The Control of Ribonucleic Acid Synthesis in Bacteria

POLYMERIZATION RATES FOR RIBONUCLEIC ACIDS IN AMINO ACID-STARVED RELAXED AND STRINGENT AUXOTROPHS OF *ESCHERICHIA COLI*

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(Received 28 January 1972)

Polymerization rates of newly formed chains of various RNA fractions were measured in *Escherichia coli* CP78 (RC^{str}) and CP79 (RC^{rel}) multiple amino acid auxotrophs, deprived of four amino acids essential for growth. Immediately after the onset of severe amino acid deprivation, in RC^{str} strains the rate of labelling of RNA by exogenous nucleotide bases was greatly diminished. At first, the initiation of new RNA chains declined faster than the rate of polymerization in RC^{str} organisms, but as starvation proceeded the rate of polymerization was eventually lowered to about 10% of that found during normal growth. In strain CP79 (RC^{rel}), on the other hand, chain-polymerization rates were unaffected by amino acid withdrawal. Artificial depletion of the intracellular purine nucleotide pools in RC^{str} or RC^{rel} strains by trimethoprim, before the onset of amino acid deprivation, showed that in the RC^{str}, but not the RC^{rel} strain, amino acid withdrawal gave rise to an inability of the cells to utilize exogenously supplied purine or pyrimidine bases for RNA synthesis. During a prolonged starvation, the observed 100-fold decrease in the total rate of incorporation of exogenous nucleotide bases into the RNA of RC^{str} organisms was ascribed to a combination of a tenfold decrease in the overall rate of RNA chain polymerization, at least a fivefold decrease in the ability of the cells to utilize exogenous bases and a preferential inhibition of initiation of stable RNA chains. None of these changes occurred in the corresponding RC^{rel} strain.

In *Escherichia coli* RC^{str} amino acid auxotrophs, the decrease in the rate of RNA synthesis that occurs as a result of amino acid deprivation (Fraenkel & Neidhardt, 1961) is intimately concerned with concomitant changes in the intracellular nucleotide pools. The unusual nucleotide (ppGpp) that accumulates in amino acid-starved *E. coli* RC^{str} cultures (Cashel, 1969; Cashel & Gallant, 1969; Cashel & Kalbacher, 1970) is an inhibitor of the enzyme IMP dehydrogenase, an essential step in the pathway of new guanine nucleotide synthesis (Gallant *et al.*, 1970). Thus the resulting major decrease in the GTP concentrations in deprived RC^{str} organisms (Gallant & Harada, 1969) could eventually act as a rate-limiting factor in RNA synthesis in these conditions. In other situations (e.g. purine starvation of purine-requiring auxotrophs of *E. coli*) the intracellular concentration of GTP is an important factor in determining the rate of RNA synthesis and the nature of the RNA produced (Gallant & Harada, 1969; Thomas *et al.*, 1970; Varney *et al.*, 1970). In RC^{rel} organisms, however, nucleotide pool depletion was not observed when the cells were deprived of amino acids (Gallant & Harada, 1969), and thus high rates of RNA synthesis were still possible (Stent & Brenner, 1961).

ppGpp can also act *in vitro* as a specific inhibitor of initiation of chains of stable RNA (rRNA+tRNA) (Travers *et al.*, 1970; Travers, 1971). Thus, in amino

acid-starved RC^{str} organisms, the synthesis of stable RNA is repressed to a relatively greater extent than is mRNA synthesis (Sarkar & Moldave, 1968; Lazzarini & Winslow, 1970; Lazzarini & Dahlberg, 1971; Stamato & Pettijohn, 1971; Gray & Midgley, 1972). Though Stamato & Pettijohn (1971) have explained the early effects of amino acid starvation in RC^{str} auxotrophs in terms of a fourfold decreased rate of rRNA+tRNA synthesis directed at the level of chain initiation, it remained possible that, as the GTP concentrations declined in starved cells, RNA chain-polymerization rates would be lowered by the secondary action of a purine deprivation. This effect would further inhibit initiation of RNA chains commencing with pppG---, rather than those commencing with pppA---, since Anthony *et al.* (1969) showed that RNA chain initiation is more sensitive to decreased concentrations of ATP or GTP than is chain elongation. In this case, if stable RNA chains generally commence with pppG--- (Altman & Smith, 1971) their initiation would be strongly inhibited, whereas mRNA chains beginning with pppA--- (Jorgensen *et al.*, 1969) would be efficiently initiated but only slowly polymerized.

In the present paper we have attempted to quantify the contributions of various parameters governing a change in the overall rate of RNA synthesis during prolonged amino acid starvation of RC^{str} amino

acid auxotrophs. In contrast with the results of Stamato & Pettijohn (1971), we have found that all RNA species in deprived cultures are eventually polymerized at a lower rate than found for normally growing cells. The combination of a decrease in the amount of stable RNA species produced, together with a fall in the ability of the cells to utilize exogenous nucleotide bases, also accounts fully for the observed decrease in the rate of RNA labelling in these conditions. Conversely, the RC^{rel} mutation allows amino acid-starved cultures to polymerize and initiate their RNA chains normally, and to incorporate exogenous labelled nucleotide bases efficiently into the cells.

Materials and Methods

Organisms

E. coli CP78 (RC^{str}) and CP79 (RC^{rel}) were used (Gray & Midgley, 1972).

The cultures were subjected to amino acid starvation either according to the method of Gray & Midgley (1972) or by a rapid filtration technique. In the latter cells were filtered from supplemented media through a large membrane filter (142 mm diam., 0.2 μ m pore size; Sartorius Membranfilter G.m.b.H., Göttingen, W. Germany). The cells on the filter were washed once with an equal volume of prewarmed medium lacking amino acids and were then resuspended in prewarmed starvation medium (glucose-salts). The whole period of filtration, washing and resuspension took not more than 90 s.

DNA-RNA hybridization

E. coli DNA was purified, denatured and immobilized on cellulose nitrate membrane filters, and RNA was hybridized as described by Gillespie & Spiegelman (1965) and Pigott & Midgley (1968). The methods of producing purified RNA for hybridization were as described by Pigott & Midgley (1968). For tRNA, unbroken cells were extracted with phenol-cresol (50:7, w/v) to avoid contamination by large rRNA molecules and by DNA (Brubaker & McCorquodale, 1963).

Ion-exchange column chromatography of RNA

tRNA was purified by column chromatography on DEAE-cellulose (BioRad Cellex D, 0.87 mequiv./g; Calbiochem., Los Angeles, Calif., U.S.A.) as described by Midgley (1962) and Gray & Midgley (1970). In these experiments a 9 cm $\times$ 1 cm column was used, and the RNA was eluted with a linear gradient (400 ml) of 0.4–0.8 M-NaCl in 10 mM-tris-10 mM-MgCl₂ adjusted to pH 7.2 with HCl. In these conditions any DNA was eluted at about 0.52 M-NaCl

(but did not usually appear in the mixture), tRNA at 0.58–0.6 M-NaCl, and 5S rRNA (the only rRNA molecule extracted) at 0.66 M-NaCl. Checks on the authenticity of the tRNA peak were initially made by chromatography with pure *E. coli* tRNA prepared from strain MRE 600 (obtained from the Microbiological Research Establishment, Porton, Wilts., U.K.). In all tests with labelled tRNA, the specific radioactivity of this fraction was constant across the peak.

Determination of DNA

Duplicate samples (20 ml) of bacterial cultures were pipetted into 1 ml volumes of ice-cold 5 M-perchloric acid. After 10 min the precipitated cells were centrifuged at 8000 g_{av} for 10 min in the 8 $\times$ 50 anglehead rotor of an MSE High Speed 18 centrifuge, cooled to 2°C. The pellet was carefully resuspended in 4 ml of 0.5 M-perchloric acid and the suspension was heated at 70°C for 20 min. It was again centrifuged at 5000 g_{av} for 10 min, and 2 ml of the resulting clear supernatant fluid was taken for assay by the modified diphenylamine test of Burton (1956).

Antibiotics

Chloramphenicol was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Rifampicin was a gift from Lepetit Pharmaceuticals, Maidenhead, Berks., U.K. Trimethoprim lactate was a gift from Burroughs Wellcome and Co., Dartford, Kent, U.K.

Radiochemicals

[2-¹⁴C]Uracil (specific radioactivity 54.5 mCi/mmol), [5-³H]uracil (specific radioactivity 1 or 28 Ci/mmol), [8-¹⁴C]adenine sulphate (specific radioactivity 51.5 mCi/mmol) and [8-¹⁴C]guanine (specific radioactivity 58 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Chain-polymerization rate for RNA in steadily growing cultures

We wished to check that the high concentrations of rifampicin used in this study were capable of inhibiting RNA chain initiation rapidly. Experiments were carried out on strains CP78 (RC^{str}) and CP79 (RC^{rel}) to compare the time for the completion of residual RNA labelling in steadily growing cultures with the corresponding time for strain MRE 600 (Gray & Midgley, 1971). In strain MRE 600 it was already known that a concentration of 0.1 mg of rifampicin/ml was sufficient to abolish RNA chain initiation within 20 s (Gray & Midgley, 1971; Vickers & Midgley, 1971).

Cultures of *E. coli* CP78 and CP79 were grown in amino acid-supplemented glucose-salts media (Gray & Midgley, 1972). When the turbidity of the cultures had reached E_{650} 0.5, [2- 14 C]uracil (0.1 μ Ci/ml; specific radioactivity 54 mCi/mmol) was injected. After 2–3 min rifampicin (0.1 mg/ml) was added. Samples (1 ml) were pipetted into either ice-cold 10% (w/v) trichloroacetic acid or 1 M-KOH. The acidified samples were filtered. After 18 h incubation at 37°C, the KOH samples, containing alkali-resistant labelled DNA, were also acidified and filtered. Thus the residual labelling of RNA and DNA could be monitored in the rifampicin-inhibited cells (Gray & Midgley, 1971). The results for the two strains of *E. coli* are shown in Fig. 1.

The kinetics of residual RNA labelling in each strain was compared with that for strain MRE 600 (Gray & Midgley, 1971). After about 2½–3 min net incorporation of radioactivity into the RNA ceased, as had been found for the RC^{str} wild-type strain MRE 600 (Gray & Midgley, 1971).

Chain polymerization rate for RNA in amino acid-starved RC^{str} cultures

Experiments similar to those described in Fig. 1 were carried out with non-growing cultures of *E. coli* CP78 (RC^{str}) suspended in amino acid-free glucose-salts defined medium. Cultures (50 ml), at a turbidity E_{650} 0.5, were either filtered rapidly through a membrane and washed with prewarmed glucose-salts medium (2 min to 10 min starvation experiments) or centrifuged from supplemented medium and resuspended in prewarmed 'starvation medium' (20 min and 30 min starvation experiments). At the appropriate time after resuspension in non-supplemented medium rifampicin (0.1 mg/ml) was added. After a

further 30 s [2- 14 C]uracil (0.1 μ Ci/ml; specific radioactivity 54 mCi/mmol) was added. At various times after this, samples (10 ml) of the inhibited culture were removed and pipetted into tubes containing the four required amino acids (50 μ g/ml final concn. for each amino acid). Residual labelling of RNA was followed in both the amino acid-supplemented and the unsupplemented cultures by rapid sampling and acid precipitation. All labelling curves were corrected for interference by DNA labelling, by measuring the alkali-stable acid-precipitable radioactivity. The corrected labelling curves are shown in Fig. 2. Other experiments were also carried out with [8- 14 C]adenine and [8- 14 C]guanine as RNA precursors. These gave results essentially the same as those obtained with [2- 14 C]uracil (J. E. M. Midgley & M. Bell, unpublished work).

The duration of amino acid withdrawal in strain RC^{str} cultures had a considerable effect on the kinetics of residual RNA labelling after rifampicin inhibition. This occurred with either unsupplemented cultures or with those supplemented with amino acids after the antibiotic was added. After the shortest period of amino acid deprivation (Fig. 2a), the labelling of the RNA after addition of rifampicin was stopped almost as quickly as in states of steady bacterial growth. Thus, if amino acids were added to a culture that had been deprived of supplements for 2 min and had been inhibited by rifampicin for a further 5 min, the labelling of the RNA was only slightly affected, compared with the corresponding large differences in cultures starved of amino acids for longer periods before addition of the antibiotic (Figs. 2b–2e). In the same experiment, there was no effect on RNA labelling if amino acids were added 10 min after rifampicin inhibition (Fig. 2a). Since amino acid-starved RC^{str} cultures were somewhat

Fig. 1. Residual labelling of nucleic acids in rifampicin-inhibited RC^{str} and RC^{rel} strains of *E. coli*

(a) Strain CP78 (RC^{str}); (b) strain CP79 (RC^{rel}). ●, DNA labelling; ○, RNA labelling. Rifampicin was added as indicated by the arrow.

slow in their response to the addition of rifampicin, compared with steadily growing cells (Gray & Midgley, 1972), it would seem that, during amino acid starvation of RC^{str} strains, chain-polymerization rates for RNA were initially unaffected, whereas the rate of chain initiation (especially those of the stable RNA species) were considerably diminished (Stamato & Pettijohn, 1971). However, as the period of severe multiple amino acid withdrawal continued, RNA polymerization rates appeared to be substantially

decreased, leading to a gradual accumulation of polymerases upon the DNA cistrons. Thus, as shown in Figs. 2(a)–2(e), the release of constraints on RNA chain polymerization during longer periods of starvation resulted in a larger residual incorporation of radioactive precursors into RNA after rifampicin inhibition. This process seemed to reach a maximum after about 20 min suspension of the culture in media lacking the required amino acids. The effect of chloramphenicol (0.1 g/l) was identical with that of amino

Fig. 2. (a-d)

Fig. 2. Residual labelling of RNA in amino acid-starved rifampicin-inhibited *E. coli* CP78 (RC^{str})

Rifampicin was added at the following times after amino acid deprivation: (a) 2 min; (b) 5 min; (c) 10 min; (d) 20 min; (e) 30 min. Amino acids were added to samples of the inhibited cultures as indicated by the arrows. The labelling of DNA was corrected for in all curves. (a)–(d) $\circ$, Culture inhibited with rifampicin and given no amino acids; $\bullet$, culture supplemented with the four required amino acids after 5 min inhibition with rifampicin; $\ominus$, culture supplemented with the four required amino acids after 10 min inhibition with rifampicin. (e) Culture inhibited with rifampicin and given no amino acids ($\circ$); cultures were supplemented with amino acids at the following times after inhibition with rifampicin at zero: $\circ$, 30 s; $\bullet$, 10 min; $\ominus$, 20 min; $\omin�$, 30 min; $\omin�$, 40 min; $\bullet$, 60 min.

acid supplementation on residual RNA synthesis in rifampicin-inhibited, amino acid-starved RC^{str} cultures (W. J. H. Gray, unpublished work).

The progressive increase in the potential amount of residual RNA labelling as amino acid deprivation continued can thus be produced by additional polymerases on the cistrons, arising from reduced rates of RNA chain polymerization. However, this explanation will not suffice to account completely for the large increases (up to tenfold) in the amount of residual RNA labelling obtained when starved cultures were given first the antibiotic, and then shortly afterwards the required amino acid supplements (e.g. Fig. 2e). In starved cultures given rifampicin, but no amino acids, the slow rate of RNA chain polymerization may be more nearly balanced by RNA degradation than in cases where the addition of amino acids momentarily accelerates polymeriza-

tion rates. As the former situation would result in a decrease in the net inflow of exogenous RNA precursors, this may in part explain some of the differences in labelling. Another factor bearing on this phenomenon may be that RC^{str} auxotrophs deprived of the necessary amino acids for growth are relatively deficient in their ability to utilize exogenous nucleotide bases for the synthesis of RNA (Edlin & Neuhaud, 1967). Further findings reported in the present paper confirm that this factor also plays a part in determining the amount of RNA labelling in starved RC^{str} cultures (see Figs. 3 and 5).

If the addition of amino acids causes a more effective utilization of exogenous labelled RNA precursors for residual RNA synthesis in rifampicin-inhibited cultures, the effect of a transient, though rapid, rate of residual RNA labelling should be mirrored in the extent to which DNA is labelled in

Fig. 3. Labelling of DNA in rifampicin-inhibited amino acid-starved culture of *E. coli* CP78 (RC^{str})

The experiment was the same as described in Fig. 2(e) except that the labelling of DNA was followed by the method described in the text. o, Culture inhibited with rifampicin and given no amino acids; cultures supplemented with amino acids at the following times, after inhibition with rifampicin at zero: ●, 30s; ○, 10min; ⊖, 30min; ⊕, 40min; ⊗, 60min.

Fig. 4. Residual labelling of nucleic acids in rifampicin-inhibited amino acid-starved *E. coli* CP79 (RC^{rel})

●, DNA labelling; ○, RNA labelling. The culture was deprived of amino acids for 30 min before the labelled uracil was added. Rifampicin was added as indicated by the arrow.

the cells. Fig. 3 shows that this was the case. In a culture of RC^{str} cells starved of amino acids for 30 min, the extent of residual DNA labelling depended on how much residual RNA synthesis was still permissible after antibiotic inhibition. When residual RNA synthesis had stopped, 25–30 min after rifampicin inhibition, the addition of amino acids still caused an increase in the ability of the cells to utilize exogenous precursors of nucleic acids for DNA synthesis. Table 1 shows that these effects did not arise from an increased production of DNA by antibiotic-inhibited amino acid-supplemented cells. There is no detectable difference in the amount of DNA produced after antibiotic inhibition of non-growing *E. coli* CP78 cultures, whether or not the required amino acids are added 5 min later.

In strain CP79 (RC^{rel}) the effects of amino acid-deprivation had little effect on the rate of RNA chain polymerization compared with the steadily growing cultures (Fig. 4). However, the inability of the cells to stabilize the new RNA formed gave rise to a slow

continuous loss of the residual labelled RNA after rifampicin inhibition. This situation can be compared with the equivalent case of RC^{str} cultures inhibited with high concentrations of chloramphenicol (Midgley & Gray, 1971).

Ability of amino acid-depleted cultures of E. coli CP78 (RC^{str}) and CP79 (RC^{rel}) to utilize exogenous nucleotide bases for RNA synthesis

The experiments described above indicated that amino acid-deprived cultures of RC^{str} strains were

deficient in their ability to utilize exogenous nucleotide bases for the synthesis of either RNA or DNA (Gallant & Cashel, 1967; Edlin & Neuhaud, 1967). Further experiments were designed to study the relationship between any changes in purine nucleotide concentrations that might occur in deprived RC^{str} cells and the observed shutdown of the uptake of exogenous bases. Growing cultures of strains CP78 and CP79 were inhibited by the addition of trimethoprim lactate (50 μ g/ml), which is known to inhibit the enzyme dihydrofolate reductase (Burchall & Hitchings, 1966). In such cells a general depletion of

Fig. 5. Residual labelling of RNA in amino acid-starved rifampicin-inhibited RC^{str} and RC^{rel} strains of *E. coli* that had been previously starved of purines by trimethoprim

(a) Strain CP78 (RC^{str}); (b) strain CP79 (RC^{rel}). Labelling of RNA: ●, in the presence of the four required amino acids + adenine + guanosine; ◐, in the presence of adenine + guanosine; ○, in the presence of the four required amino acids; ○, in unsupplemented cultures. The labelling of DNA was corrected for in all curves.

Table 1. Production of DNA in amino acid-starved cultures of *E. coli* CP78 (RC^{str}) after inhibition of RNA chain initiation by rifampicin

The amount of DNA/unit volume of the culture was taken as 100% at the time of addition of rifampicin. The culture was suspended in amino acid-free glucose-salts medium for 30 min at 37°C before the antibiotic was added. Initial turbidity of culture at zero time in the table was E_{650} 0.52. All results are the means of duplicate determinations by the Burton (1956) diphenylamine method.

Time after addition of rifampicin (min)	DNA content of culture (%)	
	Unsupplemented culture	Culture supplemented with amino acids after addition of rifampicin
0	100	100
15	113	118
30	125	120
60	127	125

tetrahydrofolate occurs, since its pool is affected by the production of the methyl group in thymidylic acid for DNA synthesis (Bertino & Stacey, 1966). Eventually this drain on tetrahydrofolate will cause a decline in the cellular ability to produce new purine nucleotides, since the involvement of tetrahydrofolate is crucial in this system (Magasanik, 1962). In the absence of exogenous purine supplementation of trimethoprim-inhibited cultures, the bacteria thus become unable to sustain RNA synthesis through a lack of purine nucleotides (R. J. Smith & J. E. M. Midgley, unpublished work). In effect, this situation is analogous to the deprivation of purine-requiring mutants of exogenously supplied purines. In this case it has been shown that decreases in especially the GTP concentrations in such mutants give rise to a crowding of DNA-dependent RNA polymerase molecules on the cistrons, owing to a decrease in the rate of RNA chain polymerization (Thomas *et al.*, 1970). We therefore designed experiments to test the ability of RC^{str} and RC^{rel} mutants to take up exogenous purines in various environments, and thus to relieve, at least temporarily, the blocks on polymerization of RNA chains induced by purine lack.

A culture (100ml) of *E. coli* CP78 (RC^{str}) was grown to a turbidity E_{650} 0.5. At this time trimethoprim lactate (5mg) was added and the cells were incubated for 10min. Net RNA and DNA synthesis had ceased by this time (J. E. M. Midgley, unpublished work), presumably the result of purine deprivation. The cells were now centrifuged and re-suspended in trimethoprim-supplemented glucose-salts media without the required amino acids (for details see Gray & Midgley, 1972). The culture was incubated for 20min at 37°C in the presence of unlabelled uracil (0.01 mg/ml). At this time $5\mu\text{Ci}$ of $[2-^{14}\text{C}]\text{uracil}$ was added, together with 5mg of rifampicin to inhibit RNA chain initiation. The culture was divided into four 25ml samples. One was a control, and to the others were added (i) the required amino acids at a final concentration of $50\mu\text{g/ml}$, (ii) 1 mg (each) of adenine and guanosine and (iii) the required amino acids+adenine+guanosine. The kinetics of residual RNA labelling are shown in Fig. 5(a). Only in the presence of required amino acids does exogenously supplied adenine+guanosine relieve the block on RNA synthesis brought about by purine deprivation. Of more interest is the finding

Fig. 6. Residual labelling of mRNA in amino acid-starved rifampicin-inhibited *E. coli* CP78 (RC^{str})

○, Culture inhibited with rifampicin and given no amino acids; cultures were supplemented with amino acids at the following times after inhibition with rifampicin at zero: ●, 30s; ◐, 10min; ◑, 20min. ●, Labelling of total RNA in unsupplemented culture. Amino acids were added to samples of inhibited cultures as indicated by the arrows. The labelling of DNA was corrected for in the production of the curve for total RNA.

that prior purine starvation, which presumably chiefly concerns guanine nucleotides, since these seem to be rate-limiting for RNA synthesis (Gallant & Harada, 1969; Thomas *et al.*, 1970), does not prevent the later effect of amino acid withdrawal on the utilization of exogenous guanosine. This suggests that the agency through which amino acid deprivation inhibits exogenous base utilization may not work directly through changes in purine nucleotide pool concentrations in the cells, since the guanine and adenine nucleotide pools were already diminished sufficiently to prevent net RNA synthesis before amino acids were removed. However, the RC^{rel} mutation abolishes these effects of withdrawal, such that even in the absence of amino acids the exogenous adenine+guanosine can relieve the prior trimethoprim-induced purine deprivation (Fig. 5b).

An experiment designed in a way identical with those described in Fig. 2 was carried out on cultures of *E. coli* CP78 (RC^{str}) suspended in amino acid-free glucose-salts media for 30 min. In this case, however, [5-³H]uracil (2 µCi/ml; specific radioactivity 28 Ci/mmol) was added with the rifampicin. At intervals samples were removed from the inhibited cells and supplemented with the amino acids. From these, and from the main unsupplemented cultures, samples were removed and the RNA was extracted. It was then hybridized with DNA (DNA/RNA ratios 5:1, w/w) as described by Pigott & Midgley (1968). The results are shown in Fig. 6.

The residual labelling of mRNA continues for about 15–20 min after rifampicin inhibition of starved RC^{str} cultures. After this time it decayed very slowly. If, at any time before the completion of residual labelling of the mRNA, the required amino acids are added back, the decay of the remaining labelled mRNA was faster (Gray & Midgley, 1972). Further, as with the total RNA labelling curve, amino acid addition before the end of residual mRNA labelling increased the actual specific radioactivity of the completed chains and increased their rate of polymerization. Thus although the rate of polymerization of mRNA and rRNA cannot be compared directly, owing to the unknown effects of the molecular heterogeneity of the mRNA fraction on the labelling patterns, it is probable that the overall rate of mRNA polymerization in RC^{str} culture is also decreased by about tenfold after a considerable period of amino acid withdrawal. The rate of polymerization of mRNA chains in the corresponding RC^{rel} strain is unaffected by amino acid withdrawal (Fig. 4; Gray & Midgley, 1972).

Rate of tRNA chain polymerization in RC^{str} and RC^{rel} strains in various conditions

Cultures (500 ml) of *E. coli* CP78 (RC^{str}) or CP79 (RC^{rel}) were grown at 30°C in glucose-salts media supplemented with the required amino acids. The

Fig. 7. Residual labelling of tRNA in amino acid-supplemented and unsupplemented cultures of *E. coli* CP78 (RC^{str}) and CP79 (RC^{rel}) after rifampicin inhibition

(a) Supplemented RC^{str} culture; (b) unsupplemented RC^{str} culture; (c) unsupplemented RC^{rel} culture.

lower temperature was chosen because of the inconveniently short time available for residual tRNA labelling after rifampicin inhibition at the higher temperature of 37°C (Vickers & Midgley, 1971). Then the bacteria were resuspended in amino acid-free glucose-salts media (Gray & Midgley, 1972) and incubated for 30 min. Rifampicin (50 mg) was added, together with 10 µCi of [5-³H]uracil (specific radioactivity 1 or 28 Ci/mmol). At intervals samples (50 ml) of the rifampicin-inhibited culture were poured on to ice and the tRNA was extracted and chromatographed on DEAE-cellulose (see the Materials and Methods section). The specific radioactivity of the tRNA

fraction was calculated as an average across the peak. The results for tRNA labelling in amino acid-starved CP78 (RC^{str}) and CP79 (RC^{rel}) strains after rifampicin inhibition are shown in Fig. 7. For comparison, residual tRNA labelling was also studied in growing cultures of amino acid-supplemented CP78 (RC^{str}) strain (Fig. 7). Whereas it took only about 30–35 s at 30°C to complete residual tRNA labelling in growing cultures of strain CP78 (RC^{str}) or starved cultures of strain CP79 (RC^{rel}), the period of labelling was extended about 20-fold in starved RC^{str} cells. Although some fraction of this time (up to 5 min) may be considered to include the time required for the full reaction of rifampicin in the cells (Gray & Midgley, 1972), it is clear that, as with total RNA, and mRNA, the rate of chain polymerization of the tRNA molecules is also considerably depressed by amino acid deprivation of RC^{str} mutants. This is not so in the corresponding situation in RC^{rel} strains.

Discussion

In RC^{str} strains of bacteria, the withdrawal of amino acids necessary for growth leads to a considerable decrease in the rate at which RNA is accumulated. A current explanation of this phenomenon suggests that the primary control mechanism in the cell is directed at the initiation of stable rRNA and tRNA chains rather than at the initiation of mRNA chains (Edlin *et al.*, 1968; Lavallé & de Hauwer, 1968; Lazzarini & Winslow, 1970; Lazzarini & Dahlberg, 1971; Stamato & Pettijohn, 1971). In addition, Stamato & Pettijohn (1971) have shown that, when an amino acid RC^{str} auxotroph is deprived of one required amino acid, the rate of polymerization of nascent RNA chains early in the period of deprivation is virtually the same as in conditions of steady growth. Therefore this situation results in the predominance of mRNA in the labelling patterns of total cellular RNA during amino acid withdrawal (Sarkar & Moldave, 1968; Lazzarini & Winslow, 1970; Lazzarini & Dahlberg, 1971). Also, since initiation of nascent chains of stable RNA species is constrained, there is a decrease in the number of polymerases specifically concerned with the transcription of stable RNA species (Stamato & Pettijohn, 1971). However, studies on the fate of the mRNA molecules formed during amino acid deprivation remain somewhat conflicting. Whereas some reports (e.g. Lavallé & de Hauwer, 1968) have indicated that specific mRNA species are degraded at normal rates in non-growing cells, others have suggested that mRNA chains may be stabilized against the degradation potentiated by unsuppressed non-terminal 'nonsense' mutations (Morse & Guertin, 1971). This may be the result of a general protection of mRNA molecules from nuclease action, arising from a decrease in ribosome translational movement caused by lack of

amino acids. Nevertheless the amount of mRNA in starved RC^{str} cells, as compared with those in normal growth, is little changed (Friesen, 1966; Lavallé & de Hauwer, 1968; Gray & Midgley, 1972).

In our studies on the rates of RNA polymerization in severely starved multiple amino acid RC^{str} auxotrophs some of the results are clearly in conflict with the above model. In agreement with the results of Stamato & Pettijohn (1971), the decrease in the rate of RNA labelling early in the period of amino acid withdrawal results principally from an inhibition in chain initiation, combined with a greatly diminished capacity for the intracellular utilization of exogenous nucleotide bases (Edlin & Neuhaard, 1967). However, as the period of deprivation lengthens, it seems that a continuous decline occurs in the rate of polymerization of nascent RNA chains, to values that are eventually 10–20% of those holding in states of steady growth. This affects all RNA species to a similar degree, and leads to a progressive accumulation of DNA-dependent RNA polymerases on the cistrons. Therefore, though initiation of RNA chains (mainly involving those cistrons for stable RNA) is preferentially inhibited at first, further amino acid deprivation leads to such a general decrease in the rate of polymerization of nascent chains that this, in turn, superimposes a secondary limitation on the rate of RNA synthesis. At the same time the production of mRNA relative to stable RNA is maintained in favour of the former, presumably because of the continuation of the primary constraint on stable RNA initiations.

Though explanations of the preferential inhibition stable RNA synthesis in such conditions have been ascribed to the elevations of intracellular concentrations of ppGpp (Travers *et al.*, 1970; Travers, 1971), an additional mechanism may come into play in especially severe deprivations where several amino acids are simultaneously withdrawn. Gallant & Harada (1969) observed that, at the onset of amino acid starvation in RC^{str} cells, considerable decreases occurred in the intracellular GTP pool, whereas the concentrations of the other nucleoside triphosphates were little changed. Although it is possible that in their conditions and in other work (e.g. Stamato & Pettijohn, 1971) the diminished GTP concentration was not sufficient by itself to affect RNA synthesis, in our studies it is probable that the observed lowering of the rate of RNA chain polymerization arose from such a decrease in GTP concentration. As it is known that the initiation of RNA chains commencing with pppG--- is more sensitive to decreased GTP concentrations than is the later process of polymerization (Anthony *et al.*, 1969), it would seem that both processes can be inhibited *in vivo* by decreases in pool concentrations if the conditions are sufficiently severe. At the present moment the exact status of ppGpp as an inhibitor of RNA chain initiation is not entirely

clear. Nor has the existence of this compound been explained unequivocally as a cause or consequence of the inhibition of RNA synthesis in bacteria (e.g. Winslow, 1971). We believe that it is at least as likely that decreases in GTP concentrations in the cells can themselves partly determine which RNA chains will be successfully initiated and transcribed, and that the extent of nucleotide pool changes is affected by the severity of amino acid withdrawal. Possibly our results are more comparable with those involving stepdown of RC^{str} organisms from a medium supporting a faster rate of growth to one allowing only slow growth, in which it might be supposed that multiple temporary deficiencies arise. Indeed, Winslow (1971) has shown that, in such conditions, the rate of RNA synthesis in RC^{str} cells is only 5% of that found in normal growth.

The RC^{rel} auxotroph is not detectably affected by multiple amino acid deprivation, either in the relative rates of synthesis of the various RNA species, or in its ability to take up exogenous base supplements in the absence of amino acids, or in its rates of RNA chain initiation and polymerization (e.g. see Winslow & Lazzarini, 1969). We find no direct relationship between changes in purine nucleotide concentrations in the cells and the onset of any cellular deficiency in the uptake of exogenous bases. The state of the RC function appears to be dominant over such changes. However, some evidence suggests that the RC function and the problem of base uptake from the medium can be expressed separately, as trimethoprim-inhibited bacteria display the RC^{str} or RC^{rel} control of RNA synthesis, at the same time showing no lack of ability to take up exogenous bases (Shih *et al.*, 1966; Edlin & Stent, 1969). Purine deprivation of cells does not prevent the later imposition of the RC^{str} uptake controls by amino acid withdrawal, and it is therefore possible that ppGpp accumulation does not occur so readily in these conditions, since it may not be produced efficiently from a very decreased GTP pool.

W. J. H. G. thanks the Medical Research Council for a postgraduate studentship. J. E. M. M. and T. G. V. are Senior Research Associates in the Medical Research Council Research Group on the Structure and Biosynthesis of Macromolecules. We thank Mrs. P. Hislop and Mrs. M. Bell for their excellent technical assistance. Thanks are also due to Professor K. Burton and others in the Department of Biochemistry, University of Newcastle upon Tyne, for their helpful comments and suggestions.

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