JOURNAL OF BACTERIOLOGY, May 1975, p. 585-591
Vol. 122, No. 2

Printed in U.S.A.

Simple Downshift and Resulting Lack of Correlation Between ppGpp Pool Size and Ribonucleic Acid Accumulation

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Received for publication 17 February 1975

The growth rate of *Escherichia coli* can be limited by the availability of carbon and energy. To impose such a limitation, α-methylglucoside (αMG), a non-metabolizable analogue, can be used to decrease uptake of glucose by competition for the transport of this sugar. Varying the ratio of glucose to αMG allowed shifts in growth rate without simultaneous qualitative changes in the growth medium and permitted examination of the immediate changes accompanying such shifts. Stringent (*rel*$^+$) as well as relaxed (*rel*$^-$) strains were able to rapidly curtail their accumulation of ribonucleic acid (RNA) after a downshift imposed by decreasing glucose transport into the cell. Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) accumulated in both *rel*$^+$ and *rel*$^-$ strains after a decrease in growth rate. However, the accumulation of ppGpp in relaxed derivatives was very slow, and there was no direct or obligatory correlation between the level of ppGpp and the rate of RNA accumulation. This latter conclusion is supported by measurements of ppGpp levels and rates of RNA accumulation after restoration of maximal growth rates by addition of excess glucose.

The macromolecular composition of bacterial cells varies as a function of growth rate. In particular, the number of ribosomes is regulated such that at any growth rate it is just adequate to sustain the corresponding rate of protein synthesis (19).

The growth rate can be varied by utilizing different carbon sources or by adding precursors for macromolecular synthesis to the medium. However, the significance of changes in cell composition during shifts between qualitatively different media is difficult to evaluate. This is particularly true for shifts from one carbon source to another and for downshifts from rich to minimal media. In these cases the cell needs to change its pattern of gene expression to be able to utilize the new carbon source or to synthesize a number of precursors. The chemostat has been widely used to distinguish which regulatory changes are exclusively a function of growth rate and which are attributable to variation in the nutritional richness of the medium (21). In the chemostat, rapid sampling, labeling, and shifts between different growth rates are, however, technically very difficult. We have therefore examined a system that allows changes in growth rate without a simultaneous qualitative change in the composition of the medium. The non-metabolizable analogue α-methylglucoside (αMG) is a competitive inhibitor of glucose uptake in *Escherichia coli* (12) and can be utilized to limit the rate of growth. The principle in this system is thus to limit the rate of entry of carbon source into the cell, in contrast to limiting the rate with which it is supplied to the medium in the chemostat.

The mechanism underlying regulation of ribosomal synthesis is at present poorly understood. Cashel and Gallant (5) first proposed the nucleotides guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) as effectors limiting the rate of synthesis of ribosomes. Results consistent with this hypothesis (6, 17, 26, 27) as well as some apparently inconsistent (2, 13, 18, 20) have been reported. In the experiments reported in this paper we limited the transport of glucose with αMG and examined the pools of ppGpp and the rate of accumulation of stable ribonucleic acid (RNA) during shifts in growth rate.

MATERIALS AND METHODS

Strain and growth conditions. Strains NF 541 and NF 542 were prepared by co-transduction of the *relA1* and *fuc*$^+$ markers into a *leu*$^-$, *pyrB*$^-$ derivative of the *E. coli* B strain AS 19 (24). Among the transductants, NF 541 (AS 19, *leu*$^-$, *pyrB*$^-$, *fuc*$^+$,

rel$^+$) and NF 542 (AS 19, *leu*$^-$, *pyrB*$^-$, *fuc*$^+$, *rel*$^-$) were selected. Strain AS 19 does not form measurable amounts of pppGpp.

Cultures were grown in tris(hydroxymethyl)aminomethane minimal medium (31) with a phosphate concentration of 1 mM. Glucose, αMG, and the αMG/glucose ratios are expressed on a weight-to-weight basis. Leucine (20 μg/ml) and uracil (20 μg/ml) were supplied when required. The amino acid mixture used in one experiment contained alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, methionine, phenylalanine, proline, serine, tryptophan, and valine, all at 50 μg/ml final concentration, and cysteine at 10 μg/ml. Turbidity was followed at 450 nm on a Beckman DB spectrophotometer.

Pool measurements. Cultures were labeled with ^{32}P (about 150 μCi/μmol) for one doubling before sampling started. Samples (100 μl) were taken into ice-cold tubes containing 10 μl of 13 M formic acid. Fractions (20 to 40 μl) were applied to polyethyleneimine-cellulose thin-layer plates (J.T. Baker). The plates were developed in 1.5 M KH_2PO_4, and the ppGpp, guanosine triphosphate (GTP), and adenosine triphosphate (ATP) locations were determined by radioautography as described by Fiil et al. (6) before the radioactivity was counted.

To determine the labeling kinetics of the GTP, ATP, and ppGpp pools with [^{3}H]guanosine, this radioisotope was given to cultures that had been labeled for one generation with ^{32}P (30 μCi/μmol). Samples were applied to polyethyleneimine-cellulose plates, which were run up twice with water and once with 21% (wt/wt) ammonium formate, 4.4% (wt/wt) boric acid at pH 7.0 in the first dimension and with 1.5 M KH_2PO_4 in the second dimension. The radiolabel was extracted from the plates with NH_4OH as described by Fiil et al. (6) before counting in Aquasol (New England Nuclear Corp.). All pool sizes are expressed as picomoles per absorbancy unit at 450 nm (A_{450}), i.e., per 1 ml of culture at 1.000 optical density unit at 450 nm.

The [^{14}C]leucine and [^{3}H]uracil incorporated into trichloroacetic acid-precipitable material was determined by filtration on membrane filters (4).

RESULTS

Limitation of growth rate by α-MG. The growth rate of *E. coli* in minimal medium with glucose as carbon source could be decreased by the non-metabolizable analogue αMG. The ratio between αMG and glucose, rather than the absolute concentration of the analogue, determined the growth rate (Fig. 1). The reduction of growth rate, as measured turbidimetrically, took effect immediately upon addition of the analogue. At extremely high αMG/glucose ratios, there was an initial period of very slow growth before the eventual steady state was established (Fig. 1). Even at these high ratios, the steady-state growth rate did not fall below approximately 0.5 doublings/h. The steady state of growth has been maintained for up to 30 doublings. When excess glucose was supplied shortly after addition of αMG, the original fast growth rate was restored instantaneously. Excess glucose caused a gradual return to the fast growth rate when supplied to cultures grown in the presence of αMG for several generations. In cultures grown with glycerol as carbon source, αMG had no effect on the growth rate.

αMG thus allowed the choice of growth rate and rapid shifts between them, whereas the source of carbon and energy, glucose, remained unchanged.

Change in cell composition in response to αMG. In terms of macromolecular composition, the RNA/protein ratio changes dramatically with growth rate of cells in different media (19). The linear relationship between growth rate and RNA/protein was identical whether growth rate was varied by the richness of the medium or by changing the αMG/glucose ratio (data not shown).

The rates of accumulation of RNA and pro-

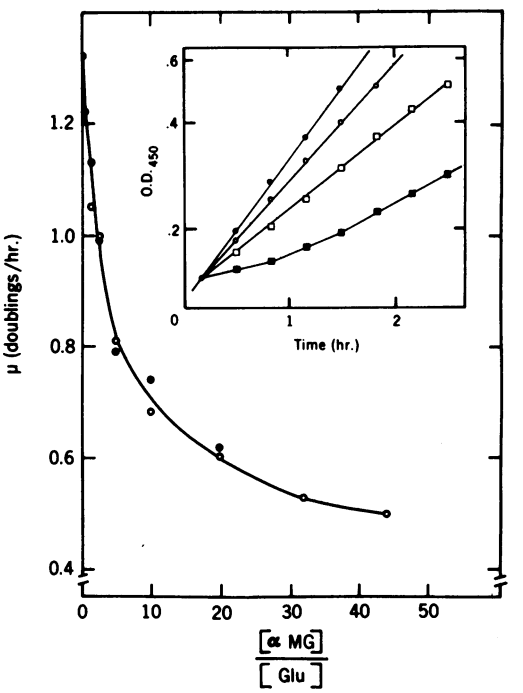

FIG. 1. *Growth rate as a function of the ratio αMG to glucose. Steady-state rates of growth are plotted for two different glucose concentrations as a function of the αMG/glucose ratio. Symbols: ○, 0.02% glucose; ●, 0.10% glucose. Insert shows the growth curves after addition of αMG at 10 min to a culture growing on 0.10% glucose. Symbols: ●, Control; ○, 0.12% αMG; □, 0.50% αMG; ■, 2.2% αMG.*

tein responded immediately when αMG was added to a culture growing at the high, unrestricted rate characteristic for glucose as carbon source. RNA accumulation in the stringent (*rel*$^+$) strain NF 541 was immediately reduced to an undetectable level (Fig. 2). After this cessation there was a very slow and gradual increase in the rate of RNA accumulation. The rate of protein synthesis was adjusted to correspond to the new growth rate immediately after addition of αMG. Thus, initially the rate of protein synthesis was not reduced nearly as drastically as the rate of RNA accumulation. In this way the RNA/protein ratio adjusted to the value characteristic of the new, lower growth rate after the downshift.

The kinetics of RNA accumulation in the relaxed (*rel*$^-$) derivative NF 542 were only slightly different. Although immediately reduced to about 20% of the preshift rate, RNA accumulation was detectable over the first 10 min after addition of αMG (Fig. 2). At this time the rate of accumulation for both strains reached approximately 2 to 5% of the preshift rate and at all times thereafter remained identical in stringent and relaxed strains. The protein synthesis throughout the shift was independent of the state of the *rel* gene.

The instantaneous rate of RNA synthesis was determined from pulse incorporation of [^{3}H]guanosine into trichloroacetic acid-precipitable material and the corresponding specific activities of the GTP and ATP pools (32). The results indicate that within 5 min of the addition of αMG the rate of RNA synthesis was reduced to approximately 30 to 40% of the preshift value in the stringent strain and to 35 to 45% in the relaxed strain. This rate changed little during the first hour after the downshift.

Accumulation of ppGpp during downshift. The nucleotide ppGpp accumulated rapidly in response to αMG in the stringent strain NF 541 (Fig. 3). The maximal concentration reached 2 min after the onset of the downshift was about 10-fold the basal level of ppGpp in the undisturbed culture growing on glucose. The behavior of the ppGpp pool in the relaxed derivative was strikingly dissimilar. The pool expanded very slowly over the first 30 min after addition of αMG. At times later than 30 min the concentration of ppGpp was relatively constant and the difference between stringent and relaxed strains was minimal (Fig. 3).

The observed rate of ppGpp accumulation, dP/dt, is the difference between its rate of synthesis [$(dP/dt)_{syn}$] and the rate of degrada-

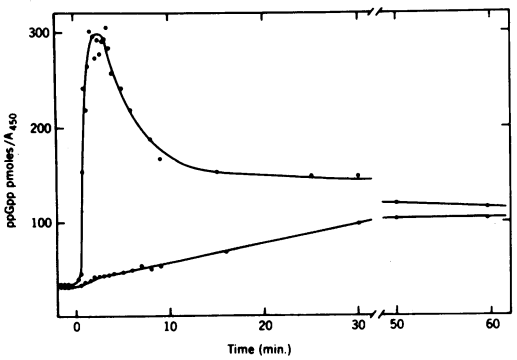

FIG. 3. *Accumulation of ppGpp during αMG-induced downshift. At time zero 2.6% αMG was added to a culture growing on 0.1% glucose. Symbols:* ●, *NF 541 (rel*$^+$*);* ○, *NF 542 (rel*$^-$*).*

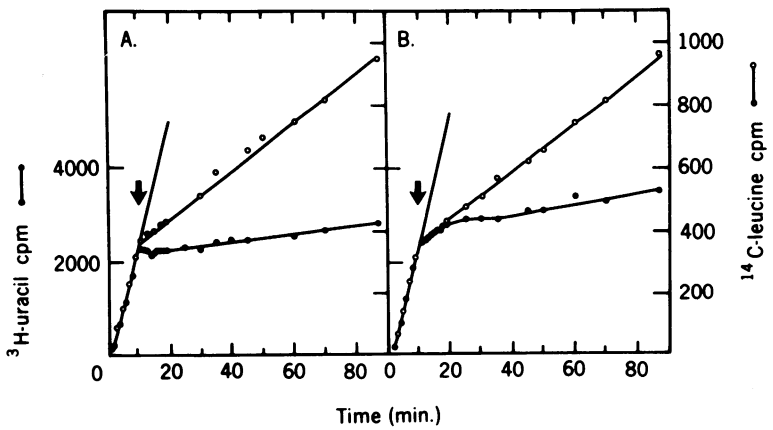

FIG. 2. *Accumulation of RNA and protein during downshift. The cultures were labeled with [*^{14}*C]leucine and [*^{3}*H]uracil at time zero. At 10 min (arrow), 2.6% αMG was added to give an αMG/glucose ratio of 26. Symbols:* ●, [^{3}*H]uracil;* ○, [^{14}*C]leucine; (A) NF 541 (rel*$^+$*); (B) NF 542 (rel*$^-$*).*

tion [$(dP/dt)_{deg}$]. To establish whether the slow accumulation of ppGpp during an αMG-imposed downshift of the relaxed NF 542 could be due entirely to inhibition of ppGpp degradation, $(dP/dt)_{syn}$ was determined in steady states of growth before the shift and at 60 min after the shift, when the ppGpp pool size was virtually constant. $(dP/dt)_{syn}$ was calculated from the kinetics with which [^{3}H]guanosine enters the GTP and the ppGpp pool of the cell (6). This calculation is based on the assumption that GTP is the precursor of ppGpp (6). In Table 1 the results are summarized and compared with the initial rate of ppGpp accumulation, dP/dt. For the relaxed strain NF 542, the rate of ppGpp synthesis in glucose-grown cells was 0.12 pmol/s × A_{450}. The rate of ppGpp accumulation, dP/dt, did not exceed 0.042 pmol/s × A_{450} during the downshift. Therefore no stimulation of ppGpp synthesis in response to the downshift would be required to account for the accumulation. Even a partial inhibition of ppGpp degradation would allow the observed accumulation.

Reversal of downshift conditions. Excess glucose caused a reversal of the slow growth rate imposed by αMG. Within 2 min the rate of RNA accumulation was restored, in both stringent and relaxed strains, to the value characteristic of glucose-grown cells (Fig. 4A). The αMG-limited growth rate could also be increased by enrichment of the medium with amino acids, and the effect on RNA accumulation was very similar to that exerted by glucose addition (Fig. 4A).

In the stringent NF 541, the ppGpp pool dropped rapidly upon addition of excess glucose. In NF 542, on the other hand, the ppGpp pool expanded during the first 4 min concomitant with restoration of rapid RNA accumulation (Fig. 4B). This response to excess glucose, a simultaneous increase in the size of the ppGpp pool and in the rate of RNA accumulation, was most marked during the initial period after the downshift. Glucose added to a culture grown for 60 min in the presence of αMG produced a barely detectable expansion of the ppGpp pool. The amino acid mixture caused a rapid shrinking of the ppGpp pool at all times in the relaxed NF 542 (Fig. 4B).

DISCUSSION

Molecular basis for the αMG-caused limitation of growth rate. More than a decade ago (12), αMG was shown to be a competitive inhibitor of glucose uptake in *E. coli*. Both glucose and the analogue are now known to be phosphorylated and transported into the cell by the phosphotransferase system (15). The same substrate-specific enzyme II of this system recognizes glucose and αMG (14). A second enzyme II capable of transporting glucose, mannose enzyme II, has been found by Adler and Epstein (1). Mannose enzyme II is incapable of transporting αMG, and its transport of glucose is not inhibited by the analogue (1). Further, the amount of mannose enzyme II is low in glucose-grown cells, presumably owing to repression of its synthesis under these conditions (1).

When glucose was used as source of carbon and energy, αMG also acted as a competitive inhibitor of growth (Fig. 1). At high ratios of αMG to glucose, the initial rate of growth was very low but accelerated to about 0.5 doublings/h. A possible explanation for these kinetics is the derepression of mannose enzyme II synthesis after addition of αMG. The mannose enzyme II activity would explain why the residual growth rate cannot be decreased below 0.5 doublings/h with αMG.

TABLE 1. *Rate of ppGpp synthesis*[a]

Strain	Time (min)	P (pmol/A_{450})	dP/dt pmol/s × A_{450}	$(dP/dt)_{syn}$ pmol/s × A_{450}	$(dP/dt)_{syn}/P$ (min^{-1})
NF 541 (*rel*$^+$)	−5	35	0	0.18	0.31
	0.5	45	6.0		
	60	116	0	1.0	0.56
NF 542 (*rel*$^-$)	−5	34	0	0.12	0.22
	2	40	0.042		
	60	104	0	0.14	0.08

[a] Abbreviations: P, ppGpp pool size; dP/dt, net rate of change in ppGpp pool; $(dP/dt)_{syn}$, rate of ppGpp synthesis; $(dP/dt)_{syn}/P$, relative turnover rate of ppGpp pool. When P is constant, $(dP/dt)_{syn} = [(dS_{ppGpp}/dt)/(S_{GTP} - S_{ppGpp})] \times P$, where S_{GTP} and S_{ppGpp} are the specific activities of GTP and ppGpp pools, respectively (6).

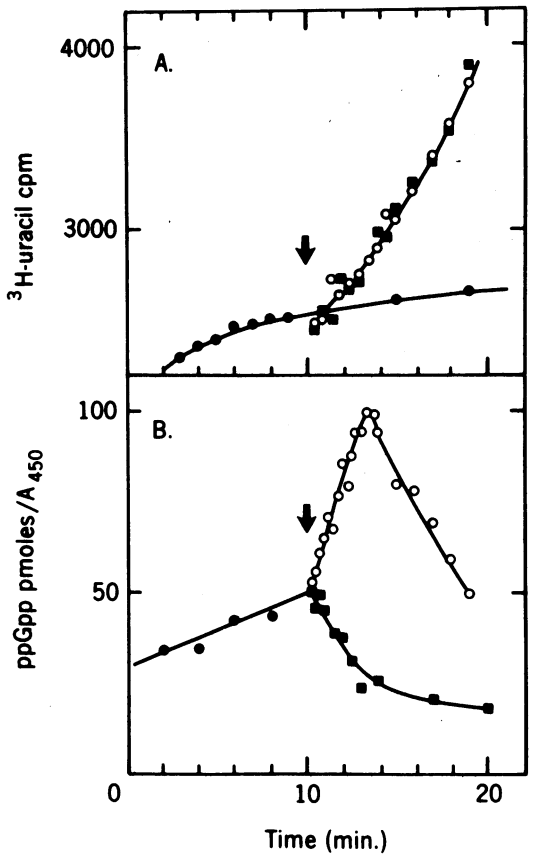

FIG. 4. *Reversal of αMG-induced downshift. A culture of NF 542 (rel⁻) was labeled with [³H]uracil at −10 min, and at time zero αMG was added to give an αMG/glucose ratio of 26. At 10 min glucose was added to give an αMG/glucose ratio of 1 in part of the culture* (○), *while another part received an amino acid mixture* (■). *The incorporation of [³H]uracil into trichloroacetic acid-precipitable material is shown in (A). Variations in the ppGpp pool size of a parallel ³²P-labeled culture are given in (B).*

αMG is phosphorylated and transported into the cell but cannot be further metabolized by the glycolytic enzymes and therefore does not support growth of *E. coli* (8). Once in the cell, αMG-6-phosphate can be dephosphorylated and an equilibrium with external αMG is established (29). The possibility that the cellular pool of αMG-6-phosphate has some kind of poisoning effect on growth cannot be entirely eliminated. It is rendered highly unlikely, however, since the inhibitory effect of αMG is very specific. RNA accumulation is preferentially reduced relative to protein synthesis, and the RNA/protein ratio is adjusted to show the same linear relationship with growth rate found for cultures grown in different media (5) and for the transport mutant CP 367 (28). Also, the rate of deoxyribonucleic acid synthesis is not affected at all initially after addition of αMG (unpublished observation). Finally, αMG has no effect on the growth of cells utilizing glycerol as carbon source or on fructose-grown cells even though the cellular pool of αMG-6-phosphate in this case reaches the same value (5 nmol/A_{450}) as in glucose-grown cells.

We therefore feel that the inhibitory effect of αMG on growth is due primarily, if not solely, to its inhibition of glucose transport into the cell.

Rate of RNA accumulation in relation to ppGpp pool size. pp Gpp was first proposed as an effector for control of RNA synthesis by Cashel and Gallant (5). In cells where growth is limited by different degrees of charging of transfer RNA, the rate of RNA accumulation correlates with the size of the ppGpp pool (6). A direct correlation between the basal levels of ppGpp and the RNA content of cells grown in different media with different growth rates has been reported (17). A more recent study, however (13), did not confirm this conclusion. The effect of shifts between different carbon sources on regulation of RNA synthesis and ppGpp accumulation has also been studied, since such regulation may differ from that observed with amino acid-starved cells (9, 17, 30). The effect of ppGpp on RNA synthesis in vitro is not well agreed upon either. ppGpp has been reported to inhibit specifically ribosomal RNA synthesis (26, 27) or to inhibit indiscriminately the synthesis of all classes of RNA (2, 18, 20). Thus the question of whether there is a causal, or even an obligatory, relation between the size of the ppGpp pool and the cell's ability to synthesize and accumulate stable RNA has found no conclusive answer.

Rates of accumulation and synthesis of RNA and the ppGpp pool sizes have been measured in stringent and relaxed strains traversing a shift from growth on glucose to growth on lactate (30). This shift and similar ones (9, 11, 17) are complicated by their diauxic nature where adaptation to utilize the new substrate is gradual. Equally important, in these experiments the precise time of the total consumption of glucose is not accurately determined and timing of the immediate cell response to the downshift condition is difficult. The αMG-induced downshift overcomes these two major complications. With this improved system, no obligatory correlation between the size of the ppGpp pool and the rate of RNA accumulation is found (Fig. 2–4); e.g., 10 min after addition of αMG to stringent and relaxed strains there is a threefold difference in the size of their ppGpp

pools, yet their rates of RNA accumulation are identical (Fig. 2, 3). Perhaps even more striking is the reversal of downshift conditions (Fig. 4) where the relaxed strain with an up to fourfold difference in its ppGpp pool shows the same rate of RNA accumulation.

The decreased rate of accumulation of RNA after downshift can be explained by a decrease in the rate of synthesis and/or an increase in the rate of degradation of normally stable RNA. The possibility that ppGpp regulates the rate of stable RNA synthesis, but that degradation of this class of RNA can be activated independent of the ppGpp pool size, remains open. Indeed, from the instantaneous rates of RNA synthesis (30 to 45% of preshift rate), protein synthesis (25%), and RNA accumulation (2 to 5%) after the shift, either turnover of ribosomal RNA or decreased translation efficiency for messenger RNA is indicated. If one assumed an unaltered translation efficiency after the downshift, the rate of messenger RNA synthesis, which constitutes approximately 60% of the instantaneous rate of RNA synthesis in the preshift condition (20), would be reduced to 15% (0.25 × 60%) of the total preshift rate. This 15%, together with the rate of RNA accumulation, 2 to 5%, cannot account for the rate of RNA synthesis observed. A measure of the instantaneous rate of ribosomal RNA synthesis will distinguish between decreased translation efficiency and turnover of newly synthesized ribosomal RNA.

The rate of accumulation of ppGpp in the relaxed strain in response to αMG is consistent with an inhibition of ppGpp degradation without interference with the rate of its synthesis (Table 1). It is not possible to completely rule out an increase in the rate of ppGpp synthesis in the initial period after the shift. During growth on glucose, the rate of ppGpp synthesis, $(dP/dt)_{syn}$, appears to be somewhat lower in the relaxed derivative than in the stringent (Table 1), and the same basal level of ppGpp in both strains seems to be maintained by a correspondingly decreased rate of ppGpp degradation in the relaxed derivative. The maximal in vivo capacity of synthesis of ppGpp with the product of the *relA1* gene might be very low, and it is not surprising that extracts from this particular mutant showed no significant stimulation of ppGpp synthesis in vitro (3).

The initial stimulation of ppGpp synthesis upon readdition of glucose to an αMG downshifted culture of the relaxed NF 542 is not yet understood. The only similar situation reported is in stringent *spoT*$^-$ strains, where degradation of ppGpp is slow on reversal of amino acid starvation (16). Even in that case no actual stimulation of ppGpp synthesis is seen, but only a slow decay. Most important, whereas the slow decrease in size of the ppGpp pool in a *rel*$^+$ *spoT*$^-$ delays the resumption of RNA accumulation (25), no trace of such delay is seen in the relaxed strain on readdition of glucose (Fig. 4).

To reach a better understanding of the role of ppGpp in regulation of stable RNA metabolism, it will be necessary to examine carefully the effects of ppGpp on both the synthesis and the degradation of stable RNA.

ACKNOWLEDGMENTS

This work was supported by a fellowship from the Danish Natural Science Research Council awarded to M.T.H. and by Public Health Service grant AI-11075 from the National Institute of Allergy and Infectious Diseases to M.L.P.

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