AMINO ACID CONTROL OVER RNA SYNTHESIS: A RE-EVALUATION*

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Communicated by James Bonner, May 23, 1968

When a stringent strain of Escherichia coli is starved of an amino acid, RNA synthesis, as usually measured, as well as protein synthesis, stops. Several explanations have been proposed for this observation. It has been suggested (1) that the discharged transfer RNA, accumulating during amino acid starvation, inhibits the RNA polymerase;1, 2 (2) that transcription and translation are coupled, through a mechanism in which the ribosomes in the process of protein synthesis pull RNA off the DNA template;3–5 and (3) that the synthesis of one of the substrates for RNA synthesis, UTP, is subject to amino acid control.6 All these theories also reflect the general belief that the synthesis of all species of RNA, messenger, transfer, and ribosomal, are equally subject to the same control. However, clear support for or against this theory of "coordinate control" has been lacking due to difficulty in obtaining an unambiguous measure of the amount of messenger RNA or unstable RNA made under conditions of amino acid starvation,7 it is to this question that this work is addressed.

In previous work it has been established that under conditions where there is little net RNA synthesis, as is the case during amino acid starvation, the entry of a radioactive base into the intracellular nucleotide pool is severely restricted.8–10 It is therefore possible that under these conditions the synthesis of unstable forms of RNA, not contributing to net RNA synthesis, may go undetected due to the failure of the labeled precursors to enter the intracellular pools.8–10 That this is in fact the case, and that considerable RNA synthesis can be measured during amino acid limitation, is shown in this paper.

Methods.—The bacterial strains used in this study were E. coli K-12, E. coli NP-2 (formerly called KB), and a mutant of the latter, NP-29 (formerly I-9), carrying a temperature-sensitive valyl-tRNA synthetase.11 These cells were cultured in a Tris-buffered medium,12 supplemented with 0.001 M K₂HPO₄, 0.4% glucose, 20 μg/ml adenine (which inhibits the interconversion reaction between guanine and adenine13), and 0.05 μg/ml thiamine-HCl. For the growth of strains NP-2 and NP-29, this medium was further supplemented with 20 μg/ml uracil and 10 mg/ml vitamin-free case amino acids (Difco).

To measure the total rate of synthesis of RNA, H₂-guanine or H₂-guanosine was added to cultures as described in the Results section. Samples of 1 ml were removed and added to 0.5 ml of cold 0.75 M perchloric acid. The cells were collected on membrane filters (the filtrate saved for isolation of GTP) and washed six times with 2 ml of 5% trichloroacetic acid containing 100 μg/ml guanine (or guanosine), followed by two washings with 2 ml H₂O. The dried samples were counted in the liquid scintillation spectrometer in a toluene-based counting mixture.

Determination of the specific activity of the tritium-labeled GTP pools requires a measurement of the total amount of GTP present in the samples. This was obtained by adding to the cultures, 15 to 25 min prior to the beginning of the experiments, either C¹⁴-guanine in the case of the experiment with E. coli K-12, or H₂P³²O₄ in the case of E. coli NP-29. The GTP was isolated from the perchloric acid extracts of the cells, after addition of 0.2 μmole of unlabeled GTP to serve as carrier. Each sample was absorbed onto
and eluted from charcoal; special care was taken to remove $\text{H}_2^3\text{PO}_4$. The GTP was then isolated by high-voltage paper electrophoresis on Whatman 3MM, in $0.06 \text{ M}$ sodium citrate buffer, pH 3.90. The GTP spots, cut from the electropherograms, were counted directly in the liquid scintillation counter. The experimental techniques have been described in detail.\textsuperscript{10, 12}

Results.—In a previous paper it was shown that the incorporation of a radioactive RNA precursor into RNA is a measure of net RNA synthesis (in growing cells this is essentially the accumulation of stable forms of RNA by the cell, since the unstable forms are normally only a small fraction of the RNA).\textsuperscript{10} For measurements carried out over long periods this conclusion is obvious since the unstable fraction, once labeled, contributes little to the isotope incorporation. On the other hand, this statement also proves true even for relatively short labeling periods. Although in principle it should be possible to measure total RNA synthesis, stable plus unstable, by following isotope incorporation over times short relative to the half life of the unstable RNA, in practice this is not the case. This follows from the fact that with \textit{E. coli} the addition of a radioactive RNA precursor to the culture medium does not lead to an immediate labeling of the intracellular pools of RNA precursors.\textsuperscript{12} One concludes in fact that the entry of isotope into the intracellular pools is subject to feedback control,\textsuperscript{14} in such a way that the entry of isotope is not significantly faster than the rate at which pool components are removed for net RNA synthesis.\textsuperscript{8, 10} Because of this limitation in isotope entry into the pools, the rate of incorporation into RNA is no faster than the net rate of RNA synthesis even over short labeling times.

In these experiments we measure the total rate of RNA synthesis by measuring the initial rates of incorporation of $\text{H}^2$-guanine into RNA. To compensate for the fact that the pool of GTP becomes labeled slowly relative to the sampling times, and at different rates under different conditions of incubation, the GTP is isolated from each sample and its specific activity measured.

Figure 1 describes an experiment to measure RNA synthesis in \textit{E. coli} K-12 during amino acid starvation. \textit{E. coli} K-12 is subject to growth inhibition by the addition of valine to the culture medium. The valine, serving as a false feedback inhibitor of the first enzyme in isoleucine biosynthesis, causes an isoleucine starvation.\textsuperscript{15} Figure 1A shows the growth of this strain and the immediate inhibitory effect of valine addition. Figure 1B shows the effect of valine addition on net rate of RNA synthesis; net synthesis is reduced to 6 per cent relative to the untreated control. Figure 1C shows the initial rate of uptake of $\text{H}^2$-guanine into the nucleic acid in an untreated culture and a culture to which valine was added 8 minutes earlier. Like the data in Figure 1B, a reduction (about 20-fold) in guanine uptake is seen. (These values are not so precise because in this experiment a small amount of $\text{H}^2$ is being counted in the presence of a large amount of C$^{14}$.) Figure 1D shows the specific activity of the H$^2$-GTP during this initial period of labeling. The data, H$^2$-GTP/C$^{14}$-GTP, having been normalized to the C$^{14}$-GTP present, are therefore corrected for a small decrease seen in size of the GTP pool on amino acid starvation,$^9$ and also for the variation in recovery of the GTP from sample to sample in the course of its isolation. From these data it is obvious that the entry of H$^2$-guanine into the GTP pools in the isoleucine-starved cells is greatly slowed; on the average, the specific activity
of the GTP pool in the experimental culture is about 20-fold lower than that of the control. This would indicate that although guanine incorporation into nucleic acid was reduced, this reduction can be largely attributed to the failure of the labeled precursor to enter the cells, and not to a reduction in the rate of RNA synthesis.

The above experiment has been repeated with a second strain of bacteria, *Escherichia coli* NP-29. This mutant strain carries a temperature-sensitive valyl-tRNA synthetase, allowing one to impose a specific aminoacyl-tRNA
starvation, independent of the amino acid pools. It is known that such strains exhibit the typical stringent control of RNA synthesis when shifted to the non-permissive temperature.\textsuperscript{11}

Figure 2A shows the growth of NP-29 in a control culture at 30.5\textdegree, and in the experimental cultures shifted first to 45\textdegree for one minute and then to 41\textdegree. The growth rate of the cultures at 41\textdegree is about 3 per cent of that of the control. (The procedure used to bring the growth of NP-29 to an abrupt halt, that is, heating for one minute at 45\textdegree, had no adverse effect on the growth of \textit{E. coli} NP-2, the strain from which NP-29 was derived.)

Figure 2B shows the incorporation of H\textsuperscript{3}-guanosine into cultures of NP-29 at 30.5\textdegree and 41\textdegree. These measurements indicate that net RNA synthesis, like growth, is reduced in the culture incubated at 41\textdegree to about 6 per cent of the control. Figure 2C shows the initial rates of incorporation of H\textsuperscript{3}-guanosine into RNA in the control and two experimental cultures at 41\textdegree. It shows that guanosine incorporation measured 8 minutes after the shift in temperature is on the order of 5 per cent of the control, and in a culture tested 18 minutes after the shift in temperature 6.5 per cent of the control. Figure 2D shows the rate at which the GTP increases in specific activity after adding H\textsuperscript{3}-guanosine in these same three cultures. Just as seen above, the H\textsuperscript{3}-guanosine enters the pools only very slowly in the two cultures incubated at 41\textdegree. The abrupt increase in the pool specific activity in the first 15 seconds following the addition of guanosine in this experiment can be attributed to pool expansion;\textsuperscript{10} this is not seen in the experiment described in Figure 1D where the pools have been pre-expanded by growth in medium containing C\textsuperscript{14}-guanine. Table 1 summarizes the data found in Figure 2. Whereas the rates of guanosine incorporation, estimated from the slopes of the uptake curves, have been reduced by valyl-tRNA starvation by about 20-fold relative to the control in the two experimental samples, the slow pool labeling largely accounts for these differences, being reduced about 10-fold relative to the control. Thus, total RNA synthesis in the experimental samples, as a fraction of that of the control, is 45 and 30 per cent, 8 and 18 minutes, respectively, after temperature shift. Finally, the table shows that, while under control conditions 40 per cent of the RNA made is unstable, under starvation conditions 90 per cent of the RNA is unstable.

\textit{Discussion}.—A basic assumption made in interpreting the above data is that the GTP extracted from the cells is representative of the GTP used for RNA synthesis; that is, that there is no significant compartmentalization or channeling within the pools of guanine ribonucleotides. Although it has been claimed that such compartmentalization exists,\textsuperscript{16} our more recent studies,\textsuperscript{12} as well as those of others,\textsuperscript{17} have failed to detect it and have provided an alternative explanation to the earlier results. As further evidence of the failure of compartmentalization to influence our measurements significantly, we see that they yield a reasonable estimate of the amount of unstable RNA, if for this purpose we assume that unstable and messenger RNA's are equivalent. A number of experiments like the control in the experiment of Figure 2 have given values for the fraction of unstable RNA relative to the \textit{total} synthesis of 0.4–0.6—values comparable to
FIG. 2.—RNA synthesis in an E. coli mutant carrying a temperature-sensitive valyl-tRNA synthetase.

To a culture of E. coli NP-29, growing at 30.5°, was added 0.4 µc/ml of carrier-free H3P32O4 to give a final specific activity of 0.4 µc/µmole. The culture was then divided into four parts to provide one control and three experimental cultures. These latter, 18 min after P32 addition, were shaken for 1 min at 45° and then transferred for further incubation to 41°. RNA synthesis was measured by adding to each flask, at the indicated times, H3-guanosine (300 µc/µmole, 0.02 µmole/ml) and sampling into perchloric acid. Two of the three experimental cultures were used to measure the initial rate of H3-guanosine incorporation into RNA (C) and into the GTP pools (D), 8 and 18 min, respectively, after the change in temperature. To the third experimental culture, H3-guanosine was added immediately after the change in temperature, and samples were taken for a measure of net RNA synthesis (B). To the control culture, H3-guanosine was added at an OD640 of 0.55; one series of samples was taken to measure the initial rate of incorporation into RNA (C) and entry into the GTP pools (D), and a second series was taken, over a longer time span, to measure net RNA synthesis (B). For (D) the H3-GTP activity was determined as the H3/P32 ratio in the isolated GTP. These data were normalized to that ratio of H3/P32 obtained on duplicate samples of cells taken from the control culture 23 min after the addition of H3-guanosine (1053 H3 cpm/4128 P32 cpm = 0.255). This time is sufficient to label the GTP with the exogenous guanosine fully, and therefore the data (relative specific activity) show the fraction of the fully labeled H3-GTP specific activity.

(A) Optical density of the control (●—●); and the experimental cultures (X—X). (B) Incorporation of H3-guanosine (net RNA synthesis) by the control (●—●) and an experimental culture (X—X). (C) Initial rate of incorporation of H3-guanosine into acid-precipitable material. Control, ●—●; 8 min after shift in temperature, +—+; 18 min after shift in temperature, □—□. (Note change in ordinate scale.) (D) Relative specific activity of H3-GTP. Symbols as for (C).
Table 1. Rates of RNA synthesis in E. coli NP-29.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial rate of guanosine incorporation (cpm/min)*</th>
<th>Initial specific activity of GTP†</th>
<th>Total rate of RNA synthesis (cpm/min)</th>
<th>Total rate fraction of control</th>
<th>Net rate of guanosine incorporation (cpm/min)†</th>
<th>Net rate fraction of control</th>
<th>Unstable RNA fraction of total§</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) 41°, 8 min</td>
<td>1,900</td>
<td>0.05</td>
<td>38,000</td>
<td>0.45</td>
<td>3,500</td>
<td>0.067</td>
<td>0.90</td>
</tr>
<tr>
<td>(B) 41°, 18 min</td>
<td>2,500</td>
<td>0.1</td>
<td>25,000</td>
<td>0.30</td>
<td>3,500</td>
<td>0.067</td>
<td>0.87</td>
</tr>
<tr>
<td>(C) Control</td>
<td>39,000</td>
<td>0.46</td>
<td>85,000</td>
<td>—</td>
<td>51,000</td>
<td>—</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Slope of incorporation curve, estimated at 30 sec.
† At 30 sec.
‡ Measured over 8 min.
§ Minimum unstable RNA: (total — net)/total.

those obtained in studies where hybridization or GC content were used to determine messenger abundance.

We conclude from these studies that although during amino acid or aminoacyl-tRNA limitation of growth there is a large reduction in stable RNA synthesis, there exists a considerable synthesis of unstable RNA. In work to be reported elsewhere, these same techniques are used to measure RNA synthesis during the transients of growth obtained by shifting cells from a rich medium, supplemented of amino acids, to a poorer minimal medium, or the reverse. In the case of shift down in growth, as in the case of amino acid starvation, what appears to be a normal synthesis of unstable RNA is seen, although there is little net RNA synthesis. In the case of the shift up, an immediate and preferential increase in the synthesis of stable RNA is seen, although there is no immediate increase in the total rate of RNA synthesis.

Stringent strains of microorganisms have been defined as those which fail to synthesize RNA during amino acid starvation. From our findings, we must conclude that the stringency of RNA control concerns primarily net RNA synthesis; thus, those theories attempting to explain this control by a central mechanism operating over the synthesis of all RNA's seem to require a re-evaluation. This interpretation is given additional support in the recent observations that the messengers for β-galactosidase and tryptophane synthetase can be made at near normal rates during amino acid starvation.

Finally, the results of these experiments may be taken as evidence that the synthesis of RNA is noncoordinate and that during amino acid starvation messenger RNA, but not ribosomal and transfer RNA, is made. Clearly this interpretation involves the assumption that the ribosomal and transfer RNA's are stable, and the messenger RNA's unstable, during amino acid starvation. This assumption is supported by studies on the RNA accumulation during amino acid starvation of a mutant of E. coli that has lost the stringent control over RNA synthesis. In this case it is shown that transfer RNA is stable, while ribosomal RNA is subject to no turnover or, at most, a slow one. Similar results are obtained as well from studies on the RNA accumulating in cells in which protein synthesis is blocked by chloramphenicol. The conclusion that RNA synthesis is noncoordinate is also obtained from studies where the RNA made
during amino acid starvation is characterized by its GC content; however, where these same measurements have been based on DNA-RNA hybridization, the opposite conclusion has been reached.

Summary.—Stringent strains of *Escherichia coli* synthesize significant amounts of RNA during amino acid limitation of protein synthesis. The inhibition in the uptake of a radioactive precursor of RNA, under these conditions, is largely explained by a failure of RNA precursors to enter the cells' ribonucleotide pools—a fact demonstrated by directly determining the specific activity of the intracellular ribonucleotide triphosphate while measuring the rate of incorporation of the precursor into RNA.

During isoleucine starvation of *E. coli* K-12, or valyl-tRNA limitation of a temperature-sensitive mutant, *E. coli* NP-29 (I-9), the synthesis of an amount of RNA, at least comparable to the amount of unstable RNA made in control cultures, is measured. This RNA, being largely unstable, is detected only by procedures which bypass the problem imposed by the preferential reutilization of degradation products of the unstable RNA in RNA synthesis.

I am indebted to Calvin McLaughlin for suggesting the use of the temperature-sensitive mutant and providing the culture.

Abbreviations: UTP, uridine triphosphate; tRNA, transfer RNA; Tris, tris(hydroxymethyl)aminomethane; GTP, guanosine 5′-triphosphate; AMP, adenosine 5′-phosphate; GMP, guanosine 5′-phosphate; GC, guanine plus cytosine.

* This research was supported by grants from the National Science Foundation (GB-4246), the National Institutes of Health (1-RO1-GM-15381-01), and the University of California Institute for Cancer Research.

1 Stent, G. S., and S. Brenner, these PROCEEDINGS, 47, 2005 (1961).
13 Magasanik, B., in *The Bacteria*, ed. I. E. Gunsalus and R. Y. Stanier (New York: Academic Press, 1962), vol. 3, pp. 295–334. Cells of *E. coli* B/5, growing as described in *Methods*, in medium supplemented with 15 µg/ml of adenine, were given C\(^{14}\)-guanine (16 µc/µmole, 0.002 µmole/ml); after 20 min, the cells were harvested and washed with perchloric acid. The RNA was subsequently hydrolyzed with 0.3 N KOH, and the nucleotides were separated by paper electrophoresis. The amount of radioactivity in the AMP was less than 7% of that in the GMP.