The Control of Ribonucleic Acid Synthesis during Amino Acid Deprivation in Escherichia coli

ROBERT A. LAZZARINI AND ALBERT E. DAHLBERG
From the Laboratory of Molecular Biology, National Institute of Neurological Diseases and Stroke, and Chemistry Branch, National Cancer Institute, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland

SUMMARY

The rate of RNA synthesis in a stringent strain of Escherichia coli was measured before and during amino acid deprivation. Upon amino acid deprivation the rate of RNA synthesis rapidly fell to 30% of the control rate and did not significantly change from that value upon prolonged starvation. The composition of RNA synthesized during amino acid deprivation was investigated by hybridization-competition, methyl labeling with 14C-methyl-labeled methionine, and polyacrylamide-agarose gel electrophoresis. Of the RNA pulse labeled during amino acid deprivation, ribosomal and transfer species comprise about 12% of the total, as opposed to about 40% in the unstarved control. Consequently, although some ribosomal and transfer RNAs are synthesized during amino acid starvation, the RNA is considerably enriched in messenger RNA, and the major effect of amino acid deprivation is the preferential curtailment of stable RNA synthesis. Our results also indicate that the rate of messenger RNA synthesis is reduced to 50% during amino acid deprivation.

When a culture of a microorganism with stringent control of RNA synthesis is deprived of a required amino acid, RNA accumulation is severely restricted (1, 2). However, some RNA synthesis is easily demonstrable. Stable RNA accumulates in the deprived culture at a rate of about 5% of that of the control (3). Similarly, some messenger RNA synthesis can be shown, since the biosynthetic pathways which yield the deprived amino acid are derepressed (4, 5). The possibility that the rate of RNA synthesis is less severely restricted than the rate of RNA accumulation during amino acid starvation has been considered by many laboratories (4–8). Under these conditions, virtually all of the RNA made would have to be unstable or messenger RNA and not accumulate. Three models describing the possible relationships between the rates of ribosomal RNA and messenger RNA synthesis during amino acid deprivation are outlined below. These models are presented in their extreme or limiting form for emphasis; the actual regulation of RNA synthesis may best be described by an intermediate model.

Restricted Synthesis with Coordinate Control—In this model the rate of RNA synthesis is severely restricted during amino acid deprivation. The small amount of messenger RNA and stable RNA synthesized has the same relative proportions of transfer RNA, ribosomal RNA, and messenger RNA as the unstarved control.

Degradative Control—RNA synthesis is unaffected by amino acid deprivation. Ribosomal RNA and messenger RNA are synthesized coordinately, but the ribosomal and transfer RNAs synthesized are rapidly degraded and do not accumulate. This kind of coordinate control of RNA synthesis has been shown in Escherichia coli deprived of uracil (9).

Noncoordinate Control—In this model the synthesis of ribosomal RNA and transfer RNA is selectively restricted, while messenger RNA synthesis is unaffected. Consequently, the rate of RNA synthesis is only moderately reduced during amino acid deprivation.

Measurements of the absolute rate of RNA synthesis during amino acid deprivation have convincingly ruled out model A (8, 10). In these studies the rate of RNA synthesis during amino acid deprivation was shown to be in considerable excess of the 5% of the unstarved control predicted by model A. Furthermore, several laboratories have shown that specific messenger RNA accumulates during amino acid starvation in abundances equal to that in unstarved constitutive mutants (4, 5). Similarly, the capacity to make β-galactosidase (11) in relaxed and stringent strains during amino acid deprivation is approximately one-half that observed in growing cells.

It is not possible to distinguish between the two remaining possibilities on the basis of published data. In either model, RNA labeled during amino acid deprivation would have a large messenger component (60% for model B; approximately 100% for model C), irrespective of the length of the labeling period. Consequently, analysis of the sucrose gradient profiles, base composition, instability of the labeled RNA in the presence of inhibitors of RNA synthesis, distribution of the labeled RNA in polysome and monosome fractions, and the size distribution of labeled RNA cannot be expected to distinguish between the two models.

In the present communication, we have reinvestigated the rate of RNA synthesis during amino acid deprivation and the relative abundances of stable and messenger RNA in pulse-labeled fractions. For these experiments we have used four
analytical methods: the determination of the instantaneous rate of RNA synthesis, the incorporation of methyl groups into ribosomal and transfer RNA, hybridization-competition experiments, and agarose acrylamide composite gel electrophoresis. The results of these studies show that during amino acid deprivation the rate of RNA synthesis falls to about 30% of the unstarved control (eliminating model A). Of the RNA synthesized during amino acid deprivation, ribosomal and transfer species comprise about 12% of the total, as opposed to the 40% in the unstarved control. Although some stable RNA species are synthesized during amino acid deprivation, the RNA synthesized is considerably enriched in messenger RNA. Consequently, the major effect of amino acid deprivation is the preferential curtailment of ribosomal and transfer RNA synthesis. We conclude that model C most correctly depicts the situation obtained during amino acid deprivation.

**EXPERIMENTAL PROCEDURE**

**Materials**

2,8-$^{3}H$-Adenosine (17.7 mCi per μmole) and $^{14}$C-methyl-labeled methionine (53.6 mCi per mmole) were purchased from New England Nuclear. $^{2}H$-Adenosine was prepared by refluxing an aqueous solution of 2,8-3H-adenosine for 5 hours and separating the adenosine from the tritiated water formed by evaporation to dryness. $^{32}$PO$_4$ was purchased from Tracerlab. E. coli deoxyribonuclease and deoxyribonuclease were purchased from Worthington. Nitrocellulose filters (144-mm diameter, type B-6) were obtained from Schleicher and Schuell. Rifampicin was a gift from Pitman Moore. Polyethyleneimine cellulose thin layer plates (cellulose-polyethyleneimine MN-Polygram cell 300 PEI) were the products of Brinkmann.

**Bacteria and Culture Conditions—**E. coli CP 78 (thi, arg, his, thr, leu, rel$^{+}$) was used in the present study. The Tris-minimal medium and culture conditions have been described previously (12). Amino acids were present at concentrations of 25 μg per ml and thiamine at 10 μg per ml. Cultures were grown at 27° with reciprocal shaking in a water bath. Under these conditions the cultures grew with a doubling time of 130 min. Growth was measured turbidimetrically at 600 μm with a Zeiss PMQ II spectrophotometer. A turbidity of 1 at 600 nm corresponds to $5 \times 10^{8}$ cells per ml.

**Methods**

**Determination of Rate of Total RNA Synthesis**—The rate of total RNA synthesis before and during threonine deprivation was estimated from the rate of $^{3}H$-adenosine labeling of trichloroacetic acid-precipitable material and the specific activity of the intracellular trichloroacetic acid precipitable material, as previously described (9, 12) and summarized below. The rate of adenine incorporation into trichloroacetic acid-precipitable material was determined in 3.2-ml aliquots of growing or threonine-deprived cultures of CP 78. At zero time 0.2 ml of $^{3}H$-adenosine (17.7 mCi per μmole, 1 mCi per ml) was added to the culture, which had been grown in the absence of exogenous adenosine. Over the course of the next 2 min 0.2-ml aliquots were removed at 15-sec intervals and diluted into 5 ml of 10% trichloroacetic acid containing 1 mg per ml of adenosine. Carrier cells were added to each sample and the cells were washed by centrifugation four times with 5% trichloroacetic acid. The final cell pellets were suspended in 0.5 ml of 0.3 M KOH and hydrolyzed overnight at 37°. The radioactivity in the hydrolysates was determined by liquid scintillation counting in scintillation fluid similar to that described by Beaven and Maickel (13), but containing equal volumes of toluene and methyl cellulose. The counting efficiency, determined by internal standardization, was 8 to 12%, depending upon the volume of hydrolysate included in the counting mixture.

To determine the specific activity of the intracellular ATP and GTP pools at various times after the addition of $^{3}H$-adenosine to E. coli CP 78, double label ($^{14}$P and $^{3}H$) experiments were performed as described previously (9, 12). The experiments determining the incorporation of $^{3}H$-adenosine into trichloroacetic acid precipitable material and those determining the incorporation into the intracellular trichloroacetic acid pools were conducted in as nearly identical a manner as possible.

The molar amounts of AMP incorporated per aliquot of cells were computed for each sample from the amount of label incorporated, the distribution of the label between adenosine and guanosine residues, and the cumulative specific activity of the ATP pool (12).

The computations involved in estimating the rate of RNA synthesis are illustrated in Table I. The incorporation of $^{3}H$-adenosine into the intracellular ATP, GTP, and RNA of a growing and an amino acid-deprived culture is shown in Columns 1, 2, and 4 of the table. In the growing culture, GTP is labeled to a small extent and consequently some of the incorporation shown in Column 4 resides in GMP residues. The amount of label residing in AMP residues only (Column 5) is calculated from Columns 4 with the specific activities of the ATP and GTP shown in Columns 1 and 2 and the relative abundance of GMP and AMP residues in pulse-labeled RNA, 29 and 95%, respectively ((0.25 ATP specific activity × 0.25 AMP specific activity + 0.29 GTP specific activity) × Column 4). In the case of threonine-
deprived cultures, conversion of adenosine to GTP is not detectable and, therefore, Columns 4 and 5 have the same value.

The average specific activity of the ATP from zero time (Column 3) is estimated for each of the labeling times as previously described (12) and is used in computing the molar incorporation of AMP from Column 6. The rates of RNA syntheses are estimated from the molar amounts of AMP (Column 6) incorporated during the first 20 to 45 min.

Rate of Methyl Group Incorporation into RNA—The rate of methyl group incorporation into RNA by whole cells was carried out as described earlier (9) with only minor modifications. In the present study, the double label procedure was not followed, since the correction for methionine residues incorporated into proteins which contain the purified RNA was shown to be negligible. Consequently, cells were labeled for 20-min periods with 14C-methyl-labeled methionine (53.6 mCi per mmole, 6 µg per ml final concentration). The cells were harvested and mixed with carrier cells and RNA was extracted from them. The purified RNA was treated with 0.1 M Tris-buffer, pH 8.9, for 4 hours at 37° to hydrolyze labeled methionine bound to transfer RNA, precipitated with NaCl and ethanol, and collected by centrifugation. When the distribution of methyl groups in the RNA preparations was studied by sucrose gradient centrifugation, the RNA was not subjected to the pH 8.9 treatment before centrifugation. After centrifugation each fraction from the gradients was adjusted to pH 8.9 and incubated for 2.5 hours at 37° and then precipitated with trichloracetic acid. The precipitated RNA was collected by centrifugation, dissolved in 0.5 ml of 1.5 M NH₄OH, and counted in Bray's liquid scintillation fluid (14).

Preparation of Pulse-labeled RNA for Hybridization— Cultures of CP 78, 50 ml, were labeled for 2 min with 3H-adenosine (8.13 mCi per µmole, 0.6 µg per ml final concentration) during normal growth or with 2,4-H adenosine (17.7 mCi per µmole, 0.6 µg per ml final concentration) for 4 min after 20 or 40 min of threonine deprivation. Cells were harvested by pouring the labeled culture over crushed ice containing enough sodium azide to yield a final concentration of 0.02 M. Cells were collected by centrifugation, washed once, and disrupted either by the freeze-thaw method (15) or by passage through a French pressure cell. RNA was prepared from these extracts as previously described (16).

Preparation of Ribosomal RNA for Hybridization-Competition Experiments—Two parallel cultures of CP 78 were grown at 37° in Tris-minimal glucose medium supplemented with 50 µg per ml of each of the 19 amino acids. At a cell density of 0.5 × 10⁹, 32P-orthophosphate was added to one culture to yield a final specific activity of 4 µCi per µmole. The cells were cultured until a cell density of 3 × 10⁹ cells per ml. Rifampicin, 250 µg per ml, was added to each culture to inhibit RNA synthesis further. Each culture was aerated at 37° for 30 min to allow existing messenger RNA to be degraded. The cells were harvested, washed twice in 0.05 M Tris, pH 7.4, containing 10⁻⁴ M magnesium acetate, and disrupted by passage through a French pressure cell. The extrudates were incubated with deoxyribonuclease (10 µg per ml) for 10 min at room temperature. The extracts were centrifuged at 30,000 × g for 30 min and the supernatant fluid was again centrifuged at 48,000 rpm for 2 hours in a Spinco model L centrifuge. The ribosome pellet obtained from the last centrifugation was resuspended in 0.05 M Tris containing 10⁻⁴ M magnesium and resedimented. RNA was prepared from the washed ribosomes as previously described (18). For hybridization-competition experiments, the specific activity of the competing ribosomal RNA was adjusted to a convenient level (1,000 to 3,000 cpm per µg of RNA) by mixing appropriate amounts of the two RNA preparations.

RNA-DNA Hybridization—The RNA-DNA hybridization experiments were carried out as described by McConnaughy, Laird, and McCarthy (17). E. coli DNA solutions were denatured according to Kennell and Kotoulas (18), except that the DNA was exposed to 0.33 M KOH at 37° for 90 min. The denatured DNA solutions were chilled to 0°, diluted, neutralized with 1 M HCl, and adjusted to 6 × SSC. The final solution had an absorbance at 260 nm of 0.25 to 0.30. The denatured DNA was fixed to nitrocellulose filters by gravity filtration in the cold. The amount of DNA adhering to the filters was determined from the difference in absorbance of the solution before and after passage through the filter. After air-drying the filters for either 4 hours or overnight, they were dried in a vacuum oven at 80° (19). Circles 22 mm in diameter were cut from these filters with a stainless steel punch and die assembly. The hybridization experiments were carried out at 42° in 48% formamide containing 5 × SSC. The hybridization vessels were either modified shell vials (9) or 6 × 50 mm test tubes (19). Hybridization was allowed to continue from 36 to 42 hours, after which time the filters were serially washed with 5 × SSAC at 42°, 5 SSC at 42°, and finally SSC at room temperature. As many as 24 filters were washed simultaneously through the use of a honeycomb basket assembly (Fluoroware, Inc., Chaska, Minnesota). After washing, each filter was mounted on an insect pin and dried under a heat lamp and counted with toluene-Liquiflor liquid scintillation fluid.

Agarose-Acrylamide Composite Gel Electrophoresis—The electrophoresis of RNA samples in mixed gels of agarose and acrylamide was carried out as previously described (20, 21). After electrophoresis the gels were stained and photographed. The distribution of radioactivity in the gels was determined by slicing the gels into 1-mm thick fractions and counting them as previously described (21).

RESULTS

Effect of Amino Acid Deprivation on Kinetics of Labeling of Intracellular Nucleoside Triphosphate Pools and Rate of Global RNA Synthesis

The rate of entry of a labeled precursor such as uracil or adenosine into its cognate nucleoside triphosphate pool is strongly dependent on the physiological state of the cell. This dependence is particularly apparent in E. coli that bear the rela allele (10, 22, 23). Fig. 1 shows the specific activity of the ATP and UTP pools after pulse labeling for 60 sec with adenosine or uracil during the course of amino acid deprivation. The data are presented as a percentage of the unstressed control. During the course of amino acid deprivation the amount of labeled adenosine or uracil that is incorporated into the ATP or UTP pools in 1 min shows a characteristic minimum at 10 min after removal of the amino acid, returning to substantially higher levels of incorporation at later times. As pointed out by Edlin and Neuhard (20), the incorporation of adenosine into ATP is much less...
sensitive to amino acid deprivation than is the incorporation of uracil into UTP. The severe restriction on incorporation of uracil into the UTP observed at 10 min after removal of threonine from CP 78 in the present study is substantially more than was found by us in a previous study (10). The alteration in our present strain of CP 78 which leads to the more severe restriction on the uracil incorporation into the UTP pool does not, however, affect the rate of RNA synthesis during amino acid deprivation. The rate of RNA synthesis estimated from the rate of incorporation of labeled uracil into RNA and the specific activity of the UTP pool is 1 to 2 µmoles per 100,000 unit per sec after 10 min of threonine deprivation. This number is in excellent agreement with our previously reported findings (10).

Because of the less severe restriction on incorporation of adenosine into the ATP pool, adenosine labeling was used to determine the rate of RNA synthesis. The incorporation of adenosine into the ATP pool and into RNA was measured at various times during the course of amino acid deprivation and the rate of RNA synthesis was computed from these data as illustrated in Table I. The results of these experiments are shown in Fig. 2. The rate of RNA synthesis does not show any of the massive fluctuations during the course of amino acid deprivation that are seen in the relative incorporation of adenosine into the ATP pool (Fig. 1). The rate of adenosine incorporation into RNA rapidly assumes a value of 25 to 35% that of the unstarved control. In an earlier study in which uracil incorporation was used, we estimated the rate of RNA synthesis to be 10 to 15% of the control after 10 min of amino acid deprivation (10). Between 20 and 60 min of amino acid starvation, the rate was 25 to 35% of the unstarved control. The difference between the rate of RNA synthesis estimated with uracil and estimated with adenosine is only demonstrable after 10 min of amino acid deprivation. At all other times the rates measured with the two precursors are the same. Since examination of the base ratios of the RNA synthesized at 10 min after amino acid deprivation did not show a significant increase in the adenosine content, we have tentatively concluded that compartmentalization or some systemic error arising from the extreme restriction on the entry of label into the pool may be responsible for this difference. Conversely, the fact that the rate of RNA synthesis determined after 20 min of amino acid deprivation is the same whether measured with uracil or adenosine (and is in good agreement with rates estimated by Nierlich (8) using guanosine incorporation) indicates that compartmentalization during this period of amino acid deprivation is not a significant factor.

Composition of RNA Synthesized during Amino Acid Deprivation

Hybridization-Competition Analysis—RNA-DNA hybridization has been used to estimate the composition of RNA pulse labeled during amino acid deprivation in the studies of Friesen (3) and Stubbs and Hall (24). In both of these studies the hybridization conditions were such that the probability of hybridization for stable RNA was reduced to 5- to 10-fold below that for messenger RNA (RNA:DNA = 0.1). At these input ratios there is sufficient DNA to form hybrids with virtually all of the messenger RNA present (25). To a first approximation,
At elevated temperatures the tritium labeling the C-8 position shown agree within 7%.

The duplicate hybridizations under these conditions. The duplicate hybridizations labeled RNA was maintained at 350 or greater to ensure complete maximum value when the input DNA:RNA ratio reached 200.

In subsequent experiments the input ratio of DNA to the pulse-labeled in a step fashion, as described by Kennel (25), and reached a maximum value when the input DNA:RNA ratio reached 300. In subsequent experiments the input ratio of DNA to the pulse-labeled RNA was maintained at 350 or greater to ensure complete hybridization. Fig. 3 also shows the reproducibility of hybridization under these conditions. The duplicate hybridizations shown agree within 7%.

The fraction of the pulse labeled RNA that hybridizes is proportional to the messenger RNA content of the RNA, the proportionality depending on the completeness of the hybridization reaction. However, the sensitivity of this method is diminished when the reaction conditions do not allow complete reaction of RNA with available DNA sites and the proportionality departs from unity. We have reinvestigated the composition of RNA pulse labeled during amino acid starvation with the more sensitive analysis of hybridization-competition developed by Hall et al. (26). In the present analysis, more than 80% of the pulse-labeled RNA forms hybrids with the DNA, since the RNA:DNA ratios of 1:350 to 1:1000 are used (25), and hybridization reaction times have been extended to afford more complete reaction. The hybridization experiments were carried out in formamide solutions, as described by McConnaughy, Laird, and McCarthy (17), since these conditions were mild enough (42°) to allow the use of 2,8-H-adenosine to pulse label the RNA. At elevated temperatures the tritium labeling the C-8 position of this compound exchanges with protons of water (27). Because of the high specific activity adenosine commercially available and the small restriction on the incorporation of adenosine into the ATP pool after 20 min of amino acid starvation, RNA of sufficiently high specific activity for this type of a hybridization-competition could be obtained. Under the conditions used the hybrids bound to filters contained approximately 100 µg of DNA and in excess of 1000 cpm of tritiated adenosine-labeled RNA.

The dependence of hybrid formation upon the concentration of DNA is shown in Fig. 3. Each hybridization mixture contained 0.2 µg of tritium-labeled RNA and the indicated amounts of DNA. The amount of RNA hybridized to the DNA increased in a step fashion, as described by Kennell (25), and reached a maximum value when the input DNA:RNA ratio reached 300. In subsequent experiments the input ratio of DNA to the pulse-labeled RNA was maintained at 350 or greater to ensure complete hybridization. Fig. 3 also shows the reproducibility of hybridization under these conditions. The duplicate hybridizations shown agree within 7%.

The composition of RNA pulse-labeled during normal growth and after 20 and 40 min of threonine starvation was determined by hybridization-competition with purified ribosomal RNA. Two methods have been described for this type of experiment: one in which the competing unlabeled RNA is mixed with the pulse-labeled RNA and presented to the DNA at the same time (26), and a second method in which the DNA immobilized on the filters is first saturated with the competing RNA and then washed free of unbound RNA. The previously treated DNA filters were then hybridized with 0.2 µg of the H-adenosine-labeled RNA in the absence of any additional competing RNA. The hybridization of pulse-labeled RNA is presented as a percentage of the uncompeted control while that of the competing P-RNA is presented as counts per min bound to the DNA filter. Simultaneous hybridization-competition: ○, pulse-labeled RNA; C, P-RNA. Sequential hybridization: ▲, pulse-labeled RNA; Δ, P-RNA.
FIG. 5. Hybridization competition with RNA prepared from amino acid-deprived cells. Escherichia coli CP 78 was deprived of threonine for 20 min and then labeled with 3H-adenosine for 4 min at 27°C. RNA prepared from these cells was used in simultaneous (■, ○) and sequential (▲, △) hybridization experiments with 32P-rRNA. Symbols and experimental conditions are the same as indicated in Fig. 4 except for the following. Hybridization mixtures contained 0.33 μg of 3H-rRNA (2 \times 10^5 cpm) and 120 μg of DNA immobilized on filters. 32P-rRNA had approximately twice the specific activity formerly used. Reaction volumes were increased to 0.75 ml.

The same type of experiment was performed with RNA pulse labeled for 4 min after 20 min of threonine starvation and after 40 min of threonine starvation (Figs. 5 and 6). In the latter experiments the difference between simultaneous (■, ○) and sequential (▲, △) hybridization-competition experiments were more striking. As before, the simultaneous competition was much more extensive, even though the amount of competing RNA bound to the DNA filters was the same or less than that found in the sequential competition case. Furthermore, the amount of competing RNA bound to the DNA filter was maximal when the input concentration of the competing RNA was 3 μg per reaction vessel. Since each reaction contained 100 μg of filter-bound DNA, this corresponds to a ribosomal RNA:DNA ratio of 0.03. Yankofsky and Spiegelman (30) and Goodman and Rich (31) have shown that stable RNA cistrons are saturated at these input ratios. Furthermore, at this level of competing RNA, the specific activity of the tritium-labeled ribosomal RNA has been reduced 10 fold. Increasing the amount of competing RNA above this level should have only a marginal effect on the competition, since the specific activity has already been reduced substantially and stable RNA sites on the DNA have become saturated. However, the data shown in Figs. 5 and 6 show that these expectations are realized only in the case of sequential hybridization. In the case of simultaneous competition the competition was decreased by 150% when the concentration of the competing RNA was increased above 3 μg per reaction vessel. On the basis of these data we conclude that sequential hybridization yields a more accurate estimate of the amount of stable RNA in the pulse-labeled fraction. From the data presented in Figs. 5 and 6, we estimate that 10% of the RNA pulse labeled after 20 and 40 min of threonine starvation consists of 16S and 23S ribosomal RNA species.

Incorporation of Methyl Groups into RNA during Amino Acid Deprivation—The amount of ribosomal RNA detected in the RNA pulse labeled during amino acid starvation by the hybridization-competition experiments borders on the limits of detectability of the method. Furthermore, since the comp-
Table II

<table>
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<th>Labeling condition</th>
<th>Incorporation of methyl groups into RNA</th>
<th>( \text{fmoles} / \text{A}260 )</th>
<th>%</th>
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<td>Plus threonine, (^{14}\text{C})-methionine present for 20 min</td>
<td>215</td>
<td>100</td>
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<tr>
<td>Minus threonine, methionine present</td>
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<tr>
<td>0th to 20th min</td>
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<td>24</td>
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<td>20th to 40th min</td>
<td>25</td>
<td>11.6</td>
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<td>40th to 60th min</td>
<td>18</td>
<td>8.4</td>
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<tr>
<td>60th to 80th min</td>
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Fig. 7. The distribution of methyl groups among the ribosomal and transfer RNA species. CP 78 was labeled with \(^{14}\text{C}\)-methyl-labeled methionine for 20 min during normal growth or between the 30th and 40th min of threonine deprivation. RNA was prepared from each of these cultures and was layered upon 25 ml of a 5 to 20% sucrose gradient and centrifuged at 25,000 rpm for 18 hours. Each gradient was fractionated into 30 fractions and the RNA of each fraction was treated at pH 8.9 for 2 hours at 37°C to remove any esterified methionine. The RNA in each fraction was then precipitated with trichloroacetic acid, collected by centrifugation, and counted in Bray's liquid scintillation counting fluid. ●, RNA labeled during normal growth; ○, RNA labeled during threonine deprivation. The radioactivity observed in the RNA labeled during amino acid deprivation is multiplied by 2.22 in the above figure so that areas under each of the two profiles could be equal.

The distribution of methyl groups between the various species of stable RNA was examined by sucrose gradient centrifugation (Fig. 7). All three major classes of stable RNA were labeled with methionine in the control as well as the amino acid-starved cultures. The relative distributions of methyl groups in these species, however, appear slightly different, with a larger percentage of the methyl groups being concentrated in the 4S species in the starved RNA. At present it is not known whether this altered distribution reflects a change in the proportion of transfer to ribosomal RNA or whether it reflects a decrease in the level of methylation of ribosomal RNA synthesized.

Electrophoretic Analysis in Agarose-Acrylamide Composite Gels—The conclusions drawn from the foregoing sections, namely, that stable RNA comprises 10 to 12% of the RNA pulse-labeled during amino acid deprivation, is further substantiated by the results of the electrophoretic analysis. Aliquots of the RNA preparations used in the hybridization experiments (Figs. 4 to 6) were electrophoresed into 2% acrylamide-0.5% agarose gels for 1 hour at 0°C. Under the conditions used, transfer RNA penetrates about 52 mm of the gel, while the 23S RNA penetrates about 13 mm. After the positions of the ribosomal and transfer RNA were determined by staining, each gel track was cut into 1-mm thick slices transversely along its length. The radioactivity in each slice was determined by liquid scintillation counting after extraction and hydrolysis of the RNA with 1.0 M NaOH.

The profiles of the pulse-labeled RNA are shown in Fig. 8. The positions of the major RNA species are indicated by the filled squares along the abscissa. Peaks of radioactivity corresponding to the ribosomal and transfer RNA species are very prominent in the profile of the control RNA, even though it was

methionine to transfer RNA. The molar amount of methyl groups incorporated into RNA during the time period was calculated from the radioactivity and the input specific activity of the \(^{14}\text{C}\)-methyl-labeled methionine. The results shown in Table II indicate that the rate of methylation falls rapidly during the course of amino acid deprivation to a level of about 10 to 12% of the unstarved control. The decreased incorporation of methyl groups cannot be attributed to degradation of newly methylated RNA, since the sum total of methyl groups incorporated during three successive 20-min periods very nearly equaled the amount incorporated during 1 hour of continuous labeling.

Since the rate of total RNA synthesis during the same period is one-third the control value (Fig. 2), these results support the conclusion that the RNA synthesized during amino acid deprivation is enriched in messenger RNA species. However, as previously indicated (9), rates of stable RNA synthesis estimated in this manner can only be considered as approximate for at least two reasons. First, the specific activity of the intracellular methionine was not measured but assumed to be the same as the input specific activity. Although evidence supporting this assumption has been obtained in the case of fully supplemented growing cultures (9), it may not be valid under conditions of amino acid deprivation when the flux of methyl groups through the intracellular pools is diminished. Consequently, the calculated rate of methyl group incorporation may be an underestimate. Second, the relative abundance of methyl groups in the various species of stable RNA synthesized during amino acid deprivation may be different from the abundance in normal RNA.

The distribution of methyl groups in the various species of stable RNA was determined by hydroxide precipitation after extraction and treatment with 1.0 M NaOH. The results obtained are shown in Fig. 9. It can be seen that the methyl groups are most strongly incorporated into the 16S RNA, while the 23S RNA contains a smaller amount. This difference is not due to differences in the specific activity of the intracellular methionine, since the specific activity of the input methionine was identical in all cases. Instead, the difference is most likely due to differences in the relative abundance of the various RNA species during amino acid deprivation.

The results obtained in this study indicate that the rate of methyl group incorporation into RNA during amino acid deprivation is significantly reduced compared to the control value. This decrease is not due to degradation of newly methylated RNA, but rather to a decrease in the rate of RNA synthesis. The specific activity of the intracellular methionine was not measured, but it is likely that the specific activity of the input methionine was similar in all cases. Therefore, the decrease in methyl group incorporation is most likely due to a decrease in the rate of RNA synthesis. The relative abundance of methyl groups in the various species of stable RNA synthesized during amino acid deprivation may be different from the abundance in normal RNA.
labeled for only 2 min at 27°. The ease with which ribosomal RNA can be detected is due principally to the fact that virtually all of the 16 S RNA and 23 S RNA were localized in bands 2 mm thick, although the total RNA profile spans 60 mm. Bands corresponding to the 5 S and 4 S RNA are also resolved but are somewhat more diffuse. A major amount of the radioactivity of the pulse-labeled control RNA is located between Fraction 20 (16 S RNA) and Fraction 48 (5 S RNA). This distribution of label is observed only with pulse-labeled RNA and not with uniformly labeled RNA, the latter having a pattern congruent with that visualized by staining. Consequently, it is assumed that the RNA migrating between the 16 S and the 5 S RNA is predominantly messenger RNA.

Small peaks of radioactivity corresponding to the ribosomal RNA species can be seen in the profiles in the RNA pulse-labeled after either 20 or 40 min of amino acid deprivation (Fig. 8). The peaks corresponding to the 5 S and 4 S RNA border on the limits of detectability with this method. The reduced levels of the stable RNA species compared to the control samples are striking, despite the fact that these RNA samples were prepared from cells labeled for 4 min rather than the 2-min period used with the control cells. If ribosomal RNA synthesized under these conditions is stable, the increased labeling period would result in a larger proportion of the label residing in the stable species.

**DISCUSSION**

The reduction in the rate of incorporation of labeled precursors into trichloroacetic acid-precipitable material that is observed during amino acid deprivation has been used both to define (2) and detect (32-34) the stringent phenotype in *E. coli*. However, the incorporation of exogenous precursors into RNA is a complex process which may be regulated at least two sites: the entry of the precursors into their cognate triphosphate pools and the incorporation of the triphosphates into RNA. Although the incorporation ratio (the amount of radioactivity incorporated in the absence of amino acid divided by the amount of radioactivity incorporated in the presence of amino acid) commonly reported ranges from 0.1 to 0.2, we previously reported (10) a ratio of 0.005 for CP 78 deprived of threonine. The explanation of the large difference between the ratios that we have observed for CP 78 and those observed by others lies in the manner in which the measurements were made. Most frequently the stringency ratios are measured over extended periods of time, approaching the doubling time of the fully supplemented culture (32, 33). The values reported by us were obtained by the comparing of the amount of label incorporated during an 80-sec period beginning at the 10th min of amino acid deprivation to that observed during a similar period in growing cells. From Fig. 1 it is apparent that the incorporation was measured at a time when the restriction on the entry of uracil into the UTP pool was greatest. Measured at other times after threonine removal, ratios of 0.02 are obtained with uracil. Measured with adenosine, which more readily enters its cognate triphosphate pool, the stringency ratios are substantially larger—of the order of 0.14.

The restriction in incorporation of labeled precursors observed during amino acid deprivation is at best only a very approximate measure of the reduction of the rate of RNA accumulation. The direct relationship between the incorporation of precursors and the rate of “net RNA synthesis” (RNA accumulation) originally described by Nierlich (35) holds only if several conditions are met, including the absence of any significant pool expansion upon the addition of the label precursor (35, 36). That these conditions have not been met in the work presented here is apparent, since incorporation ratios differing by a factor of 7 to 10 can be measured, depending only on whether uracil or adenosine was used.

During threonine deprivation the rate of RNA synthesis estimated from the rate of adenosine incorporation and the specific activity of the ATP pool is reduced to one third of that observed in growing cells. This value agrees well with that estimated by uracil incorporation (10). A similar reduction was observed by Nierlich (8) in a stringent strain of *E. coli* possessing a thermolabile valyl-tRNA synthetase.

It should be pointed out that the rate of RNA synthesis observed during amino acid deprivation is likely to depend on the particular amino acid withheld as well as on the strain of *E. coli* used. When *E. coli* CP 78 is deprived of isoleucine by the addition of valine to the culture medium, the rate of RNA synthesis is reduced by only 10 to 20%, although RNA accumulation abruptly ceases. However, as in the case of threonine deprivation, hybridization competition experiments indicate that the RNA synthesized during deprivation is likely to depend on the particular amino acid withheld as well as on the strain of *E. coli* used. When *E. coli* CP 78 is deprived of isoleucine by the addition of valine to the culture medium, the rate of RNA synthesis is reduced by only 10 to 20%, although RNA accumulation abruptly ceases. However, as in the case of threonine deprivation, hybridization competition experiments indicate that the RNA synthesized during
valine inhibition is substantially enriched in messenger RNA (90% mRNA compared to 60% in the uninhibited control).  

The rate of RNA synthesis observed during threonine deprivation is clearly incompatible with all three models presented in the introductory section. The measured rate of RNA synthesis is much faster than that predicted on the basis of model A (5% of the unstarved rate) and substantially slower than those predicted by models B and C (100% and 60% of the unstarved control). Although these measurements convincingly rule out model A, the possibility must be considered that the rate of RNA synthesis might be reduced during amino acid deprivation by secondary phenomena (37), and that the basic regulatory response of amino acid deprivation might be that indicated by model B or C.

The hybridization-competition experiments indicate that ribosomal RNA species comprise about 10% of the RNA pulse-labeled during threonine deprivation. The stable RNA measured by these experiments does not include any contribution of transfer RNA, since the competing RNA was isolated from washed ribosomes. However, the synthesis of transfer RNA is clearly indicated by the incorporation of methyl groups into 4 S as well as 16 S and 23 S RNA during amino acid deprivation (Fig. 7). Adjusting the estimate of stable RNA upward to include a proportional amount of transfer RNA, the estimated stable RNA content of RNA pulse labeled during amino acid deprivation is 12.5%.

Although a precise quantitative interpretation of the incorporation of methyl groups into RNA is not possible, the results suggest a similar composition for RNA synthesized during amino acid deprivation. During threonine starvation the rate of total RNA synthesis decreased to one-third the control value while the rate of methyl group incorporation decreased to one-tenth. Assuming that the specific activity and the distribution of the methyl groups are the same in the starved and control cells, these results suggest that stable RNA accounts for only 12% of the RNA synthesized during threonine deprivation ((1/10)/(1/3) x 40/100). Since stable RNA comprises approximately 40% of the RNA pulse labeled during normal growth, the values obtained from the hybridization, electrophoresis, and methyl group incorporation experiments indicate that RNA synthesized during amino acid deprivation is considerably enriched in messenger RNA species.

The failure of label to accumulate in stable RNA species during the 4-min labeling of amino acid-deprived cells can conceivably be due to a rapid turnover of newly synthesized ribosomal RNA, as predicted by model B. However, several observations make this possibility unlikely. First, for degradation of newly synthesized ribosomal RNA to account for the hybridization results shown in Figs. 4 to 6, the half-life time of ribosomal RNA would have to be much shorter than that of messenger RNA. Assuming the messenger RNA has a half-time of 4 min at 27°C (38), the calculated half-life time of ribosomal RNA necessary to account for the results is 0.5 min. Although degradation of ribosomal and transfer RNA has been observed under certain conditions (16, 39, 40), the estimated half-life times range from 50 to 500 times longer than that required in the present case. Second, the ribosomal RNA that is labeled and survives during a 4-min labeling period must be resistant to degradation. This conclusion stems from the fact that the measured rate of RNA accumulation in CP 78 deprived of threonine is precisely that predicted from the rate of RNA synthesis (Figs. 2 and 5) and the composition of RNA labeled for 4 min (Figs. 5 and 6). Finally, the data shown in Table I indicate that ribosomal and transfer RNA synthesized and methylated during amino acid deprivation are stable and do not turn over.

In summary, our results indicate that the rate of RNA synthesis during amino acid deprivation is reduced to one-third that of the control and that the RNA synthesized under these conditions is substantially enriched in messenger RNA. Although some ribosomal and transfer RNAs are synthesized, we have been unable to show any degradation of the species. We conclude, therefore, that upon threonine deprivation the rate of messenger RNA synthesis is reduced to 40% and the rate of ribosomal RNA to 10% that observed in unstarved cells. These results are most easily reconciled with the model similar to C but allowing a general reduction of messenger RNA synthesis during amino acid deprivation.

The conclusion of noncoordinate control of RNA synthesis has been reached by several laboratories. These conclusions are based on the behavior of either the general (total) messenger RNA fraction (4, 6, 41) or specific messenger RNAs (4, 5, 11) during amino acid deprivation. However, in two detailed and well documented reports, the opposite conclusion, that of coordinate control of RNA synthesis, is drawn (3, 42). In the first of these, Gros et al. (42) used dinitrophenol to terminate the incorporation of labeled precursors into RNA. The amount of trichloracetic acid-precipitable label that became solubilized during a subsequent 40-min incubation (DNP-labile fraction) was assumed to be proportional to the messenger RNA. However, in the light of more recent evidence, it seems likely that the level of dinitrophenol used did not completely inhibit RNA synthesis and that the dinitrophenol-labile fraction contained ribosomal as well as messenger RNA. The exponential decay of pulse-labeled RNA in the presence of dinitrophenol observed by Gros et al. had a half-life of 6 to 8 min at 37°C. At the same temperature, β-galactosidase messenger RNA (43) or general messenger RNA (44) exhibits a half-life of 1 to 1½ min. This discrepancy suggests that dinitrophenol, while preventing the further incorporation of additional labeled precursors, did not completely inhibit the synthesis of RNA and the recycling of labeled bases. Furthermore, Friesen concludes on the basis of his studies that the dinitrophenol-labile fraction contains ribosomal RNA (3). We believe that the inherent inaccuracy of the dinitrophenol method is sufficient to obscure the change in the proportion of stable RNA predicted by our results (approximately 40% changing to 12% during amino acid deprivation).

In the same report Gros et al. also estimated messenger RNA from the ability of the RNA preparations to stimulate RNA synthesis in vitro. Their results indicated a substantial reduction in the template activity of RNA isolated from amino acid-deprived cells. Although our results also predict a reduction in template activity of RNA-deprived cells, the predicted reduction is substantially less (perhaps one-half of the unstarved control) than that observed by Gros et al. (approximately one-tenth of the unstarved control). We are unable to present a reasonable explanation for this discrepancy.

In the second report Friesen (3) estimates the composition of pulse-labeled RNA isolated from growing and amino acid-deprived cells by hybridization at RNA:DNA input ratios of 0.1 to 0.3. It seems likely that the conditions used by Friesen did

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not allow complete reaction between the RNA fraction and the DNA, since only 20 to 27% of RNA pulse labeled during normal growth formed hybrids, and sequential hybridization of the RNA increased the amount of RNA hybridized by half. Consequently, the sensitivity of the method may not have been sufficient to detect the 1.5-fold increase in mRNA content observed by us (Figs. 4 to 6). Using the same type of analysis as Friesen but using conditions in which a larger fraction of the pulse-labeled RNA hybridized, Stubbs and Hall observed that the percentage of labeled RNA forming hybrids was substantially greater with RNA pulse labeled during amino acid deprivation than with the control RNA (Table 3 of Reference 24).

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