INAPPARENT CORRELATION BETWEEN GUANOSINE TETRAPHOSPHATE LEVELS AND RNA CONTENTS IN ESCHERICHIA COLI

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Received May 8,1974

SUMMARY

We have reexamined the intracellular levels of guanosine 5'-diphosphate 3'-diphosphate in a prototrophic stringent strain of Escherichia coli during balanced growth at eight different growth rates. No regular inverse relationship between the basal levels of this nucleotide and the RNA content per unit DNA was observed. Thus, it is concluded that the cellular capacity to accumulate RNA is not directly related to the basal level of this nucleotide. It was also observed that significant quantities of this nucleotide accumulated extracellularly to varying degrees depending on the carbon source used.

Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) (1, 2) rapidly accumulates in bacteria when RNA accumulation is inhibited during various physiological stress conditions such as (i) amino acid starvation or restriction of aminoacylation of tRNA (3 - 5); (ii) carbon source starvation (6, 7); (iii) nutritional stepdown (6, 8, 9); (iv) salt shock (10). The excellent correlation between the kinetics of ppGpp accumulation and the cessation of RNA accumulation suggests a possible inhibitory role of this nucleotide in the regulation of synthesis of stable bacterial RNA. However, ppGpp causes a modest inhibition of global RNA in vitro, and its presumed specific inhibitory action on stable RNA synthesis has not been demonstrated (11, 12). On the other hand, Lazzarini et al. (6) reported that the basal levels of ppGpp observed during balanced growth vary inversely with the growth rate and RNA content per unit DNA. Such data has led to a generalization that ppGpp may also

be involved in the regulation of stable RNA accumulation during balanced growth.

Because of its relative importance, we have reinvestigated this relationship using a prototrophic stringent strain of Escherichia coli over a wider range of growth rates created by utilizing different carbon sources. Although the RNA content per unit DNA increases linearly with increasing growth rate, the basal levels of ppGpp do not exhibit a regular inverse relationship to the RNA content.

MATERIALS AND METHODS

Bacteria and Culture Conditions: A prototrophic and stringent E. coli K-12 strain was provided by Dr. Ira Pastan, National Cancer Institute, Bethesda, Md. This strain which we have designated HY 1 was used throughout. During valine-imposed isoleucine starvation, RNA accumulation in this strain is severely inhibited, which is accompanied by the accumulation of both MS I (ppGpp) and MS II (guanosine 5'-triphosphate 3'-diphosphate: pppGpp).

Tris-maleate minimal medium (TMM) (13) containing 1 mM phosphate was used throughout. Eight carbon sources were used to create eight different growth rates: 0.4% glucose plus 20 common amino acids (100 µg each per ml); 0.4% glucose; 0.9% sodium succinate hexahydrate; 0.5% sodium lactate; 0.5% sodium pyruvate; 1% sodium acetate trihydrate; 0.4% alanine; 0.5% aspartic acid. The cells were grown by shaking at 37° in the same carbon source throughout. Growth was followed by measuring absorbance at 500 nm (A_{500}) with a Bausch and Lomb Spectronic 20 spectrophotometer using a round cuvette (11 mm inside diameter).

Colorimetric Assay of RNA and DNA: Triplicate samples (10 ml) were removed from a culture during exponential growth and were assayed for RNA and DNA according to the procedure of Lazzarini, Cashel

and Gallant (6). Samples were thoroughly washed with CCl_3COOH to ensure removal of nucleotides and interfering substances. The average color density due to RNA (orcinol assay) was divided by the average color density due to DNA (diphenylamine assay) and the ratio was taken as a relative measure of RNA accumulation per unit DNA (RNA/DNA). The use of arbitrary colorimetric standards was purposely avoided since it likely introduces unnecessary errors (14) and the absolute value for the RNA/DNA ratio is not essential for the present analysis.

Determination of Total and Extracellular Nucleotides: All determinations described here were made using the same batch of [^{32}P]-orthophosphate (Atomic Energy of Canada, Ltd.; radio-purity greater than 99%) after ensuring by one-dimensional thin-layer chromatography (4) that it was free of possible contaminants that co-migrate with ppGpp. Carrier-free [^{32}P] orthophosphoric acid was added to an exponentially growing culture to final specific activities of 350-500 Ci per mole. After one doubling, duplicate samples (50 µl) were withdrawn and mixed with an equal volume of 2 M formic acid at 0°. After at least 30 min at 0°, samples were centrifuged. The extracted ppGpp, GTP and ATP in the supernate were assayed by means of two-dimensional thin-layer chromatography (1). Growth was monitored in the parallel unlabeled culture: we have previously determined that the addition of [^{32}P] phosphoric acid up to a specific activity of 500 Ci per mole causes no significant radiation effect on growth. The amount of phosphate in the nucleotides present in one ml of culture at A_{500} = 1.0 is defined as "total" nucleotides.

In order to determine the "extracellular" amount of nucleotides, 0.2 ml portions of the labeled culture described above are suction-filtered through a membrane filter (0.45 microns pore) on a Swinnex-

13 filter unit (Millipore Corporation). An aliquot (50 µl) of the filtrates was mixed with an equal volume of 2 M formic acid at 0°. After at least 30 min at 0°, samples were centrifuged and assayed for ppGpp, GTP and ATP as before (1). This was taken as a measure of the "extracellular" nucleotides. The "intracellular" amounts of nucleotides were calculated by subtracting the "extracellular" amounts from the "total" amounts of the nucleotides.

RESULTS AND DISCUSSION

A prototrophic strain was chosen for the present study because of the possibility that an auxotroph, if used, may utilize its growth requirement as a preferred carbon source rather than a poor carbon source. Each of the carbon sources used yielded a single definite exponential growth phase except aspartate. The doubling times are shown in Table I. Aspartate yielded an initial faster but short exponential phase which was followed by a final slower and longer exponential phase with the doubling time shown in Table I.

Autoradiography of two-dimensional chromatograms indicated that ppGpp was well resolved from other ^{32}P-containing compounds thus permitting reliable determination of this less abundant nucleotide, whereas ATP and GTP regions suffered varying but minor degrees of contamination from unidentified compounds.

It has been previously assumed that ppGpp accumulates only intracellularly. Thus all the ppGpp found in the acidified culture ("total" ppGpp as shown in Part A of Fig. 1) has been taken as the intracellular level of ppGpp (1, 3 - 10, 12, 13). However, we found that significant quantities of ppGpp (as well as trace quantities of triphosphates) accumulated also extracellularly as shown in Table I. The extracellular amounts of ppGpp varied considerably with the carbon source used. Its extracellular presence is likely due to the excretion of this nucleotide rather than to the lysis of a fraction

TABLE I

Intra- and extracellular amounts of ppGpp, GTP and ATP

[^{32}P]Orthophosphate was added to final specific activities of 350 to 500 Ci per mole to cultures of HY 1 growing exponentially on various carbon sources with the doubling times shown below. After one doubling, samples were withdrawn in duplicate and assayed for intra- and extracellular ppGpp, GTP and ATP. The amounts of these nucleotides are presented as picomoles of phosphate in the nucleotides present in one ml of culture at A_{500} = 1.0.

Carbon Source	Doubling Time (min)	ppGpp		GTP		ATP	
		Intra-	Extra-Cellular	Intra-	Extra-Cellular	Intra-	Extra-Cellular
Glucose + amino acids	35-40	86 86	14 25	2,530 2,730	90 130	7,180 7,520	340 400
Glucose	55	137 126	14 12	3,800 3,460	70 50	9,110 8,350	170 100
Succinate	70-80	68 68	23 23	2,460 2,470	20 20	7,290 7,500	100 90
Lactate	90	62 50	40 50	1,890 1,690	90 60	4,830 4,870	200 110
Pyruvate	95	68 71	54 62	1,630 1,680	40 70	4,320 4,410	180 220
Acetate	120	153 --	23 --	1,410 --	20 --	5,770 --	80 --
Alanine	130	195 153	61 43	2,510 2,670	90 40	7,350 7,940	130 140
Aspartate	300-330	115 119	49 50	821 634	35 42	3,460 2,890	110 100

of the cells, since its relative extracellular distribution was much greater than that of ATP or GTP. If it had been a result of lysis, the extracellular distribution of all three nucleotides would have

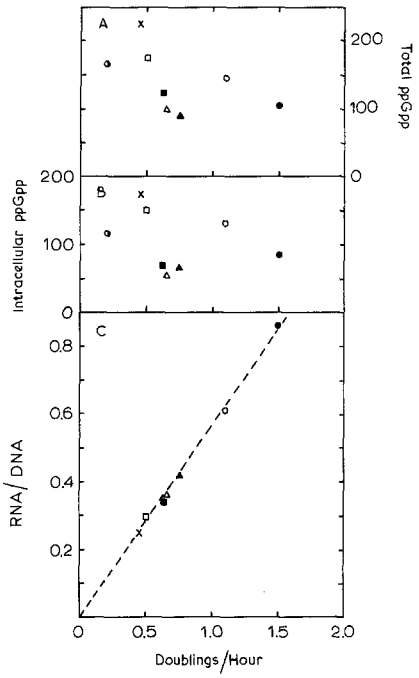

Fig. 1. Levels of ppGpp and RNA/DNA ratios at different growth rates. Strain HY 1 was cultured in each of the following carbon sources: glucose plus 20 common amino acids (●); glucose (○); succinate (▲); lactate (△); pyruvate (■); acetate (□); alanine (×); aspartate (◑). The data in Part A (total ppGpp) and Part B (intracellular ppGpp) represents the picomolar quantities of phosphate in ppGpp per ml of culture at A_{500} = 1.0 (average of two determinations). For the determination of the RNA/DNA ratios (Part C), samples were removed in triplicate and assayed for RNA (orcinol assay) and DNA (diphenylamine assay) as described previously (6). The data (average of three determinations) represents the relative color intensities (orcinol color/diphenylamine color). The RNA/DNA ratio for aspartate is not included since growth on this carbon source consisted of an initial fast exponential phase followed by a final slow exponential phase, resulting in an overestimated value of RNA.

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been similar. It is now obvious that the intracellular ppGpp levels should be used in an attempt to study its possible role in the regulation of RNA synthesis.

Fig. 1 illustrates that there is no definite correlation between the intracellular level of ppGpp (Part B) and the growth rate (correlation coefficient = -.248; not significant at 5% level). Since the RNA/DNA ratios are directly correlatable with the growth

rate (Part C), no regular inverse relation can be drawn between the intracellular ppGpp levels and the RNA/DNA ratios during balanced growth. More specifically, the ppGpp levels in succinate, lactate and pyruvate were lower than the levels in glucose and glucose plus amino acids. Similarly, the level in aspartate was lower than the levels in acetate and alanine but similar to that in glucose. Our conclusion does not, however, rule out the possibility that growth on these carbon sources may be unusual in that RNA accumulation may be primarily limited by some step in RNA precursor (e.g. ribose) biosynthesis, whereas RNA accumulation during growth on other carbon sources is indeed regulated by the ppGpp levels. The possibility that the turnover rate of ppGpp is related to the rate of RNA accumulation is also not ruled out by the present observation.

The inverse relationship between the ppGpp levels and the RNA contents found in E. coli strain, NF 161 (6) is, therefore, not obligatory. The strain used in the present study is prototrophic and wild type with respect to the metabolism of guanosine polyphosphates, whereas NF 161 (rel$^+$, arg, met) possesses additional mutation (designated spoT) which appears to decrease the rate of conversion of ppGpp to pppGpp (R.A. Lazzarini and J. Gallant, personal communication). This spoT$^-$ mutant maintains an abnormally high basal level of ppGpp as compared to wild type spoT$^+$ strains such as HY 1 and accumulates only ppGpp during amino acid starvation. Furthermore, the previous conclusion (6) is based upon the examination of four different carbon sources: glucose plus Casamino acids; glucose; succinate; alanine.

Fig. 1 (Part C) also demonstrates that the RNA/DNA ratio can be extrapolated to zero when the growth rate reaches zero. This is consistent with the theoretical consideration of Koch (14) that the RNA/DNA ratio would be directly proportional to the growth rate.

Similar extrapolations by other workers have yielded significant finite values for the RNA/DNA ratios (15-17). The reason for this discrepancy is not known.

The main purpose of the present communication is to demonstrate that the RNA content per unit DNA in E. coli can be regulated without having a definite inverse relationship to the ppGpp levels during balanced growth.

ACKNOWLEDGEMENTS

We thank Dr. Robert A. Lazzarini for suggesting to us the possibility of extracellular accumulation of ppGpp and Dr. Jonathan Gallant for critical reading of our manuscript. This work was supported by a Grant from the National Research Council of Canada (A - 4698). One of us (S.R.K.) was a holder of the National Research Council of Canada Postgraduate Scholarship.

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