

Decay of Ribonucleic Acid Synthesis in Amino Acid-Starved *Escherichia coli* After Rifampin Treatment

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The concentration of rifampin necessary to affect the initiation of ribonucleic acid (RNA) synthesis quickly in *Escherichia coli* strains K-12 and 15TAU was about 200 $\mu\text{g/ml}$, as determined by extrapolation of the effect of the drug on the induction of β -galactosidase synthesis. A lag in the action of rifampin of about 10 s was confirmed. Rifampin was then used as a probe to compare RNA synthesis in growing and amino acid-starved *E. coli*. Restoring arginine to arginine-starved strain 15TAU immediately after rifampin inhibition did not detectably restore the rate of uracil uptake to that of uninhibited cells. The residual rate of RNA synthesis (corrected for acid-soluble triphosphate specific activities) after rifampin treatment of both growing and isoleucine-starved (valine-inhibited) cultures of strain K-12 showed similar decay kinetics. These findings support the notion that amino acid starvation blocks the initiation of some RNA transcription units, but do not rule out other possibilities.

If growing cells of a "stringent" strain of *Escherichia coli* are deprived of an amino acid, net ribonucleic acid (RNA) synthesis is drastically reduced (3, 20). Most workers now accept the conclusion that this reduction in accumulation is primarily due to a decrease in the net synthesis of ribosomal RNA (rRNA) and reflects a two- to fourfold reduction in gross synthetic rate (4, 7, 11, 12, 15, 17, 22, 23). Some messenger RNA (mRNA) synthesis may also be controlled, although not as completely as rRNA (4, 5, 12, 17).

What step or steps in RNA metabolism are affected by amino acid starvation? The labeling pattern of rRNA made after the re-addition of amino acid to previously starved cells suggests that its synthesis resumes from the beginning of the linked 16 to 23S transcription units (10, 23). This inference is consistent with the notion that amino acid starvation promotes a reduction in the number of RNA-polymerase initiations per unit of time.

In contrast to these experiments, another report indicates that there is no reduction in the number of growing RNA chains in amino acid-starved cultures of *E. coli*, yet confirms that the rate of RNA synthesis is reduced (25). One interpretation of these results (not necessarily favored by the authors) is that amino acid starvation slows the rate of transcription.

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The work reported here examines the point at which RNA synthesis may be damped by amino acid starvation by comparing the residual RNA synthetic capacity of growing and amino acid-starved cells after inhibition of new RNA polymerase initiations (14) with rifampin. Evidence supporting the notion of inhibition of RNA polymerase initiation during amino acid starvation is presented.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strain K-12, prototrophic, *rel*⁺, and valine sensitive, was originally obtained from L. P. Clarke. *E. coli* strain 15TAU, requiring thymine or thymidine, arginine and uracil, *rel*⁺, was obtained from S. S. Cohen. Either Davis and Mingioli (2) minimal medium or a tris(hydroxymethyl)aminomethane (Tris) low-phosphate medium (13) was used. Growth was at 37 C in shake flasks or 27 C in bubbler tubes. One percent glucose (not noted) or 1% glycerol (as noted) was used as carbon sources. Supplements were added at 50 $\mu\text{g/ml}$, except that thiamine was added at 1 $\mu\text{g/ml}$. Growth was measured with a Bausch and Lomb Spectronic 20 Spectrophotometer at 660 nm. All experiments were conducted at a cell concentration of about 5×10^8 cells/ml (approximate optical density at 660 nm = 0.35) after at least three doublings. β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) was induced with 0.2 mM (final concentration) isopropyl β -D-thiogalactopyranoside (IPTG). Assays were performed according to Kepes (9), except that 1 ml of toluene was added instead of 1 drop to insure reproducibility.

Chemicals, radiochemicals, and materials. [2-¹⁴C]uracil (3 to 5 mCi/mmol) was from Schwarz Bio-Research, Inc., or New England Nuclear Corp. [2,8-³H]adenosine (30 Ci/mmol) was from New England Nuclear Corp. [³²P]H₂PO₄ was from Tracerlab or New England Nuclear Corp. Rifampin was a generous gift of Pitman-Moore. It was dissolved at 1 mg/ml in distilled water just before use. Chloramphenicol was a kind gift of Parke, Davis & Co. DL-Amino acids were used throughout. Polyethylene cellulose plates (cellulose polyethyleneimine MN Polygram cel 300 PEI, Brinkmann Instruments, Inc.) were rinsed 0.5 h in distilled water and dried before use.

Estimate of the rate of RNA synthesis. The following procedures were based on the triphosphate separation technique described by Cashel et al. (1) and the methods of Winslow and Lazzarini for determining the rate of RNA synthesis in *E. coli* (24). Two parallel cultures were grown in Tris minimal medium (27 C), one with 5 to 15 μCi/μM ³²P (approximately 2 to 5 μCi of ³²P/ml of medium), the other without added ³²P. The culture grown without ³²P was used to determine the adenosine uptake into trichloroacetic acid-precipitable material. A 100-μliter portion of the culture was mixed with 5 or 10 μliters of [³H]adenosine (5 to 10 μCi) to initiate uptake. At the end of 1 min, 5 ml of ice-cold 5% trichloroacetic acid containing carrier cells was added. After a minimum of 1 h at 0 to 4 C, the suspension was centrifuged at 13,000 × *g* for 10 min. The pellet was resuspended in 5% trichloroacetic acid without added carrier, and re-centrifuged after a minimum of 0.5 h at 0 to 4 C. This wash procedure was repeated three times. After the last wash, the pellet was allowed to drain and was then suspended in 0.5 ml of 1 N NaOH. This suspension was mixed with 10 ml of scintillation fluid consisting of 950 ml of toluene, 950 ml of 2-ethoxyethanol and 12 g of 5-bis-(5-*tert*-butylbenzoxazolyl)-thiophene. The ³H was determined in a Packard liquid scintillation spectrometer.

The cultures grown with ³²P were used to determine the relative specific activities of the triphosphate pools of adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP). After [³H]adenosine labeling as above, 50 μliters of 4 M NaCOOH, sodium formate, pH 3.4, was added to terminate incorporation and extract the triphosphates. After a minimum of 15 min on ice, the suspension was centrifuged for 2.5 min in a Beckman "Microfuge" to sediment the cells. Ten-μliter samples of the clarified formate extract were applied to polyethyleneimine plates, to which 5 μliters of a mixture of authentic ATP, GTP, cytidine 5'-triphosphate, and uridine 5'-triphosphate (10 mM each) had been previously applied. The plates were developed by ascending chromatography with distilled water, dried, and redeveloped with 0.75 M K₂HPO₄, pH 4.0 to 4.5. The areas corresponding to ATP and GTP were located with an ultraviolet lamp, cut out, and placed in scintillation vials with 5 ml of scintillation fluid consisting of 0.5% 2,5 bis-(5-*tert*-butylbenzoxazolyl)-thiophene in toluene. The ³²P and ³H activities were determined in a Packard liquid scintillation spectrometer with appropriate correction for channel overlap. The ratio of ³H to ³²P gave a relative specific activity for each nucleotide. Because

the average adenosine/guanosine ratio in *E. coli* RNA is approximately 1:1 (22), the specific activities were averaged.

The rate of RNA synthesis was then estimated as the trichloroacetic acid-precipitable uptake divided by the pool relative specific activity. This method approximates the pool specific activities during 1 min by measuring them at the end of 1 min after initiation of uptake. Because the specific activities and incorporation rates are approximately linear for longer than 1 min at 27 C (24), the method will give an estimate of the rate of RNA synthesis. Because of the error introduced by estimation, and because absolute pool sizes and the specific activity of [³H]adenosine in RNA are not determined, an absolute rate of RNA synthesis cannot be derived.

Other isotope incorporation studies were done by using a "batch" filter paper method as previously described (6).

RESULTS

Concentration of rifampin necessary to affect *E. coli* immediately. Expression of the lactose operon was used to determine the amount of rifampin needed to inhibit new RNA polymerase initiations immediately in growing *E. coli* cells. A concentration of rifampin sufficient to prevent any induction of the lactose operon when added simultaneously with inducer should be sufficient to block all cellular RNA polymerase initiations, assuming an equal sensitivity of all cellular RNA polymerase molecules to rifampin.

Samples of glycerol-supplemented, growing cultures of *E. coli* strain 15TAU (at 37 C in minimal medium) or of *E. coli* strain K-12 (at 27 C in Tris low-phosphate medium) were mixed with IPTG (0.1 mM final concentration) and increasing final concentrations of rifampin (Fig. 1). The samples were incubated 20 to 30 min to allow the completion and expression of β-galactosidase mRNA. The β-galactosidase activity in each sample and in two uninduced (basal) samples was determined. The amount of enzyme expressed in each sample was plotted against the log of the rifampin concentration. This gave a convenient straight-line intercept with the basal level. This intercept was deemed the minimal inhibitory concentration of rifampin necessary to block RNA synthesis immediately. It was below 200 μg/ml for both the tested strains. These results cannot be generalized for all *E. coli* strains, as a copy of *E. coli* strain CP78 required more than 600 μg of rifampin per ml for inhibition by this test (Rogerson and Ezekiel, unpublished observation).

Speed of rifampin action. How quickly can a high concentration of rifampin act on cellular RNA polymerase? To answer this question, rifampin was added to samples of a culture of *E.*

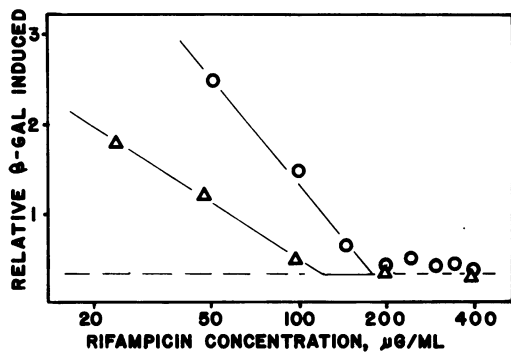


FIG. 1. Inhibition of induction of β -galactosidase synthesis by increasing amounts of rifampin in *E. coli* strains 15TAU and K-12. Portions of glycerol-grown cultures of *E. coli* 15TAU in Davis minimal medium, 37 C (O) or of K-12 growing in Tris low-phosphate medium at 27 C (Δ) were mixed with IPTG at a final concentration of 0.2 mM and rifampin at the various concentrations indicated on the abscissa. The tubes were incubated for 20 min and then assayed for β -galactosidase (ordinate). The dashed line indicates the basal enzyme level, and the points indicate the relative amounts of enzyme observed at the corresponding rifampin concentrations.

coli strain 15TAU growing at 37 C in minimal-glycerol medium at various times shortly before, at the point of, and shortly after, addition of IPTG.

After further incubation, β -galactosidase was assayed in each tube. The amount of β -galactosidase formed at each point was plotted relative to the time of IPTG addition.

If rifampin can block initiation of RNA polymerase as quickly as IPTG can induce β -galactosidase, then there should be no induction when the two are added simultaneously. This is not the case (Fig. 2). Extrapolating from points obtained from rifampin addition after IPTG addition, it is obvious that rifampin will allow 10 s of synthesis of β -galactosidase after addition. The points obtained before addition of IPTG diverge from this extrapolation and indicate that some β -galactosidase synthesis is possible for even longer times (about 30 s). Other experiments, similar in design to those of Kepes (10), confirmed a lack of effect of rifampin on protein synthesis (9, 17).

Effect of rifampin on uracil uptake in starved cultures around the time of arginine re-addition. If amino acid starvation were promoting a slowing of the RNA polymerase molecules as they add nucleotides to growing RNA chains, then re-addition of amino acid to previously starved cultures should promote an almost instantaneous increase in the rate of transcription. Because this rate increase would be almost independent of new initiations, it

should be apparent in cells just after inhibition with rifampin.

Samples of growing culture of *E. coli* strain 15TAU were starved for arginine for approximately 0.5 h. Zero time in each experiment was taken as the time of re-addition of arginine. At 7.5 min before zero time, $[2-^{14}\text{C}]$ uracil was added, and net RNA synthesis was measured as trichloroacetic acid-insoluble counts per 100 μ liters of culture. In a series of experiments, rifampin (300 μ g of final concentration per ml) was added at: -0.5 min, -0.25 min, +0.5 min, +1.0 min, and no addition (Fig. 3). Addition of arginine alone caused an almost immediate increase in the rate of uracil uptake (apparent lag: 10 to 20 s). Addition of rifampin before arginine completely abolished this increase in rate. Addition of rifampin after arginine allowed continued synthesis at a rapid rate for 1 to 2 min after rifampin addition. Experiments where rifampin and arginine were added simultaneously (not shown) gave equivocal results, sometimes giving no increase in rate, and sometimes allowing a small increase.

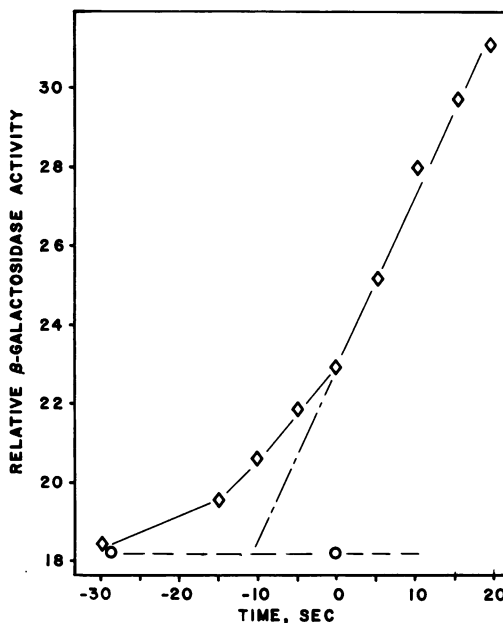


FIG. 2. The time required for rifampin to shut off the capacity for the induction of β -galactosidase. *E. coli* strain 15TAU cells were grown at 37 C in Davis-glycerol minimal medium and induced with 0.2 mM IPTG at time zero. At the times indicated on the abscissa (both before and after zero), 300 μ g of rifampin per ml was added. Each sample was incubated 20 min longer and then assayed for β -galactosidase (ordinate). Each point is an average of four determinations. The horizontal dotted line and circles indicate the basal (uninduced) enzyme level.

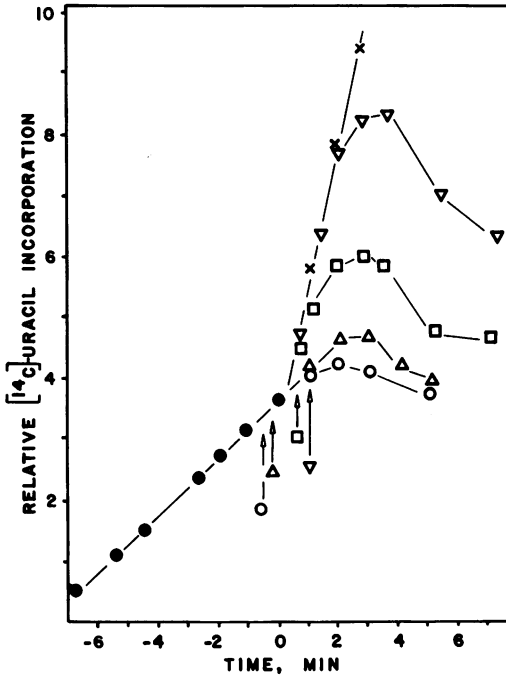


FIG. 3. Effect of rifampin on the stimulation of uracil uptake by arginine in arginine-starved *E. coli* strain 15TAU. *E. coli* 15TAU was grown in minimal medium at 37 C. The cells were centrifuged, washed once, and suspended in medium lacking arginine. After 30 min, 1.5-ml samples were removed for experimentation. First, 1 μ Ci of [14 C]uracil per ml and, after 7.5 min, 200 μ g of arginine per ml were added. At various times before and after the addition of arginine (0 time on the abscissa), 300 μ g of rifampin per ml was added: (O), -0.5 min; (Δ), 0.25 min; (\square), +0.5 min; (∇), 1.0 min; (x), no rifampin; and (\oplus), "average" points before 0 time. Ordinate, trichloroacetic acid-insoluble isotope incorporation (normalized to counts at 0 time). Small arrows indicate rifampin addition corresponding to appropriate points.

Determination of rates of RNA synthesis in growing and amino acid-starved cultures after rifampin addition. Uracil labeling can only be taken as a measure of net RNA synthesis. Furthermore, during amino acid starvation, entry of this base is restricted (25). Thus, it is possible that the above experiments failed to detect slowed transcription because of uracil uptake restrictions. We have, therefore, estimated the actual rates of RNA synthesis by measuring the specific activity of the nucleotide pools and simultaneously measuring short-term incorporation of [3 H]adenosine into RNA (see Materials and Methods).

The idea of these experiments does not depend on the re-addition of amino acid, but rather on measuring the rate at which RNA

synthesis declines in growing and amino acid-starved cultures after the addition of rifampin. If transcription were slowed in amino acid-starved cells, then the rate at which RNA synthesis would decline in these cells should be proportionately much less (in a given time interval) than in growing cells. Implicit in this argument is the hypothesis that the average cistron length transcribed in amino acid-starved cells is approximately the same as in growing cells.

Table 1 presents the data used in estimating the rates of RNA synthesis in growing and isoleucine-starved (valine-inhibited) cultures of *E. coli* strain K-12. Points were taken just before, and at intervals after, the addition of rifampin to 300 μ g/ml.

Figure 4 plots the log of the estimated rate of RNA synthesis divided by the rate of synthesis before rifampin was added versus the time after rifampin addition. It must be emphasized that the absolute RNA-synthetic rate in the amino acid-starved cultures is actually less than in the growing cultures and that the pool sizes cannot

TABLE 1. Parameters used in determining rates of RNA synthesis in growing and amino acid-starved cells of *E. coli* K-12 after rifampin treatment^a

Condition	Time ^b (min)	³² P counts/min in		³ H counts/min in		
		ATP	GTP	ATP	GTP	RNA
Growing ^c	Control	314	172	569	157	7,907
	0.5	300	138	516	120	4,703
	2.0	362	187	617	155	2,707
	3.5	405	235	564	131	1,481
	5.0	335	228	314	96	404
	6.5	451	318	308	98	217
	8.0	529	376	375	113	146
	9.5	504	357	373	108	160
	Starved ^{c, d}	Control	64	27	103	41
0.5		70	24	132	72	1,926
2.0		61	23	110	68	1,768
3.5		94	29	147	68	1,140
5.0		60	22	91	68	740
6.5		65	23	95	65	427
8.0		63	23	80	67	277
9.5		77	29	89	74	160

^a The details of growth, labeling, and measurement are given in Materials and Methods.

^b Rifampin (300 μ g/ml) was added at zero time. Time (min) indicates the time at which a 1-min [3 H]adenosine pulse was begun. Control points were taken in duplicate just before rifampin was added.

^c Growing, average of two experiments; starved, average of three.

^d Starvation for isoleucine was accomplished by the addition of 400 μ g of valine/ml 0.5 h before starting the experiment.

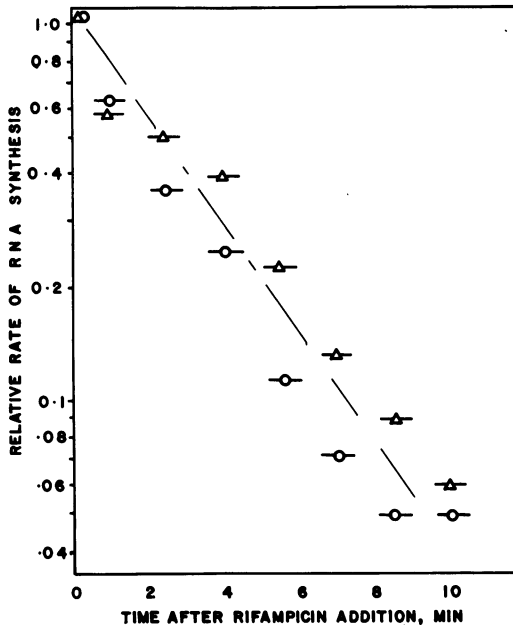


FIG. 4. Rates of RNA synthesis in growing and amino acid-starved *E. coli* K-12 after rifampin addition. The rates were derived from data in Table 1 according to Materials and Methods. Symbols: O, growing culture; Δ, amino acid-starved culture. A 300- μ g portion of rifampin per ml was added at 0 time. Horizontal lines through each point indicate duration of pulse. Points are placed at the center of each pulse period. Note that the experiment was conducted at 27 C.

be determined from the amount of ^{32}P , as no correction for ^{32}P decay was made.

The fall in the relative rate of RNA synthesis in growing and amino acid-starved cultures is virtually identical. These observations indicate probably no uniform inhibition of RNA chain elongation in amino acid-starved cells.

DISCUSSION

An experiment very similar to our experiments probing the effects of the addition of rifampin to arginine-starved *E. coli* 15TAU, both just before and after re-addition of arginine (Fig. 3), has been reported (23). Stamato and Pettijohn used permeabilized cells and re-added amino acid 4 min after the addition of rifampin. Our experiment probed points as close as 15 s before and 30 s after rifampin treatment. The principle of both sets of experiments was the same: if the amino acid control of RNA synthesis in *E. coli* operates by slowing the step of transcription (RNA chain elongation), and if the measure of RNA synthesis used can reflect a rapid increase in rate upon the re-addition of

amino acid, then this rise in rate should be transiently visible in rifampin-treated cells. No such increase in uracil labeling was detected in either experiment, even when, in our experiments, amino acid was re-added 15 s after rifampin inhibition. We demonstrated that, in the absence of rifampin, the apparent rate of RNA synthesis increased within 10 to 20 s after the addition of amino acid and that rapid synthesis would continue for at least 1 min after rifampin addition. Our experiments were done with a minimum period of prelabeling with uracil (7.5 min) in order to obtain the greatest possible relative increase in counts incorporated into RNA, although still allowing some equilibration of the added uracil with the internal triphosphate pool. Although experiments similar to these have been shown to be sensitive enough to measure the ratio of stable to unstable RNA in *E. coli* (18), any experiment utilizing simple uptake of a radioactive precursor of RNA synthesis can be criticized if only the net rate of [^{14}C]uracil incorporation is determined, and no correction is made for the specific activities of the nucleoside triphosphate precursors (17, 25).

We performed experiments of a slightly different sort which do take these parameters into account (Fig. 4). In any case, we must point out that unless rifampin is interfering with uracil uptake in some unknown manner, the re-addition of amino acid should reverse all of the changes caused by amino acid starvation and thus allow detection of a change in the rate of uracil incorporation. Moreover, it has been shown that rifampin does not interfere with the synthesis of guanosine-5'-diphosphate-3'-diphosphate in amino acid-starved cells (5), which makes it appear unlikely that uracil utilization would be impaired by the drug.

In our final series of experiments (Fig. 4), we estimated the gross rate of RNA synthesis by correcting for internal trinucleotide pool specific activity. We determined the kinetics of the decline in rate of RNA synthesis after the addition of rifampin to both growing and amino acid-starved cells. Reid and Speyer (19) have also examined the rifampin-promoted decay kinetics of RNA synthesis in growing cultures, but not in amino acid-starved cultures of *E. coli*. Their results differ from those reported here by indicating a lag before the decay kinetics become exponential. It is difficult to reconcile their results with ours, except to note that they did not determine by an independent test the amount of rifampin necessary to cause nearly instantaneous inhibition.

If transcription (RNA chain elongation) were slowed by amino acid starvation, and if the

length of the transcribed cistrons in amino acid-starved cells is similar to those in unstarved cells, then the residual rate of RNA synthesis should take a proportionately longer time to decline by a given percentage after rifampin inhibition of the amino acid-starved cells than the time required for a similar decline in the unstarved controls. If, on the other hand, the RNA polymerase molecules are inhibited at any point other than chain elongation, the few molecules left free to transcribe should complete their passage along the open cistrons in approximately the same time as those in unstarved cells, and the times needed to complete a similar percentage of RNA synthesis should be similar. The experimental results demonstrate that quite similar times are needed to complete given percentages of RNA synthesis after rifampin inhibition of growing and amino acid-starved cultures. Apparently the step of chain elongation is not uniformly slowed during amino acid starvation.

The amino acid control of RNA synthesis in *E. coli* seems to be partially or completely non-coordinate, i.e., the synthesis of stable RNA is either the primary or the exclusive target of this mechanism. Then our measurements of gross RNA synthesis measure two different capacities, depending on whether or not the cells are growing or are amino acid starved. In the first case, we would measure a sum of stable plus unstable RNA synthesis. In the second, we would measure primarily unstable RNA synthesis. An understanding of how this difference might affect our conclusions could only be reached if we knew the average size of the cistrons being transcribed in each case, as the time to complete RNA synthesis after rifampin inhibition is a function of both chain growth rate and cistron length. The presumably linked 16 to 23S ribosomal RNA cistrons have a length of approximately 5,000 nucleotides. This would require, at a transcription rate of 23 nucleotides/s (at 27 C), about 3.6 min for total transcription, or about 1.8 min for 50% transcription. Our results indicate a half-time for completion of transcription in *E. coli* at 27 C of approximately 2.1 min, indicating that, during growth, the average length of the transcribed cistrons must be greater than 5,000 nucleotides. If the growth rate of RNA were reduced to a uniform 10 nucleotides/s by amino acid starvation, consistent with about a twofold reduction in gross RNA synthesis, the average cistron length would have to be correspondingly reduced to about 2,000 nucleotides. This could occur if ribosomal RNA transcription were halted and if a group of shorter cistrons were

transcribed at a fortuitously slower rate. We consider this possibility unlikely.

Our results suggest that the RNA polymerase is "trapped" by amino acid starvation in such a manner that it must pass through a rifampin-sensitive step (presumably initiation) in order to achieve the rate of RNA synthesis typical of the unstarved cell. This implicates the involvement of some step of RNA synthesis subsequent to the step of chain elongation, such as termination or new initiation. Although these results are necessary to any hypothesis implicating RNA chain initiation as the target of the amino acid regulation of RNA synthesis, they are not sufficient to demonstrate that initiation is the target of this control.

We conclude that most current evidence favors the hypothesis that amino acid starvation of *E. coli* cells damps RNA synthesis by a mechanism that reduces the number of RNA polymerase initiations. This conclusion cannot, however, account for the observations of Winslow and Lazzarini (25), which have been interpreted to indicate no reduction in RNA polymerase initiations during amino acid starvation. It is difficult to postulate a single, unifying control mechanism which would include these puzzling conclusions, but they do suggest that more than one regulatory mechanism may be operating during amino acid starvation.

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