JOURNAL OF BACTERIOLOGY, May 1972, p. 554-561
Vol. 110, No. 2

Control of Nucleotide Metabolism and Ribosomal Ribonucleic Acid Synthesis During Nitrogen Starvation of *Escherichia coli*

JOSEPH D. IRR

Department of Biology, Marquette University, Milwaukee, Wisconsin 53233

Received for publication 14 January 1972

Ribosomal ribonucleic acid (RNA) synthesis and ribonucleoside triphosphate metabolism were studied in cultures of *Escherichia coli* subjected to starvation for inorganic nitrogen. In a strain that was under stringent control, a 50-fold reduction in the formation of both 16*S* and 23*S* RNA was accompanied by a severe restriction on nucleotide biosynthesis. These inhibitions were relieved in part by incubating the starved cells with amino acids. This result suggests that regulation by the functional RNA control (RC) gene is involved in the effect. This suggestion was confirmed by showing that the effector of the stringent response, guanosine-5'-diphosphate-2'- or 3'-diphosphate ($_{pp}G_{pp}$), accumulated at the onset of starvation and disappeared immediately when the amino acids were added. Ribosomal RNA synthesis was severely restricted and the same nucleotide, $_{pp}G_{pp}$, accumulated at the onset of nitrogen starvation of a relaxed mutant too. These findings suggest that a control mechanism other than the one provided by the functional *rel* gene might operate to regulate RNA synthesis and that this mechanism is expressed through the synthesis of $_{pp}G_{pp}$.

Bacteria are usually starved by the removal of an essential nutrient from the culture medium. As a result, the culture more or less ceases to grow and enters the resting state. Whether the cells remain physiologically "balanced" or "unbalanced" once starvation starts will depend upon the nature of the limiting nutrient (7) and the genetic constitution of the organism (1). There have been numerous studies on changes in the physiological state of cells following the onset of starvation. A frequent observation is that net protein synthesis is reduced to a minimum, but select enzymes are preferentially formed even when there is some degradation of existing proteins. As an example, Klein (13) studied resting cultures of *Pseudomonas saccharophila* and found preferential synthesis of α-amylase in cells incubated with starch as a carbon source. Klein then suggested that there exists in all resting cells a generalized repressor which affects the transcription of most genes except those responding to the presence of high levels of their specific inducers. He went on to propose that the only direct imposition on protein synthesis was brought about by the state of the amino acid pools in the starving cells. These ideas imply the existence of a specific control mechanism, operating at the level of transcription, which facilitates the cessation of growth by merely terminating the synthesis of unneeded messenger ribonucleic acids (RNA).

Studies on the physiological state of nitrogen-starved cells, however, argue against such a basic model for control. Under these conditions, there is a distortion of normal ribosome patterns (7); specific enzymes cannot be induced (16); there is an increased rate of degradation of proteins (11); and there is preferential inhibition of ribosomal protein and ribosomal RNA synthesis (2).

Except for a preliminary report in which it was noted that the inhibition of RNA synthesis was approximately the same in both RC^+ (RNA control) and RC^- strains of *Escherichia coli* (Irr, Bacteriol. Proc., 1970), there have been no attempts to demonstrate whether nitrogen starvation results in the expression of a unique control mechanism. It will be shown here that starvation for nitrogen does trigger the stringent response in RC^+ cells and that this effect on RNA synthesis and nucleotide metabolism can be partially overcome by incubating starved cells with a solution of all 20

amino acids. Whether any additional controls on nucleic acid metabolism are expressed under these conditions or whether synthesis is merely impaired by the exhaustion of available substrates will also be examined.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain CP78 is RC$^+$, *his, leu, thr, arg, vitB1*. Strain CP79 is isogenic to CP78 except for a mutation at the *rel* locus to yield the "relaxed" phenotype; it is RC$^-$ (8).

Culture conditions. All cultures were incubated at 37 C with forced aeration in a tris(hydroxymethyl)-aminomethane (Tris)-glucose minimal medium of the following composition: 0.1 M Tris-hydrochloride, *p*H 7.4; sodium citrate·$4H_2O$, 422 mg per liter; $MgSO_4$, 0.1 g per liter; $Na_2SO_4 \cdot 10H_2O$, 2.42 g per liter; $FeCl_3$, 0.4 mg per liter; 2.0 mM potassium phosphate, *p*H 7.0; and glucose, 2 g per liter. All required amino acids were added at 40 mg per liter, and thiamine was added at 0.1 mg per liter. Three variations of this basic medium were used: complete medium (CM) contained $(NH_4)_2SO_4$ at 1.0 g per liter; limiting nitrogen medium (LN) contained $(NH_4)_2SO_4$ at 50 mg per liter; and nitrogen-free medium (NF) was the basic medium lacking ammonium sulfate.

Growth was monitored by readings taken in a Leitz model M photometer at 415 nm in 10-mm cuvettes. An optical density (OD) reading of 0.10 equals about 10^8 glucose-grown, log-phase cells per ml.

In all experiments exponential cultures growing in CM medium were harvested at an OD of 0.40 and centrifuged for 1 min at 12,000 × *g* in an SS-34 rotor for the RC2B Sorvall refrigerated centrifuge. The cell pellet was washed with one volume of 0.1 M Tris-hydrochloride buffer (*p*H 7.4), centrifuged again, and suspended at the desired OD in NF medium. To effect nitrogen starvation directly, cells were suspended at an OD of 0.40 to 0.50 and incubated with forced aeration at 37 C. Starvation was considered complete when the change in OD was 0.01 or less in 10 min and when a portion of the culture resumed exponential growth in 10 min or less when incubated with added ammonium sulfate at 1.0 g per liter. Growth in LN medium was achieved by suspending the cells in NF medium at an OD of 0.10, after which sufficient ammonium sulfate was added to complete the medium. Under these conditions exponential growth usually terminated at an OD of about 0.45 to 0.49. A portion of the LN-grown culture was then tested for nitrogen starvation as described above.

Isotopic labeling. Essentially two types of isotope incorporation experiments were performed: (i) direct incorporation studies, and (ii) measures of the concentration of nucleotide pools. The direct incorporation experiments were performed as follows. A portion of nitrogen-starved cells was transferred to a chilled test tube and mixed with the desired isotope as described in the legends of the figures and tables. A portion of the labeled culture was then transferred to a small test tube in a 37 C water bath which contained any desired additives. The culture was then incubated with forced aeration, time zero being when it was added to the small tube in the 37 C bath.

Nucleotide pools were fully labeled by growing cultures from 0.10 OD unit for at least two generations in medium containing from 70 to 100 μCi of $^{32}P_i$ per ml. Any measurements of the nucleotide pools were made after this period of growth. In the case of pool determinations for nitrogen-starved cultures, cells were grown in LN medium and incubation was continued for a minimum of 1 hr beyond the time when nitrogen starvation was verified by the procedures described above.

Biochemical techniques. RNA synthesis was estimated by studying the incorporation of ^{3}H-uracil into alkali-sensitive acid-precipitated material by the methods of Gallant and Harada (9). At any desired time duplicate 20-μliter samples were removed from a labeled culture to two small tubes. One was chilled and contained about 1 ml of 10% trichloroacetic acid plus 1% Casamino Acids, and the other contained 0.5 ml of 0.5 N KOH. The alkali-treated sample was hydrolyzed by incubation at 37 C overnight, neutralized, and then precipitated with the 10% trichloroacetic acid-1% Casamino Acid solution and chilled. Both samples then were collected on Schleicher & Schuell Co. type B6 membrane filters, washed three times with the 10% trichloroacetic acid-1% Casamino Acid solution, washed with 5 ml of 95% ethanol, dried in a 150 C oven, and counted in a Mark II Nuclear-Chicago scintillation counter by using toluene base Liquifluor (New England Nuclear Corp.) as the fluor. Radioactively labeled RNA was calculated from the loss of acid-precipitable counts on alkaline hydrolysis. Raw counts were converted to molar quantities by first correcting the data to disintegrations per minute (dpm) by the channels ratio procedure and then by dividing the dpm values by the specific activity of the labeled culture as determined by assaying a 5-μliter portion of the culture for total dpm. In order to equate one experiment with another, the molar quantity of RNA determined in any one experiment was normalized by dividing that value by the OD of the culture at time zero.

Analysis of nucleotides. Nucleotides were extracted from radioactively labeled cultures by the methods of Cashel and Gallant (4). Samples of 100 μliters were taken from cultures, mixed with 50 μliters of 2 M formic acid, chilled for 30 min in an ice bucket, and either frozen or used immediately for thin-layer chromatography.

Ribonucleoside triphosphates were separated from other materials in the formic acid extract by a previously described two-dimensional thin-layer technique (12). The formic acid extracts were clarified by a 1-min centrifugation in a Beckman microfuge. Twenty microliters of the supernatant material was placed onto a polyethyleneimine thin-layer plate which was then dried in front of a fan, soaked in methanol, dried, and developed through the solvent systems of the deoxy separation of Randerath and Randerath (17). Positions of the nucleotides on the

thin layers were detected by co-chromatographing sufficient quantities of unlabeled compounds so that they could be seen under an ultraviolet lamp. In addition, the thin layers were used to expose Kodak no-screen X-ray film to assist in the localization of the radioactivity. The localized spots were then scraped into vials and prepared for scintillation counting. The concentrations of nucleotides labeled with radioactivity were then computed in a manner similar to that described above for RNA synthesis.

Analysis of ribosomal RNA species. Extracts of labeled cultures were prepared for zone sedimentation by the following modification of the methods of Salivar and Sinsheimer (18). A 1-ml culture was mixed with 0.1 ml of 0.2 M potassium phosphate buffer, *p*H 7.0, and chilled in an ice bucket for 5 min; the cells were harvested by centrifugation in the cold at 3,000 × *g* for 10 min and washed twice with lysing buffer (0.15 M NaCl; 0.05 M Tris-hydrochloride, *p*H 7.4; 0.005 M ethylenediaminetetraacetic acid). The final cell pellet was suspended in 0.2 ml of lysing buffer and 40 μg of freshly prepared lysozyme was added. After 5 min of incubation at 37 C, 10 μliters of 10% (w/v) sodium lauryl sulfate was added, and incubation was continued at 37 C until lysis of the culture was obvious. Deoxyribonucleic acid (DNA) was degraded enzymatically by adding 20 μliters of 1 M $MgCl_2$ and 10 μg of deoxyribonuclease with subsequent incubation at 37 C for 10 min. The extracts then were either frozen or immediately layered onto sucrose gradients.

Sucrose gradients. Linear 5 to 20% sucrose gradients in 0.1 M NaCl plus 0.01 M Tris-hydrochloride, *p*H 7.5, were prepared from Mann Ultra-Pure sucrose which is ribonuclease-free. Up to 0.20 ml of extract was layered onto 4.8-ml gradients which were then centrifuged at 41,000 rev/min for 4 hr in a Beckman L3-50 ultracentrifuge with an SW50.1 rotor. Fractions of 0.15 ml were collected from the bottom of the tube directly into tubes containing 10% trichloroacetic acid. One milligram of yeast RNA was added to each fraction as carrier, and the fractions were chilled for 15 min, collected onto Whatman GF/A glass fiber filters, washed with 10% trichloroacetic acid twice, and 95% ethanol once, dried, and prepared for scintillation counting.

Each gradient was layered with ^{32}P-labeled RNA (prepared for the specific experiment) and with ^{3}H-uridine-labeled RNA (extracted from an exponential culture) which served as reference markers. An estimate of the amount of ^{32}P-labeled RNA contained in a series of fractions giving rise to a peak was calculated from the formula used to compute the area of a polygon: $A = [(X_1 + X_2)(Y_1 - Y_2) + (X_2 + X_3)(Y_2 - Y_3) + \ldots + (X_n + X_1)(Y_n - Y_1)]/2$, where A = area of the peak, X_1 = fraction number, and Y_1 = counts/min in the fraction X_1.

RESULTS

RNA synthesis during nitrogen starvation. Nitrogen-starved cells incorporate ^{3}H-uracil into RNA much more slowly than exponential cultures (Fig. 1). This effect on the accumulation of RNA is comparable in both strains CP78 and CP79, yet one is RC^+ and the other RC^-. Since the rate of turnover of stable RNA is not stimulated by nitrogen starvation (2), the experiment presumably reflects a marked reduction in the synthesis of ribosomal RNA in both the stringent and relaxed strains.

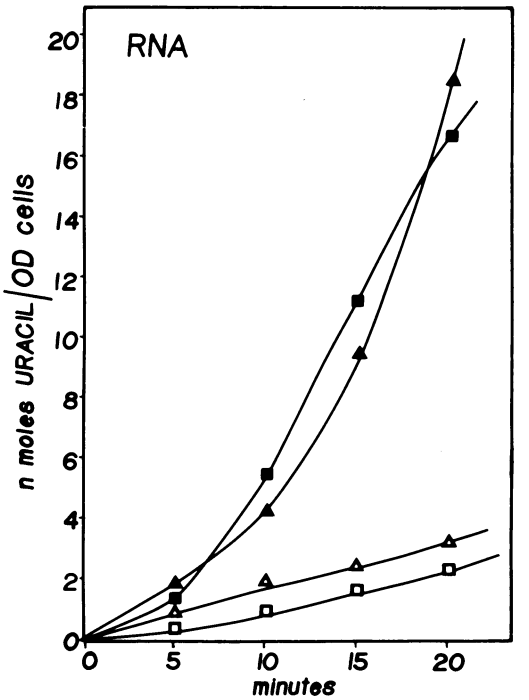

FIG. 1. *Incorporation of uracil into ribonucleic acid in strains CP78 and CP79 during nitrogen starvation. Log-phase cells were nitrogen-starved and divided into two portions at time zero. Each was labeled with ^{3}H-uracil (4 μCi/0.05 μmole/ml), and ammonium sulfate (1 mg/ml) was added to one portion as a control (filled symbols). Nitrogen-starved portions (open symbols); CP78 (triangles); CP79 (squares).*

Table 1 shows the effect of nitrogen and amino acid starvation on ribosomal RNA synthesis in the stringent and relaxed bacteria. Exponential cultures were starved for nitrogen as described in Materials and Methods. Separate portions of the same cultures were amino acid-starved by suspending them in CM medium which lacked leucine. Two 1.0-ml portions of the nitrogen-starved cells and 1.0-ml portions of the leucine-starved cells were chilled and then labeled with 20 μliters of a stock solution of $^{32}P_i$ (approximately 7 mCi per ml). Ammonium sulfate (1.0 mg per ml final concentration) was added to one of the

TABLE 1. *Incorporation of $^{32}PO_4$ into ribosomal RNA in strains CP78 and CP79*

RNA fraction	^{32}P Disintegrations/min[a]			Ratios	
	Exp[b]	-Leu	$-NH_4$	-Leu/Exp	$-NH_4$/Exp
CP78 23*S*	345,059	4,909	6,782	0.014	0.019
CP78 16*S*	216,775	3,994	4,652	0.018	0.021
CP79 23*S*	219,377	46,074	3,350	0.21	0.015
CP79 16*S*	156,629	49,028	3,287	0.31	0.021

[a] The ^{32}P content of each ribosomal RNA species was calculated as described in the text. Cultures were labeled with the same quantity of stock $^{32}P_i$ for 30 min at 37 C. The differences in the disintegrations per minute between the two strains was due in part to isotope decay because each was assayed on different days.

[b] Cultures were incubated in complete medium for exponential growth (Exp), medium lacking leucine (-Leu) to effect amino acid starvation, or medium lacking ammonium sulfate ($-NH_4$) to effect nitrogen starvation.

nitrogen-starved cultures for a control. After 30 min of incubation of the labeled cultures at 37 C, RNA was extracted from each, and identical quantities of each extract were analyzed for ribosomal RNA by sucrose density gradient centrifugation. In strain CP78, leucine deprivation caused a 50-fold reduction in the amount of phosphorus that was incorporated into both 16 and 23*S* RNA when the amounts were compared to the control which resumed exponential growth during the 30-min incubation. The nitrogen-starved portion of the culture showed a similar effect. On the other hand, strain CP79 incorporated 21 and 31% as much phosphorus into 23*S* and 16*S* RNA, respectively, under conditions of leucine starvation as it did during exponential growth, whereas the nitrogen-starved portion of this relaxed mutant was similar to the stringent strain with about a 50-fold reduction in 23 and 16*S* RNA. It appeared that the limited supply of inorganic nitrogen affected ribosomal RNA synthesis to the same extent in both RC^+ and RC^- strains of bacteria. Nitrogen starvation must therefore do something more than trigger the stringent response.

Accumulation of guanosine-5′-diphosphate-2′- or 3′-diphosphate. Studies relating nucleotide metabolism with RNA synthesis in amino acid-starved RC^+ strains demonstrated the production of a novel guanosine nucleotide (3, 5) which has been reported to be an inhibitor of ribosomal RNA synthesis (19) and of the guanylate and adenylate biosynthetic pathways in *E. coli* (10). This nucleotide was formerly designated MS I, but its structure is now known to be guanosine-5′-diphosphate-2′- or 3′-diphosphate (6) and it is abbreviated $_{pp}G_{pp}$. Recently, low levels of this nucleotide were detected in both stringent and relaxed strains during growth, and it accumulates in both strains during periods of carbon source starvation and at the time of step-down transitions (14).

Table 2 shows the relationship of $_{pp}G_{pp}$ concentrations to growth and the synthesis of RNA before and at the onset of nitrogen starvation in both strains CP78 and CP79. The nucleotide is present during growth of the stringent strain and accumulates very rapidly at about the same time the OD curve breaks, and the entry of phosphorus into RNA is impaired. The level of $_{pp}G_{pp}$ in strain CP79 is much lower during growth, and it accumulates at a much slower rate at the onset of nitrogen starvation with a yield of only one-third that found in the stringent strain. In both strains the level of $_{pp}G_{pp}$ gradually declines, but even after more than 2 hr of starvation it does not return to the concentrations found during exponential growth. The appearance of excess amounts of this nucleotide at the onset of nitrogen starvation suggests that there is a control mechanism working to regulate RNA synthesis in both RC^+ and RC^- cells.

Effect of amino acids on RNA synthesis. The inhibition of RNA synthesis imposed by nitrogen starvation on strain CP78 was relieved partially by the presence of all 20 amino acids at a concentration of 0.0125 mM. The amino acid mixture was added to NF medium which already contained the amino acids needed for growth at 40 μg per ml. As a result, the incorporation of uracil into RNA was stimulated about fivefold over the starved control (Fig. 2). Because the incorporation of uracil is always linear in this type of experiment, it seems unlikely that the addition of the amino acids allowed the resumption of growth. It is assumed that a nitrogen-starved culture exhausts one or more of its amino acid reserves and, as a result, the stringent response typical of amino acid starvation is triggered.

Effect of amino acids on nucleotide metabolism. Most conditions which favor the accumulation of high levels of $_{pp}G_{pp}$ also result in an impairment of nucleotide metabolism which parallels inhibition of ribosomal RNA synthesis, and nitrogen starvation is no different. When strain CP78 was deprived of ammonium ions, $^{32}P_i$ entered all four ribonucleoside triphosphates much more slowly than was observed in a growing culture (Fig. 3). One

TABLE 2. *Accumulation of $_{pp}G_{pp}$ at the onset of nitrogen starvation*[a]

CP78 RC+				CP79 RC-			
Time (min)	OD_{415}	RNA (nmoles of $^{32}PO_4$/ml)	$_{pp}G_{pp}$ (nmoles of $^{32}PO_4$/ml)	Time (min)	OD_{415}	RNA (nmoles of $^{32}PO_4$/ml)	$_{pp}G_{pp}$ (nmoles of $^{32}PO_4$/ml)
100	0.395	53.8	0.21	100	0.250	88.2	0.04
105	0.417	58.5	0.32	110	0.280	101.3	0.03
110	0.430	63.9	0.37	120	0.312	114.9	0.07
115	0.450	68.1	0.25	130	0.365	131.0	0.13
120	0.460	73.3	0.72	140	0.398	135.2	0.22
125	0.464	73.9	1.59	150	0.435	136.7	0.45
130	0.468	74.6	1.66	160	0.458	137.4	0.57
135	0.475	75.3	1.53	170	0.470	135.9	0.47
140	0.478	76.3	1.12	180	0.480	137.4	0.17
145	0.481	77.0	0.83				
150	0.486	77.6	0.77				

[a] At time zero, growth was initiated in limiting nitrogen medium at an OD of approximately 0.10. Within a few minutes a portion of each culture was labeled with $^{32}P_i$ (100 μCi per ml). At the indicated times samples were taken for thin-layer chromatography to detect $_{pp}G_{pp}$, and samples were also taken into 10% trichloroacetic acid and into 0.5 N KOH to estimate the incorporation of phosphorus into RNA. Abbreviations: $_{pp}G_{pp}$ = guanosine-5'-diphosphate-2'- or 3'-diphosphate; RNA = ribonucleic acid; RC = RNA control.

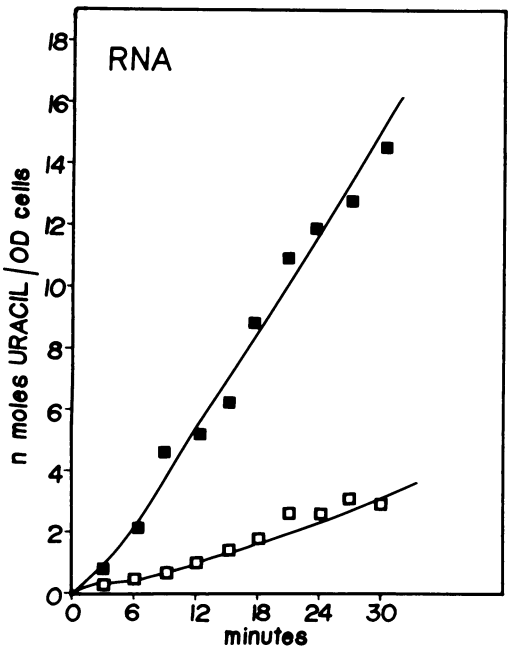

FIG. 2. *Effect of amino acids on incorporation of uracil into ribonucleic acid in CP78 during nitrogen starvation. An exponential culture of strain CP78 was nitrogen-starved and at time zero was labeled with ^{3}H-uracil (6 μCi/0.05 μmole/ml) and divided into two portions. One part was incubated with a mixture of 20 amino acids (0.0125 mM final concentration of each amino acid) (filled squares), and the other part was nitrogen-starved (open squares).*

unexpected finding which accompanied the studies of the triphosphates was the very slight incorporation of phosphorus into $_{pp}G_{pp}$. Cashel (3) found a marked increase in ^{32}P labeling of this nucleotide when he starved the same strain for any or all of its required amino acids. In other preliminary experiments, when incorporation of phosphorus was initiated within 2 to 3 min after suspension of the cells in nitrogen-free medium, I found more radioactivity in the $_{pp}G_{pp}$ region of the chromatogram, but there was no test made to assure that the cells were fully nitrogen-starved. There is no doubt that this nucleotide accumulates during nitrogen starvation, however, for its presence was clearly shown above.

Also shown in Fig. 3 are the results obtained when the mixture of all 20 amino acids was added to nitrogen-starved cells. Within 20 min there was approximately three times as much $^{32}P_i$ incorporation into each of the four triphosphates than was found in the starved culture. There was even more labeling of $_{pp}G_{pp}$, but at a much slower rate. No attempt was made to determine the position of the ^{32}P label in these nucleotides, but some of the phosphorus must enter the alpha position, for there was a coordinate increase in the rate of ^{32}P labeling of acid-precipitable material during this same period of time. It seems likely that the addition of amino acids relieves in part the restriction on nucleotide metabolism which is imposed by nitrogen starvation. Since the same conditions allow nucleotides to be withdrawn into stable RNA at an accelerated rate, it is presumed that these results show an increased rate of synthesis of nucleotides rather than just an enhanced rate of turnover.

Nucleotide pools. The apparent inhibition of nucleotide synthesis noted above should af-

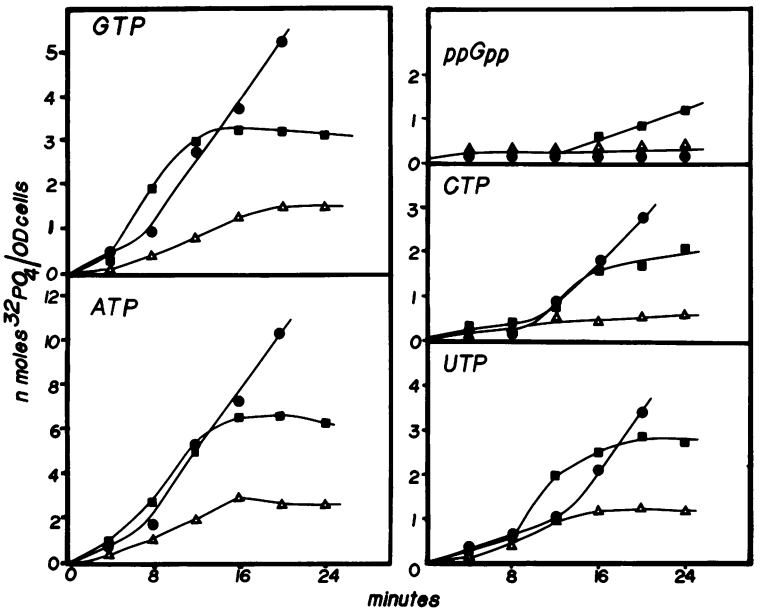

FIG. 3. *Incorporation of $^{32}P_i$ into ribonucleoside triphosphates and $_{pp}G_{pp}$ during nitrogen starvation and the effects of amino acids on the starved cells. An exponential culture of strain CP78 was nitrogen-starved and divided into three parts. Each was labeled with $^{32}P_i$ (50 μCi/ml) at time zero. Ammonium sulfate (1 mg/ml) was added to one part as a control (filled circles); a mixture of 20 amino acids (0.0125 mM for each) was added to another portion (filled squares); and the third portion was allowed to starve for nitrogen (open triangles).*

fect the intracellular concentrations of the ribonucleoside triphosphates, especially if these compounds are still being withdrawn from their pools at a rate which exceeds their continued production as a result of the starvation. To test this possibility, triphosphate pools were measured in log-phase and nitrogen-starved cultures of strain CP78.

Exponential cultures were grown from an OD_{415} of 0.05 in fresh CM medium containing $^{32}P_i$ at 100 μCi per ml. After the OD_{415} reached 0.30, samples were withdrawn at 5-min intervals for 45 min and analyzed for nucleotides and cell turbidity. Nitrogen-starved cultures were prepared by growing log-phase cells from an OD_{415} of 0.10 in LN medium containing 100 μCi of $^{32}P_i$ per ml. Turbidity readings were made at intervals and incubation was continued for 1 hr beyond the point when it was ascertained that the cultures were nitrogen-starved. Samples were then removed at 5-min intervals for 45 min for nucleotides and cell turbidity. Table 3 represents grouped data from two experiments with exponential cultures and three experiments with nitrogen-starved cultures. In both cases the nucleotide levels per OD_{415} of cells remained fairly constant throughout the period of sampling, but the concentrations in the starved cultures were on the average only one-half the levels found during growth. The fluctuation between two sequential points seldom exceeded 7% in both starved and log-phase cultures, which is about the same degree of variation found for replicate thin-layer chromatograms of the same sample. A considerable degree of scatter and variation of nucleotide concentrations was observed in samples taken during the first hour of starvation. The only pattern observed during the early starvation period was that the nucleoside triphosphates never exceeded the levels found in the log-phase cultures. These results suggest that both the entry of nucleotides into the triphosphate pools and their withdrawal must be curtailed by nitrogen starvation.

The effect of amino acids on the flow of nucleotides through the triphosphate pools is shown in Fig. 4. Strain CP78 was grown in $^{32}P_i$-containing LN medium and starved for 1 hr as described above. At time zero the 20 amino acids were added and samples were taken at 1-min intervals to determine nucleotide concentrations. Within the first minute $_{pp}G_{pp}$ almost disappears, and there is an initial increase in all four triphosphate pools followed by a gradual decline and a subsequent return to approximately the levels found prior to the

TABLE 3. *Ribonucleoside triphosphate pools in strain CP78*

Nucleotide	Exponential	Starved	S/E[a]
GTP	6.72 ± 0.17[b]	3.00 ± 0.07	0.45
ATP	13.62 ± 0.16	8.08 ± 0.13	0.59
CTP	4.56 ± 0.17	2.11 ± 0.08	0.46
UTP	4.64 ± 0.19	2.43 ± 0.12	0.52

[a] Ratio of the average nucleotide content in starved cultures to the average nucleotide content in the exponential cultures.

[b] Units are nanomoles of $^{32}PO_4$ per nucleotide per optical density unit of culture. Cultures were grown in complete medium to achieve exponential growth and in limiting nitrogen medium to effect nitrogen starvation. Details are in the text.

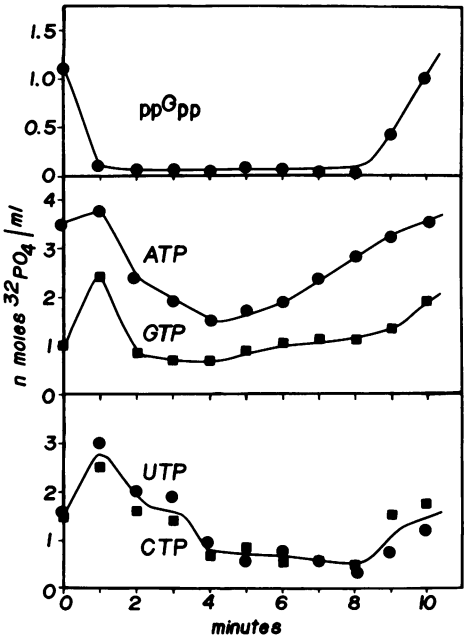

FIG. 4. *The effect of amino acids on the pools of ribonucleoside triphosphates and $_{pp}G_{pp}$. Strain CP78 was grown from an OD_{415} of 0.10 in the presence of $^{32}P_i$ (100 μCi/ml) in limiting nitrogen medium. Time zero on the graph represents the nucleotide levels found after the cells grew to an OD_{415} of 0.45 and then starved for nitrogen for 90 min. At this point, a mixture of 20 amino acids (0.0125 mM of each) was added to the culture.*

addition of the amino acids. During this same period of time, the inhibition of entry of uracil into RNA is partially relieved by amino acids. It seems therefore that the addition of amino acids releases the inhibition of nucleotide synthesis simultaneously with the change in the rate of accumulation of stable RNA in the starved culture. Interestingly, the compound thought to inhibit purine nucleotide metabolism and ribosomal RNA synthesis disappears almost instantaneously and then accumulates again at the time the nucleotide pools appear to return to their steady-state levels characteristic of nitrogen starvation.

DISCUSSION

The results of the experiments with the relaxed mutant strain CP79 indicate that nitrogen starvation has a marked effect upon the synthesis of ribosomal RNA. This finding, of course, is consistent with previous studies which presumably were carried out with RC^+ strains (2). If this relaxed mutant is not leaky in respect to the stringent response, then the inhibition of ribosomal RNA synthesis found can be attributed to either the expression of a different type of control mechanism under these conditions or to the exhaustion of available substrates required for RNA synthesis as in the case with uracil starvation (15). Preliminary experiments indicate that the nucleotide pools contract only by about twofold, as reported here for the stringent strain, when strain CP79 is nitrogen-starved. Hence, total exhaustion of one or more of the substrates required for RNA synthesis seems unlikely. Also, because this relaxed mutant accumulates excessive amounts of $_{pp}G_{pp}$ at about the time ribosomal RNA synthesis is impaired, it seems quite likely that the starving cells respond by generating a situation which favors the specific regulation of stable RNA synthesis. This raises the possibility that the relaxed mutant possesses some other type of control mechanism which exerts a regulatory function on nucleic acid metabolism by causing a known effector of ribosomal RNA synthesis and purine nucleotide metabolism to be synthesized at times when continued formation of these molecules is not desirable. Note also that $_{pp}G_{pp}$ accumulates in relaxed mutants during carbon deprivation and step-down transitions. Both of these conditions are also situations which do not sustain continued ribosomal RNA synthesis during their initial stages (14).

The experiments with strain CP78 demonstrate what is believed to be an expression of the stringent response during nitrogen starvation. This is thought to be so because the addition of amino acids to the nitrogen-starved cultures relieves in part the inhibition on the synthesis of ribonucleoside triphosphates and allows a partial restoration of stable RNA synthesis. The relief of these inhibitions occurs as the concentration of the suspect effector, $_{pp}G_{pp}$, falls to almost undetectable levels. The

amino acids do not replace the requirement for inorganic nitrogen because cultures treated in this manner do not resume growth.

Within a few minutes after addition of amino acids to the stringent strain, $_{pp}G_{pp}$ again starts to accumulate, and it appears that the nucleotide pools start to level off at concentrations found during nitrogen starvation. In the experiments reported here, it is not clear whether this is due to another round of amino acid starvation or whether some other mechanism is involved in causing this sequence of events. One important observation can be made however. Even when the level of $_{pp}G_{pp}$ is again elevated, RNA synthesis continues for at least another 20 min at a rate which is considerably greater than that measured during nitrogen starvation. This finding suggests, but certainly does not prove, that the synthesis of nucleotides may be far more sensitive to elevated levels of $_{pp}G_{pp}$ than is the RNA polymerase when it is programmed to synthesize ribosomal RNA.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant GB-16785 and by a generous gift from the Peter M. Stanka Foundation for Cancer Research.

Dan Simonsen's expert technical assistance made this work possible.

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