 μg/ml). Growth was monitored turbidimetrically at 720 nm. Nucleotides were labeled with carrier-free $H_3{}^{32}PO_4$ (15 μCi/ml) which was added to cultures at least two generations prior to extraction. Cultures of PC-0207 were labeled with [8-^{14}C]guanine (0.5 μCi/ml) at the onset of growth. Valine induced isoleucine starvation was used to produce large amounts of ppGpp in strains JC-355 (Table I) and PC-0207. Valine (400 μg/ml) was added at 0.3 A_{720} (4 X 10^8 cells/ml) and all samples were withdrawn seven minutes later. All reagents and vessels used to process the extracts and all manipulations were performed at 0° unless otherwise stated. The Corex tubes used to collect culture samples were chilled in a dry ice-acetone bath and immediately before sampling they were all wrapped in aluminum foil and packed in ice. 10 ml aliquots of culture, all removed at the same time, were pipetted down the sides of the glass tubes. All samples were immediately centrifuged at 23,500 x g, placed on ice, and the supernatants were carefully removed and discarded. The cell pellets were then individually treated as indicated in the legend to Table I. Commercially prepared polyethyleneimine (PEI) cellulose sheets were predeveloped for two-dimensional chromatography (19). 3 μl of each unlabeled nucleotide standard (2.5 mg/ml) were spotted at the origin together with the desired amount of radioactive sample (1 μl at a time). Plates containing lysozyme samples were also spotted with 3 μl of water at the origin prior to development in the first dimension to prevent streaking. The chromatography solvent system used was 1.5 M LiCl & 2 M HCOOH in the first dimension and 1.5 M KH_2PO_4 (pH 3.4) in the second dimension (20).

Results and Discussion

Cultures of the stringent E. coli strain JC-355, labeled with carrier-free $H_3{}^{32}PO_4$ (15 μCi/ml) at least two generations prior to sampling, were subjected to isoleucine starvation by the addition of 400 μg/ml of valine. 1 M formic acid nucleotide extracts of culture samples taken before and after isoleucine starvation were prepared (21,22). Two-dimensional thin-layer chromatography of these extracts on PEI-cellulose (20) revealed the presence of ppGpp and an unidentified phosphorylated compound (X) in the samples taken after isoleucine starvation but not in those made before the addition of valine (Fig. 1). Since compound X was

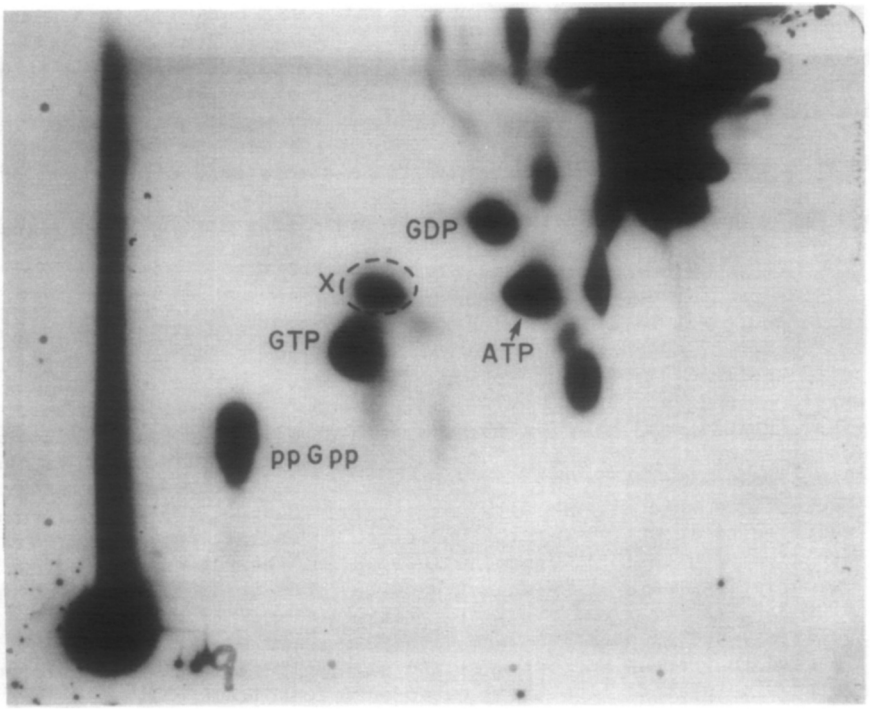

Figure 1. Radioautograph of a 1 M formic acid nucleotide extract of E. coli JC-355. The bacterial culture was labeled with $H_3{}^{32}PO_4$ (15 μCi/ml) and was starved for isoleucine by the addition of valine (400 μg/ml) prior to sampling. Two-dimensional thin-layer chromatography on PEI-cellulose was carried out with 1.5 M LiCl & 2 M HCOOH in the first dimension (bottom to top) and 1.5 M KH_2PO_4, pH 3.4 in the second dimension (left to right). The locations of authentic, unlabeled standards are indicated along with the position of the unidentified phosphorylated compound (X).

never found in the formic acid extracts of amino acid starved relaxed bacteria (which contain no ppGpp) it was suspected that it was derived from ppGpp. To determine the nature of this compound an E. coli purine auxotroph (PC-0207) was then labeled with [8-^{14}C]guanine and subjected to valine induced isoleucine starvation. These studies revealed that compound X was a guanine derivative (figure not shown).

Compound X was subsequently found to comigrate with authentic guanosine 5'-diphosphate, 3'-monophosphate in two-dimensional thin-layer chromatography in six different solvent systems (19,20,23,24). It is also sensitive to 3'-nucleotidase digestion (producing GDP) without prior treatment with zinc-activated yeast inorganic pyrophosphatase. This is consistent with a structure

which involves a monophosphate esterified to the 3'-position of the ribose moiety but we have not been able to completely rule out the possibility that it is actually a racemic mixture of the 3'- and 2'-monophosphates which is known to exist under acidic conditions (25). This compound shall therefore be refered to by the general term ppGp. Guanosine 5'-monophosphate, 3'-diphosphate (pGpp), however, was never detected in any nucleotide extract using comigration with an authentic standard as the criterion for its presence.

To determine whether or not the extraction procedure was responsible for the production of ppGp, a survey of those techniques which have been used to extract ppGpp from E. coli was made in an attempt to find one which might prevent its degradation (Table I). A comparison of these procedures (Table I, columns 1 through 3) reveals substantial differences in the amount of ppGpp recovered and these differences appear to be dependent upon the pH of the reagent used during extraction. The largest amount of ppGpp was obtained, not by any of the conventional methods reported previously (21,22,26,27), but by the lysozyme freeze-thaw nucleotide extraction procedure reported here (Table I, column 4). This procedure is a modification of the technique originally designed to extract polysomes from E. coli (28) and produces a final pH of 6.8.

The nucleotide extract made with sodium formate at pH 3.4 resulted in the recovery of substantially less ppGpp than had been obtained by the lysozyme procedure. It did, however, yield more than twice as much ppGpp as the 1 M formic acid extract which had a pH of 1.8. Extraction with 1 M monochloroacetic acid (pH 1.4) resulted in the lowest amount of ppGpp recovered.

Since all of the samples used in Table I were processed in the same manner there are two possible explainations for these results. Either ppGpp continued to be synthesized at the higher pH values after cell lysis or it was being broken down at the lower ones. To eliminate the possibility that ppGpp continued to be produced during the extraction procedure, 0.5 μCi of a [8-^{3}H]GTP and [8-^{3}H]GDP mixture were added to pellets derived from unlabeled culture aliquots of isoleucine starved E. coli JC-355 before the lysozyme mixture was added. The fluorograph in

TABLE I

The Influence Of Various Extraction Procedures

Upon The Content And In Vitro Degradation Of ppGpp

Nucleotide	Extraction Reagent & Final pH							
p moles/A_{720}	1 M $CH_2ClCOOH$	1 M HCOOH	1 M HCOONa	Lysozyme-DOC	Lysozyme-DOC & 1 M HCOOH	Lysozyme-DOC & 0.3 M HCOONa	1 M HCOOH adjusted to pH 3.4	1 M HCOOH adjusted to pH 6.8
	pH 1.4	pH 1.8	pH 3.4	pH 6.8	pH 1.8	pH 3.4	pH 3.4	pH 6.8
GDP	461	465	433	273	890	960	516	458
ppGp	55	36	63	29	142	165	40	60
ppGpp	240	252	595	1,000	358	425	184	291

Table I. A culture of E. coli JC-355, labeled with $H_3{}^{32}PO_4$ (15 μCi/ml), was subjected to valine induced isoleucine starvation at 0.3 A_{720} (4 X 10^8 cells/ml). Seven minutes later all samples (taken from the same culture at the same time) were collected according to the procedure described in Materials and Methods. The samples used in columns 1, 2, 3, 7 and 8 were processed by adding 200 μl of the appropriate reagent to each pellet (26), mixing for 3 sec, and then subjecting them to two cycles of freezing in a dry ice-acetone bath and thawing in ice water with intermittent vortexing. The tubes were then centrifuged at 23,500 x g and the supernatants were removed. The pH adjustments (columns 7 & 8) were made by first pooling the 1 M formic acid extracts from three tubes and then adding either cold NaOH (for pH 3.4) or NaOH with Tris-HCl (for pH 6.8) solutions to measured amounts of the pooled extracts with constant vortexing at 0°. The final pH values were checked with pH paper. Lysozyme extracts (columns 4-6) were prepared by adding 200 μl of lysozyme mixture [lysozyme (1 mg/ml) in 0.01 M Tris-HCl (pH 7.8) and 0.015 M magnesium acetate] to each pellet and vortexing for 3 sec. The suspensions were subjected to two cycles of freezing and thawing as detailed previously and then 15 μl of a 10% deoxycholate (DOC) solution were added to each tube with vortexing. The samples were centrifuged and the supernatants were pooled. Treatment with acid was carried out by adding a measured aliquot of lysozyme extracted nucleotides to a cold centrifuge tube containing the appropriate reagent. The samples were mixed and then immediately centrifuged at 23,500 x g. The resulting supernatants were carefully removed and saved. The extracts were chromatographed in two dimensions on PEI-cellulose and were then located by radioautography. The labeled compounds were then cut from the thin-layer chromatographs and counted in 10 ml of Bray's Solution. The nucleotide levels, expressed as p moles/A_{720}, were adjusted to compensate for dilution differences which arose during the various procedures.

Figure 2 shows that neither ppGpp nor pppGpp (guanosine 5'-triphosphate, 3'-diphosphate) was produced after cell lysis during nucleotide extraction. Similar results were obtained with the E. coli stringent spoT mutant NF-161 (figure not shown)

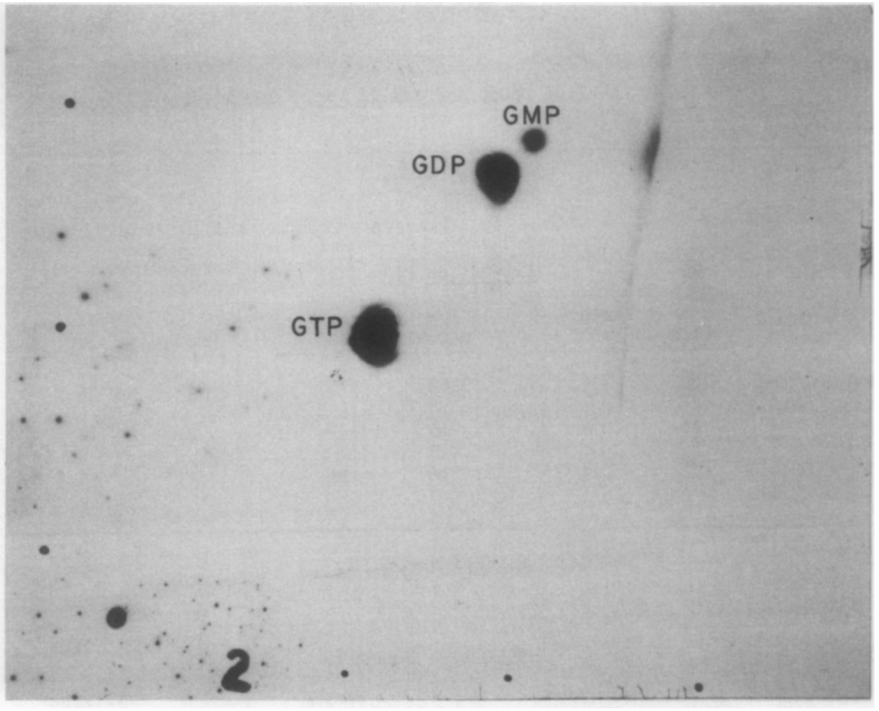

Figure 2. Fluorograph of a lysozyme nucleotide extract of E. coli JC-355. The bacterial culture was starved for isoleucine and a mixture of [8-^{3}H]GTP and [8-^{3}H]GDP (0.5 μCi) was added to the cell pellet prior to extraction. Chromatography was the same as in Figure 1 and fluorography was accomplished by pouring a 5% solution of 2,5 diphenyloxazole (PPO) in anhydrous ethyl ether over the thin-layer sheet and allowing it to dry before exposure to X-ray film at -70°. The locations of nucleotide **standards are indicated. Note the complete lack** of both pppGpp and ppGpp.

To determine the influence of low pH upon ppGpp content, aliquots of the lysozyme extracted nucleotides were treated with either formic acid (pH 1.8) or sodium formate (pH 3.4). The data presented in columns 4, 5, and 6 of Table I show that ppGpp is rapidly degraded upon brief exposure to low pH at 0°. The data also indicate that it is the initial exposure to acid that causes this breakdown because adjusting the pH of 1 M formic acid nucleotide extracts to either pH 3.4 or 6.8 could not prevent ppGpp degradation (Table I, columns 2, 7, and 8).

The ability to extract large amounts of ppGpp from E. coli by using the lysozyme procedure and at the same time drastically reduce the proportion of ppGp produced, as compared to the most commonly used formic acid procedure, indicates

TABLE II

The Influence Of Extraction Procedure Upon

The Basal Level Of ppGpp And Its *In Vitro* Degradation

E. coli JC-355

Nucleotide	A		B		C	
	Extraction Procedure					
p moles/A_{720}	1 M HCOOH	Lysozyme-DOC	1 M HCOOH	Lysozyme-DOC	1 M HCOOH	Lysozyme-DOC
GDP	360	334	368	330	415	357
ppGp	15	6	16	9	21	10
ppGpp	11	57	16	58	13	65

E. coli NF-161

Nucleotide	A		B		C	
	Extraction Procedure					
p moles/A_{720}	1 M HCOOH	Lysozyme-DOC	1 M HCOOH	Lysozyme-DOC	1 M HCOOH	Lysozyme-DOC
GDP	536	344	473	411	579	384
ppGp	39	16	34	23	58	22
ppGpp	51	218	48	225	75	375

Table II. Cultures of *E. coli* JC-355 and NF-161, both labeled with $H_3{}^{32}PO_4$ (15 μCi/ml), were grown in minimal supplemented media at 37°. At three different time points (A, 0.175 A_{720}; B, 0.3 A_{720}; and C, 0.5 A_{720}) duplicate samples of each culture were taken and prepared for cell pellet nucleotide extraction. One of each pair was extracted with 1 M formic acid while the other was processed according to the procedure for lysozyme extraction as described in the legend to Table I. All extracts were chromatographed in two dimensions on PEI-cellulose and were quantitated as described previously. The p moles/A_{720} values were adjusted to compensate for dilution differences between the two extraction procedures.

that ppGp is the result of *in vitro* degradation. It cannot be the only product of this breakdown, however, because its level does not increase linearly as the amount of ppGpp recovered decreases. If the levels of guanosine 5'-diphosphate (GDP) produced by the various extraction procedures are also examined it can be seen that they are inversely related to the amount of ppGpp recovered. The amount

of GDP obtained by the lysozyme procedure is the lowest and as the pH of the extraction reagent is lowered, the level of GDP increases (Table I, columns 1 through 4). Guanosine 5'-monophosphate, 3'-monophosphate (pGp), on the other hand, was never detected in any nucleotide extract.

To establish whether or not the extraction procedure also influences the basal level of ppGpp in E. coli during normal growth on defined medium, a comparison was made between the 1 M formic acid method and the lysozyme freeze-thaw technique. Two different stringent E. coli strains, JC-355 and NF-161, were used. JC-355 is $spoT^+$ while NF-161 carries a mutation in the spoT locus and is therefore defective in ppGpp catabolism. Table II shows that in both JC-355 and NF-161, considerably higher basal levels of ppGpp were obtained throughout the normal growth cycles of both cultures by using the lysozyme procedure.

A comparison of the data in Tables I and II (columns 2 and 4 from Table I and column B for JC-355 from Table II) reveals that in JC-355 valine induced isoleucine starvation causes a 15 to 20 fold increase in the basal level of ppGpp. The lysozyme procedure, however, recovered from four to five times as much ppGpp from JC-355 as compared to the amount obtained by formic acid extraction.

In light of these findings the kinetic values assigned to ppGpp synthesis and degradation by other workers (7-14) may have to be reexamined. The recent work concerning the mechanism of in vivo degradation of ppGpp (29-31) and the quantitation of in vivo degradation products (29) must also be reevaluated. The use of formic acid to extract the nucleotides in these studies may have influenced their conclusion that GDP is the in vivo degradation product of ppGpp.

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