The Control of Ribonucleic Acid Synthesis in Escherichia coli

III. THE FUNCTIONAL RELATIONSHIP BETWEEN PURINE RIBONUCLEOSIDE TRIPHOSPHATE POOL SIZES AND THE RATE OF RIBONUCLEIC ACID ACCUMULATION*

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SUMMARY

We have examined the functional relationship in vivo between purine ribonucleoside triphosphate pool size and the rate of RNA accumulation. Our findings are as follows.

The imposition of stringency leads to a roughly 40% drop in the GTP pool which occurs very quickly; it is complete in less than 10 min, and occurs simultaneously with, or just before, the shutdown of RNA accumulation. A somewhat slower 30% drop in the ATP pool was also observed. In a relaxed mutant, there is a momentary shrinkage of both pools, but they quickly return to normal and thereafter expand.

The shrinkage of the ATP and GTP pools during stringency cannot be accounted for as a feedback response to blockade of RNA accumulation; when RNA accumulation is blocked directly by uracil starvation, the ATP and GTP pools expand rather than contract.

Shrinkage of the purine ribonucleoside triphosphate pools may be sufficient to account for the reduced rate of RNA accumulation during stringency. When the synthesis of ATP and GTP is blocked directly by purine starvation, the rate of RNA accumulation falls drastically with only about a 40% shrinkage of the two pools. Thus net RNA synthesis in vivo is much more sensitive to purine ribonucleoside triphosphate concentration than is RNA polymerase activity in vitro.

Shrinkage of the GTP pool alone may be sufficient to account for the response of RNA accumulation to stringency. When GTP synthesis is blocked directly by guanine or guanosine starvation, a moderate decrease in the GTP pool results in a disproportionate reduction in net RNA synthesis.

Despite virtually complete inhibition of net RNA synthesis during guanosine starvation, protein synthesis proceeds at about 25% of the normal rate. This suggests that the synthesis and turnover of messenger RNA is much less affected by a moderate decrease in GTP than is the accumulation of stable forms of RNA.

RNA accumulation in wild type strains of Escherichia coli is sharply reduced by amino acid starvation (1-4). This control

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1 The terminology for the RC locus is that employed in Reference 4 and that for MS nucleotides is defined in Reference 15.
eralised reduction in energy metabolism whose effects are not mimicked by specific inhibition of the RNA polymerase reaction. The simplest interpretation appears to be that stringent control operates somewhere in energy metabolism, and that stringent control of the synthesis of various phosphorylated metabolites, including RNA, is in each case a response to a primary effect on energy metabolism. Our next step was, therefore, to examine the effect of stringency on the pool sizes of the purine ribonucleoside triphosphates, which are the major repositories of high energy phosphate; and to determine the functional relationship in vivo between these pool sizes and the rate of net RNA synthesis. This is the subject of the present communication.

Edlin and Neuhard (8) have previously observed that amino acid starvation led to a significant decrease in both purine pools in a stringent strain, but not in an isogenic relaxed derivative. This finding appears grossly consistent with our hypothesis. However, their experiments left unresolved the functional relationship between triphosphate pool shrinkage and stringent control of RNA accumulation: which is cause and which is effect. They also concluded that pool shrinkage occurred gradually, which would imply that it is an indirect effect of the quite rapid stringent response of RNA accumulation. We have accordingly re-examined this question, employing more frequent sampling and an improved method of nucleotide extraction. We find that pool shrinkage, at least in the case of GTP, certainly occurs as soon as or before the deceleration of net RNA synthesis.

As an approach to the cause and effect problem, we have asked two questions. (a) Can contraction of the ATP and GTP pools be a secondary consequence of blocked RNA synthesis? We find that when RNA synthesis is blocked by the direct agency of uracil starvation, the ATP and GTP pools expand rather than contract. This finding is inconsistent with the notion that shrinkage, at least in the case of GTP, certainly occurs as soon as or before the deceleration of net RNA synthesis. (b) Is the moderate shrinkage of the purine pools occasioned by the stringent response sufficient to account for the large decrease in the rate of RNA accumulation? We find that it could be: a similar moderate decrease in the purine ribonucleoside triphosphate pools, brought about through the direct agency of purine starvation, elicits a major reduction in the rate of net RNA synthesis. This finding is consistent with the hypothesis of substrate level control. Furthermore, when RNA accumulation is completely blocked by purine starvation, protein synthesis continues at about one-quarter of the normal rate. This finding suggests that the synthesis of messenger RNA and the accumulation of stable RNA are affected to very different extents by purine ribonucleoside triphosphate limitation.

MATERIALS AND METHODS

_E. coli_ CP78 and CP79 are an isogenic pair which are RC<sup>++</sup> and RC<sup>rel</sup>, respectively. They require histidine, leucine, threonine, arginine, and B<sub>2</sub>, and are sensitive to valine inhibition. _E. coli_ BD1 and BD6 are sibling derivatives of strain B3; BD1 requires thymine, uracil, and histidine, BD6 requires thymine, uracil, and isoleucine, and both are stringent. _E. coli_ DB429 is a stringent K-12 strain requiring adenine and thymine; _E. coli_ 156-2a is a stringent K-12 strain requiring adenine, thymine, and proline. (Both of these purine auxotrophs are blocked before IMP.) R257 is a guanine auxotroph, its RC allele is unknown. AT2465 is a derivative of Hfr H (RC<sup>++</sup>) which requires guanine and B<sub>1</sub>. We have confirmed its relaxed phenotype by showing that valine inhibits growth but does not inhibit uridine incorporation into RNA.

Cultures were grown in Tris-glucose medium at 37° (unless otherwise indicated) under forced aeration. Nutritional supplements are indicated in the legends. In all experiments, <sup>32</sup>P-phosphoric acid was added to exponentially growing cells. Samples were removed for triphosphate determination no earlier than about 0.7 generation after addition of isotope to the medium; complete equilibration of the triphosphate pools and exogenous <sup>32</sup>P<sub>4</sub> is attained within about half a generation (30 min), as judged by a constant ratio of <sup>32</sup>P in any triphosphate to bacterial density. For measurement of triphosphate levels, 100-μl samples were acidified with 20 μl of 2 N sodium formate, pH 3.4, extracted, and chromatographed as previously described (6, 9). As pointed out previously (8), our formate extraction procedure provides considerably better recovery of triphosphates and far greater reproducibility than the trichloroacetic acid extraction procedure used by Edlin and Neuhard (8). Bagnara and Finch (10) have recently recounted the deficiencies of trichloroacetic acid extraction procedure used by Edlin and Neuhard (8).

RESULTS AND DISCUSSION

**Stringent Response**—In order to obtain a unified picture of the stringent response, we have measured the levels of <sup>32</sup>P<sub>4</sub> in

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**Fig. 1.** Stringent response of CP78 to valine inhibition. To an exponential culture of CP78 growing in Tris-glucose medium containing 2 × 10<sup>-3</sup> m K<sub>3</sub>HPO<sub>4</sub>, 100 μg of arginine per ml, 200 μg of threonine per ml, 20 μg of histidine per ml, 40 μg of leucine per ml, and 1 μg of vitamin B<sub>1</sub> per ml, 10.0 μCi of <sup>32</sup>P<sub>4</sub> per ml were added. Valine (VAL) (400 μg per ml) was added at the point indicated. The _abscissa_ is time after addition of <sup>32</sup>P<sub>4</sub>; the _ordinate_ is millimicromoles of <sup>32</sup>P<sub>4</sub> found in the compounds indicated per ml.
FIG. 2. Stringent response of BD6 to isoleucine exhaustion. To an exponential culture of BD6 growing in Tris-glucose medium containing $2 \times 10^{-4}$ M K$_2$HPO$_4$, 20 $\mu$g of uracil per ml, 20 $\mu$g of thymine per ml, and 4 $\mu$g of isoleucine per ml, 13.8 $\mu$Ci of $^{32}$P$_4$ per ml were added. (In a few experiments, including this one and that of Fig. 5, the phosphate concentration was reduced to $2 \times 10^{-4}$ M in order to raise the specific activity and obtain significant radioactivity in minor nucleotides not discussed in this paper. The lower level of phosphate is still in excess with respect to bacterial growth, and in each type of experiment virtually identical results were obtained at the two phosphate concentrations.)

DNA, RNA, and the purine ribonucleoside triphosphates simultaneously in each experiment. These experiments provide a true account of the levels of the compounds measured, uncomplicated by effects on the uptake or phosphorylation of precursors, because sampling was not begun until virtually complete equilibration of the nucleotide pools with $^{32}$P$_4$ had been attained (see “Materials and Methods”). Amino acid starvation was imposed either by addition of valine, which acts as a feedback inhibitor of isoleucine biosynthesis in E. coli K-12 (12, 13) or by cultivation of an isoleucine auxotroph in a limiting supply of that amino acid. We avoided filtering the cells out of amino acid sufficient medium because filtration itself tends to perturb the ATP pool.

The response of CP78 to valine inhibition is shown in Fig. 1. Within a few minutes of valine addition, the prior conditions of steady state growth are disturbed as follows: net synthesis of ATP and GTP stops; there is a small drop in both pools which is especially abrupt in the case of GTP; RNA accumulation comes to a virtual stop; DNA synthesis proceeds, but at a slightly decreased rate, as has been observed by Friesen and Maaløe (14).

To be sure that isoleucine starvation is responsible for these effects, we have examined the behavior of an authentic isoleucine auxotroph. Fig. 2 shows the response of strain BD6 to cultivation in a limiting supply of isoleucine. The amino acid was exhausted from the medium at between 100 and 110 min, judging by the sharp break in the optical density curve. ATP, GTP, RNA, and DNA respond in much the same way as they do to valine addition in CP78.

To confirm that this pattern of events is due to the operation of stringency, we have examined the effect of valine addition on CP79, the relaxed derivative of CP78. Fig. 3 shows the result. On a gross time scale, our results are in agreement with those of Edlin and Neuhard (8). The relaxed mutant is capable of continued net synthesis of ATP, GTP, and RNA, demonstrating that the very different behavior of CP78 (and presumably BD6) is dependent on the presence of a wild type RC gene product. During the first few minutes following valine addition, however, the relaxed mutant shows a remarkable transient response, in which the purine ribonucleoside triphosphate pools drop significantly, then rapidly recover. This unusual kinetics, which has been observed in each of three experiments, might mean that the relaxed mutant is not defective in stringent control after all, but is on the contrary able to override it.

Our results differ from those of Edlin and Neuhard (8) primarily in the rapidity with which the GTP pool shrinks during the stringent response. In six experiments, three with CP78 and three with BD6, the GTP pool fell an average of 38% within a few minutes of the addition of valine or (in the case of BD6) the break in the optical density curve signalling exhaustion of isoleucine from the medium. In four of six experiments, the GTP pool dropped significantly before RNA accumulation was

$^2$ G. Edlin, personal communication.
shut off (for example, Figs. 1 and 2); in two experiments, the drop in GTP, and shutoff of net RNA synthesis appeared to be simultaneous. Shrinkage of the GTP pool is therefore certainly rapid enough to qualify as a possible cause of stringent control of RNA synthesis.

The case of the ATP pool is less clear. ATP dropped in each experiment, but with kinetics which was less reproducible, and generally more sluggish, than that shown by GTP. ATP fell by an average of 28% over a variable time period (from as little as 8 min to as much as 20 min) that spanned the shutoff of RNA accumulation. Our data are not sufficiently precise to make it clear whether or not shrinkage of the ATP pool is already under way by the time RNA accumulation shuts off. On the other hand, it is clear that net synthesis of ATP stops at least as soon as net synthesis of RNA. For reasons that will become clear in connection with the next experiment, the failure of ATP to accumulate during the stringent response reveals a significant aspect of the mechanism of stringency.

**Direct Blockade of RNA Accumulation by Uracil Starvation**—If pool shrinkage were an indirect consequence of the cessation of RNA accumulation, then the same effect should be produced when RNA accumulation is restrained by the direct agency of uracil starvation. But this is not the case as shown in Fig. 4. Uracil exhaustion is reflected in the declining rate of net RNA synthesis and the depletion of the UTP pool. (The CTP pool,

**Fig. 4.** Response of BD1 to uracil exhaustion. To an exponential culture of BD1 growing in Tris-glucose medium containing \(2 \times 10^{-5} \text{ M} \text{KHPO}_4\), 20 \(\mu\)g of thymine per ml, 20 \(\mu\)g of histidine per ml, and 4 \(\mu\)g of uracil per ml, 9.1 \(\mu\)Ci of \(^{32}\text{P}\) per ml were added. A shows \(^{32}\text{P}\) found in RNA and the four triphosphates, as in the preceding figures. Increase in turbidity is also shown (upper panel, right-hand ordinate). B, we have divided the levels of phosphate in ATP and GTP at each point by the optical density at that point, to give a value proportional to the intracellular pool size. The normal pool sizes for exponentially growing cells are indicated by the dashed lines, which were averaged from the data of two experiments (open symbols) in which uracil was present in excess (20 \(\mu\)g per ml).
FIG. 6. Response of 156-2a to adenine exhaustion. To an exponential culture of 156-2a growing in Tris-glucose medium containing $2 \times 10^{-4}$ M $K_2HPO_4$, 20 $\mu$g of thymine per ml, 20 $\mu$g of proline per ml, and 13.3 $\mu$g of adenine sulfate per ml, 61.8 $\mu$Ci of $^{32}$P$_4$ per ml were added.

FIG. 7. Response of R257 to guanosine exhaustion. To an exponential culture of R257 growing in Tris-glucose medium containing $2 \times 10^{-3}$ M $K_2HPO_4$ and 7.1 $\mu$g of guanosine per ml, 31.8 $\mu$Ci of $^{32}$P$_4$ per ml were added.

Fig. 6. Response of 156-2a to adenine exhaustion. To an exponential culture of 156-2a growing in Tris-glucose medium containing $2 \times 10^{-4}$ M $K_2HPO_4$, 20 $\mu$g of thymine per ml, 20 $\mu$g of proline per ml, and 13.3 $\mu$g of adenine sulfate per ml, 61.8 $\mu$Ci of $^{32}$P$_4$ per ml were added.

Surprisingly, is not depleted by uracil starvation, presumably (the decrease in net synthesis of RNA compensates for decreased CTP formation.) The ATP and GTP pools, far from contracting as they do during the stringent response, instead expand to several times their normal sizes (Fig. 4B). Thus, the contraction of these pools occasioned by stringency cannot be accounted for as a consequence of a decreased rate of RNA accumulation, which leads instead to the opposite result. The large expansion of which these pools are capable shows that the pathways of ATP and GTP biosynthesis are not tightly feedback-inhibited at normal pool sizes. The ATP and GTP pools are expanded in response to purine starvation. The ATP pool drops by a factor of eight, which, assuming a cellular volume of a cubic micron, means an intra-

Direct Blockade of Purine Nucleotide Synthesis by Purine Starvation—If pool contraction cannot be a consequence of the decreased rate of RNA accumulation associated with stringency, could it be, on the contrary, the cause? Naively, this would seem unlikely, because the pools drop only by 30 or 40%, and most studies of RNA polymerase activity in vitro give no indication of cooperative substrate kinetics. Furthermore, the intracellular concentrations of ATP and GTP (assuming a cellular volume of a cubic micron, our data specify values of about $6.3 \times 10^{-6}$ M and $1.3 \times 10^{-4}$ M, respectively) are much higher than the $K_m$ values that have been reported for RNA polymerase.

However, RNA polymerase reaction mixtures are not cells. We have therefore sought to determine the functional relationship between purine nucleoside triphosphate pool size and the rate of RNA accumulation in vitro. We did so by limiting ATP and GTP synthesis through the direct agency of purine starvation. The results with two different purine auxotrophs are shown in Figs. 5 and 6. Exhaustion of adenine from the medium greatly decreases the rate of RNA accumulation, but the purine pools decrease only moderately and stabilize at a level which would be nearly saturating in vitro.

Purine starvation results in a decrease in both the ATP and GTP pools. It is thus not clear from these experiments whether it is the level of ATP or of GTP or of both which critically determines the rate of net RNA synthesis. Because it is the GTP pool which shrinks most rapidly in response to stringency, we were particularly interested in the functional relationship between the size of this pool and the rate of net RNA synthesis. Accordingly, we examined the behavior of guanine-less mutants in which the synthesis of adenine nucleotides is impaired.

As A similar expansion of the purine ribonucleoside triphosphate pools has been observed in a different uracil auxotroph by R. A. Lazarini, K. Nakata, and R. M. Winslow (personal communication).
cellular GTP concentration of about $1.6 \times 10^{-4}$ M. This is about the same as the highest $K_m$ values that have been reported for RNA polymerase (and well above the lowest values), but it is clear that net synthesis of RNA is very severely inhibited. The severe effect of guanosine starvation on RNA synthesis cannot be due to the operation of stringency itself, triggered accidentally by some consequence of the reduced GTP level. We find that guanine starvation of a relaxed guanine-less mutant (AT 2465, derived from Hfr H) elicits a similar shutoff of net RNA synthesis with only a 4-fold decrease in GTP (Fig. 8). Thus, net synthesis of RNA in vivo is exceptionally sensitive to the level of GTP alone.

The stringent response is strikingly mimicked by purine starvation in two other respects. First, stringency results in a small reduction in the rate of DNA synthesis (Figs. 1 and 2). A similar small reduction in the rate of DNA synthesis is observed during adenine or guanosine starvation (Figs. 5 to 7). This continued DNA synthesis, which may proceed on the basis of RNA breakdown, continues for about one doubling and then stops.

Second, there is reason to believe that stringency and purine starvation both affect the synthesis of different species of RNA non-coordinately. Although there have been remarkably contradictory reports on this question in the case of stringency (reviewed by Edlin and Broda (15)), recent experiments by Nierlich (16) and Kjeldgaard and Forchhammer (17) argue convincingly that the synthesis of messenger RNA is at least much less severely inhibited by stringency than is the synthesis of stable RNA. During purine starvation, the continued synthesis of protein at an appreciable rate strongly suggests that messenger RNA synthesis and turnover are much less severely inhibited than the synthesis of stable species of RNA. Fig. 9 shows the effects of guanosine starvation on protein and RNA synthesis. Guanosine was exhausted between 135 and 150 min, producing an 8-fold drop in the level of GTP. After this time, uridine incorporation into RNA was severely inhibited, as shown in the lower panel. But protein synthesis, as reflected both in turbidity increase and the incorporation of tryptophan into trichloracetic acid-precipitable (TCA precipitable) material, was reduced by only about a factor of 4. Similar results have been obtained in the case of adenine starvation. Unless purine starvation results in the stabilization of pre-existing messenger RNA, which we have no reason to expect, this continued protein synthesis must reflect the translation of messenger RNA molecules whose synthesis is proceeding at at least one-quarter of the normal rate.

**CONCLUSIONS**

The experiments described in the first section of "Results and Discussion" demonstrate that the stringent response brings...
about an abrupt shrinkage of the GTP pool, and a somewhat slower shrinkage of the ATP pool. The experiments described in the next two sections consider the logical relationship between this aspect of the stringent response and the shutoff of RNA accumulation. Direct blockade of RNA accumulation by uracil starvation leads to a large expansion of the GTP and ATP pools. Thus, the absence of such pool expansion during the stringent response demonstrates that the synthesis of GTP and ATP is inhibited. This can scarcely be due to feedback inhibition of the purine nucleotide pathways by their end products (16), because both end products decrease, rather than increase, in level. Nor can it be due to a hypothetical feedback response to the rate of RNA accumulation (8), because uracil starvation blocks RNA accumulation while allowing the purine ribonucleoside triphosphate pools to expand. We are therefore led to conclude that the stringent control system plays a direct role in regulating purine nucleotide biosynthesis.

We have previously proposed a mechanism by which the stringent control system could play such a role (6, 18). We postulated that some reaction normally involved in protein synthesis “idles” in the absence of one or more species of aminoacylated transfer RNA; and that this idling reaction, carried out by the RC gene product, generates an inhibitor of ATP synthesis. The synthesis of the MS nucleotides has been shown to be specific to the stringent response (18), and these nucleotides show the kinetic properties to be expected of the postulated inhibitor or inhibitors (10). Experiments to determine whether the MS nucleotides are in fact regulators of purine nucleotide metabolism are in progress.

We might note that the behavior of the pyrimidine ribonucleoside triphosphate pools seems to depend on the manner in which stringency is imposed. We have measured the effect of isoleucine starvation on these pools in several experiments, and found little if any decrease. This is to be contrasted with the decrease in pyrimidine ribonucleoside triphosphates observed in the case of arginine starvation (8) and histidine starvation (6). We suspect that in these latter instances, the operation of stringency is complicated by a metabolic drain that may arise when an auxotroph is starved for an amino acid whose biosynthetic pathway consumes high energy phosphate. Arginine and histidine are such amino acids, as are proline, threonine, lysine, and the aromatic amino acids.

No such consideration applies to isoleucine starvation, since high energy phosphate is not consumed in the isoleucine pathway. Furthermore, valine inhibits the second enzyme of the pathway (12, 13) and our isoleucine auxotroph is defective in threonine deaminase, the first enzyme of the pathway. Thus we impose stringency under conditions where the isoleucine pathway is essentially inoperative, and consequently there can be no metabolic drain of precursor material. For this reason, the shrinkage of the purine ribonucleoside triphosphate pools should reflect only the operation of stringency. In addition, we have observed very similar decreases in GTP and ATP when valine activation is blocked at high temperature in a mutant (NP29, see Neidhardt (20)) with a thermosensitive activating enzyme. Edlin and Broda (15) have obtained similar results with the same mutant. This method of imposing stringency, like isoleucine starvation, should impose no metabolic drain.

Our purine starvation experiments show that net synthesis of RNA in vivo is extraordinarily sensitive to the pool sizes of the purine ribonucleoside triphosphates. This finding raises the possibility that purine pool shrinkage, especially the abrupt shrinkage of the GTP pool, is partly or wholly responsible for the reduced rate of RNA accumulation during the stringent response. It further raises the possibility that “growth rate” control of RNA synthesis, which is independent of stringent control (22), reflects nothing more mysterious than the levels of purine ribonucleoside triphosphates. Now that adequate methods have been developed for the rapid extraction and quantitation of nucleotides, this question can be investigated.

The unusually critical relationship between purine ribonucleoside triphosphate concentration and the rate of RNA accumulation is entirely different from the substrate dependence of RNA polymerase activity in vitro. The reason for this difference is not known, but may be worth speculating upon briefly.

One obvious possibility, compartmentalization of the triphosphate pools, is unattractive because kinetic studies have shown that GTP behaves as a single homogeneous pool in its labeling by exogenous guanine and its equilibration with the products of messenger RNA breakdown (23).

Furthermore, the persistence of protein synthesis at a substantial rate in the absence of RNA accumulation, in purine-starved cells, suggests that purine ribonucleoside triphosphate limitation affects messenger RNA synthesis and the accumulation of stable species of RNA in very different ways. A direct substrate effect on RNA polymerase activity in vivo would not produce such discriminate effects if a single polymerase were responsible for the synthesis of all types of RNA, as is evidently the case (24).

Substrate level control of initiation, however, could well differ from one RNA species to the next, depending on the properties of individual promoters. Thus, one possibility is that one or both of the purine ribonucleoside triphosphates exercise substrate level control over the initiation of transcription for ribosomal and transfer RNA molecules, which account for nearly all of the RNA accumulation measured in our experiments, and conceivably some but not all messengers. By substrate level control, we mean nothing more than initiation substrate kinetics which is cooperative and has dissociation constants much higher than the apparent K_m for RNA chain growth. Anthony, Zeszotek, and Goldthwaite (25) have reported suggestive evidence for a relationship of this kind in the formation of the initiation complex in vitro.

An alternative hypothesis is that purine nucleotides regulate RNA accumulation at the level of stabilization, rather than initiation of synthesis. The mechanism of such a control is not clear, but several possibilities can be imagined. One (suggested by Dr. R. Lazzarini) is that shrinkage of the purine ribonucleoside triphosphate pools produces a moderate coordinate decrease

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6 Unpublished experiments.

8 H. Erlich, personal communication.

4 We are grateful to Dr. Fred Neidhardt for bringing this point to our attention.

7 In another stringent strain carrying a different temperature-sensitive lesion in the valine-activating enzyme, contradictory results have been obtained. Edlin and Broda (15) reported a moderate but rapid decline in the GTP pool upon transfer from 30-42°, the kinetics of which resemble our results on valine inhibition. More recently, however, Edlin and Stent (21) report that transfer of this mutant from 30-42° produces no change in the GTP pool. The reason for the discrepancy between these two reports is not clear.
in the rate of RNA chain growth, and that slow chain growth renders nascent ribosomal and transfer RNA, ordinarily stable, vulnerable to breakdown.

Our results are consistent with either of these general hypotheses. Experiments designed to distinguish between them are in progress.

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