Turnover as a Control of Ribonucleic Acid Accumulation in Bacteria Undergoing Stepdown

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The synthesis of ribosomes was compared in *rel*⁺ and *rel*⁻ strains of *Escherichia coli* undergoing 'stepdown' in growth from glucose medium to one with lactate as principal carbon source. Two strains (CP78 and CP79), isogenic except for *rel*, showed similar behaviour with respect to (1) the kinetics of labelling total RNA and ribosomes with exogenous uracil, (2) the proportion of newly formed protein that could be bound with nascent rRNA in mature ribosomes, and (3) the rate of induction of enzymically active β -galactosidase (relative to the rate of ribosome synthesis). It was concluded that, as there was no net accumulation of RNA during stepdown in either strain, rRNA turnover must be occurring at a high rate. The general features of ribosome maturation in *rel*⁺ and *rel*⁻ cells were almost identical with those found in auxotrophic *rel*⁺ organisms starved of required amino acids. In both cases, there was a considerable delay in the maturation of new ribosomal particles, owing to a relative shortfall in the rate of synthesis of ribosome-associated proteins. Only about 4-5% of the total protein labelled during stepdown was capable of binding with newly formed rRNA. This compared with 3.5% for *rel*⁺ and 0.5% for *rel*⁻ auxotrophs during amino acid starvation. The turnover rate for newly formed mRNA and rRNA was virtually the same in 'stepped-down' *rel*⁺ and *rel*⁻ strains and was similar to that of the same fraction in amino acid-starved *rel*⁺ cells. The functional lifetime of mRNA was also identical. It seems that in the *rel*⁻ strain many of the characteristics typical of the isogenic *rel*⁺ strain are displayed under these conditions, at least as regards the speed of ribosome maturation and the induction of β -galactosidase. Studies on the thermolability of the latter enzyme induced during stepdown indicate that inaccurate translation, which occurs in *rel*⁻ strains starved for only a few amino acids, is less evident in this situation than in straightforward amino acid deprivation.

In bacteria the synthesis of stable RNA (rRNA + tRNA) is under the control of the regulatory RC (*rel*) function (Stent & Brenner, 1961; Edlin & Broda, 1968). The expression of this function is determined by the supply of amino acids for protein synthesis (Pardee & Prestidge, 1956; Edlin & Broda, 1968). *rel*⁺(RC^{str}) organisms cannot accumulate stable RNA at a high rate in the absence of required amino acids (Sands & Roberts, 1952; Pardee & Prestidge, 1956), but *rel*⁻(RC^{rel}) cells continue to do so (Borek *et al.*, 1955; Ryan & Borek, 1971). During amino acid starvation, *rel*⁺ strains accumulate high concentrations of ppGpp (Cashel, 1969; Cashel & Gallant, 1969). Either coincidentally, or as a direct result of increased ppGpp concentrations (Haseltine *et al.*, 1972; Lazzarini & Johnson, 1973; Murooka & Lazzarini, 1973; Travers, 1973), the initiation of stable RNA chains is inhibited preferentially (Stamato & Pettijohn, 1971; Lazzarini & Dahlberg, 1971; Gray & Midgley, 1972). There may also be smaller effects

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on the initiation of certain fractions of mRNA (Gallant *et al.*, 1970; Gallant & Margason, 1972). On the other hand, ppGpp does not accumulate in *rel*⁻ strains in the absence of specific amino acids, and normal rates of stable RNA synthesis continue (Borek *et al.*, 1955; Ryan & Borek, 1971; Cashel, 1969; Cashel & Gallant, 1969). The current explanation of the difference in behaviour of *rel*⁺ and *rel*⁻ cells is that in *rel*⁺ cells there is sufficient stringent-factor protein to permit the idling reaction in the protein biosynthetic machinery on the ribosome to produce high amounts of ppGpp (Cashel & Gallant, 1969; Lund & Kjeldgaard, 1972). In *rel*⁻ cells the decreased concentration of stringent factor results in no ppGpp accumulation during starvation, and mistranslation or premature termination of nascent protein chains can occur (Hall & Gallant, 1971, 1972).

Though the behaviour of *rel*⁺ and *rel*⁻ strains is readily distinguishable under conditions where one or two amino acids are specifically withdrawn, their responses to diauxic lag (that is, to a stepdown from a

medium supporting a high rate of growth to one supporting a lower rate) are very similar (Lazzarini & Winslow, 1970; Winslow, 1971; Sokawa *et al.*, 1971). In both cases the accumulation of RNA is stopped until the new, lower, rate of growth is established (Lazzarini & Winslow, 1970; Winslow, 1971; Sokawa *et al.*, 1971). In the lag period ppGpp accumulates, though there are significant differences in *rel*⁺ and *rel*⁻ strains in the kinetics of its synthesis and breakdown (Winslow, 1971; Lazzarini *et al.*, 1971; Harshman & Yamazaki, 1971; Gallant *et al.*, 1972). Levallorphan, which can cause a general amino acid loss from the cells by affecting their permeability (Simon *et al.*, 1970), has the same effect as stepdown (Simon *et al.*, 1970; Harshman & Yamazaki, 1972).

In amino acid-starved *rel*⁺ cells, the accumulation of ppGpp is accompanied by a shortfall in the syntheses of ribosome-associated proteins, so that ribosomal maturation is severely delayed (Midgley & Smith, 1973; Midgley, 1974). This allows considerable rRNA turnover, diminishing still further the already lowered rates of stable RNA accumulation (Midgley, 1974). RNA turnover is also evident in *rel*⁻ cells during amino acid starvation. In such strains of *E. coli* an especially dramatic effect of the mistranslation in starved cells results in the almost complete prevention of new ribosome synthesis (Midgley & Gray, 1971; Gray & Midgley, 1972; Midgley & Smith, 1973). The RNA formed is not stable, turning over with a half-life, at 37°C, of about 20 min (Midgley & Gray, 1971; Midgley & Smith, 1973).

In view of the ability of the *rel*⁻ strain to mitigate the effect of amino acid starvation on stable RNA synthesis, by avoiding reactions giving rise to ppGpp, it is not clear why, in the more searching conditions of stepdown, *rel*⁻ cells should respond like *rel*⁺ cells in shutting-off RNA accumulation and raising their ppGpp concentrations. The lack of stringent factor in such mutants should again avoid the consequences of the idling reaction, and ppGpp concentrations should not alter.

In the present paper I compare the ability of *rel*⁺ and *rel*⁻ strains of *E. coli* to synthesize ribosomes in the period of stepdown, with a view to assessing their ability to form enzymically active proteins. I have chosen as an example of stepdown the shift from glucose/salts to lactate/salts medium, as Burton (1971) has shown that glucose-grown cultures have a considerable capability for utilizing lactate for energy production through oxygen uptake. Other evidence (J. E. M. Midgley, unpublished work) has shown that lactate can support a high rate of RNA accumulation in glucose-grown cultures when chloramphenicol is added, again demonstrating the ability of lactate to supply energy and carbon for nucleic acid synthesis at the earliest stages of stepdown.

The choice of ribosome synthesis as a measure of mistranslation was made because this complex pro-

cess should be an especially sensitive assay for the phenomenon, since inaccurate synthesis of any of a number of ribosome-specific proteins could lead to severe disturbances in ribosomal maturation. From the results it seems that, in nearly all aspects of ribonucleic acid synthesis in the stepdown period, *rel*⁺ and *rel*⁻ strains behave identically. Just as in amino acid-starved *rel*⁺ cells (Midgley & Smith, 1973; Midgley, 1974), there are large delays in the maturation of ribosomes. In both cases, the turnover of uncoated rRNA must contribute largely to the lack of RNA accumulation. When the two strains were compared in the respect of competent protein synthesis, it seems that ppGpp in *rel*⁻ organisms arises during stepdown from normal idling reactions in translation and that there is much less mistranslation than found in the same organisms starved of only a few amino acids.

Materials and Methods

Bacterial strains

Throughout this work the following strains of *Escherichia coli* were used: CP78 (F⁻, *arg*⁻, *his*⁻, *leu*⁻, *thr*⁻, *mal*⁻, *xyl*⁻, *ara*⁻, *gal*⁻, *Sm*^r, *rel*⁺) and CP79 (isogenic with CP78 except *rel*⁻). The strains were grown at 37°C in media previously described (Gray & Midgley, 1972). Cultures believed to be *rel*⁻ were as a routine tested by labelling with [5-³H]uracil of suitable specific radioactivity before and after inhibition of bacterial growth by trimethoprim (Smith & Midgley, 1973a). In the presence of exogenous adenine and guanosine, *rel*⁻ cultures continued to accumulate labelled RNA at a high rate when trimethoprim was added, whereas *rel*⁺ strains did not (Smith & Midgley, 1973a,b).

Mechanics of stepdown

In all cases, stepdown of bulk cultures of *E. coli* from glucose to lactate medium was carried out by centrifugation. Cultures were centrifuged at room temperature (18°C) for 5 min at 50000g and were then resuspended in lactate medium. They were again centrifuged and resuspended in lactate medium, the whole procedure taking not more than 15 min. At room temperature this period is equivalent to only a few minutes of stepdown at 37°C. Such cultures (whether *rel*⁺ or *rel*⁻) did not resume growth over a further period of 70–100 min, when a slower growth rate, typical of the lactate medium, then began. Other, more severe, stepdown procedures employed a shift from a broth-grown culture (Gray & Midgley, 1970, 1972) to lactate medium. This shift produced a lag period of some 2 h in the *rel*⁺ strain before growth restarted, but the *rel*⁻ strain suffered a greatly extended lag of up to 8 h (Neidhardt, 1963).

Isolation and hybridization of RNA and DNA

All methods pertaining to the isotopic labelling and harvesting of cultures, the isolation of pure RNA and DNA and techniques of hybridization were described previously (Pigott & Midgley, 1968). Ribosomes and their precursors were fractionated on DEAE-cellulose [Serva (Entwicklungslabor), Heidelberg, Germany] by the method of McCarthy *et al.* (1962) as modified by Midgley & Smith (1973). As described by these workers, ribosomes were eluted from the columns by 0.4 M-NaCl, ribonucleoprotein precursors to ribosomes and tRNA at 0.5 M-NaCl and nearly pure rRNA+mRNA at 0.6 M-NaCl. A linear gradient of 0–1 M-NaCl was used to fractionate cell extracts, the eluting buffer also containing 0.05 M-KCl, 0.01 M-MgCl₂ and 0.01 M-Tris (adjusted to pH 7.2 with HCl).

Radiochemicals

[2-¹⁴C]Uracil (specific radioactivity 54 mCi/mmol), [5-³H]uracil (sp. radioactivity 28 Ci/mmol) and L-[U-¹⁴C]valine (sp. radioactivity 270 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Labelling of RNA in *rel*⁺ and *rel*⁻ strains of *E. coli* through a growth transition from glucose to lactate medium

Cultures (100 ml) of *E. coli* CP78 (*rel*⁺) and CP79 (*rel*⁻) were grown in glucose/salts medium supplemented with required amino acids and thiamin (Gray & Midgley, 1972). At a suitable time, [5-³H]uracil (sp. radioactivity 0.6 mCi/mmol; concn. 0.1 µCi/ml) was added, and the kinetics of incorporation of the base into nucleic acids (almost entirely RNA) was followed by sampling and acid precipitation. The cultures were stepped-down in this instance by rapid filtration through a nitrocellulose membrane filter (Sartorius Membranfilter G.m.b.H., 34 Göttingen, West Germany; 0.2 µm pore size, 142 mm diam.). The cells were washed with prewarmed lactate/salts/amino acids medium (Midgley & Gray, 1971) containing labelled uracil of the same concentration and specific radioactivity as used for the previous labelling. They were then resuspended in a further portion (100 ml) of the same medium and samples were taken at intervals to monitor the synthesis and accumulation of labelled nucleic acids. The results are shown in Fig. 1. Over a period of 100 min after the onset of the growth transition, there was no further incorporation of radioactivity into nucleic acids, but after this time the cell mass and nucleic acid content per ml of culture began to increase at the slower rate, typical of the poorer growth medium. This result agrees with those

obtained by others (Lazzarini & Winslow, 1970; Winslow, 1971; Sokawa *et al.*, 1971). If any new RNA molecules are being synthesized over the step-down period, turnover must ensure that the amount of RNA per cell does not alter until further growth begins.

Ribosome synthesis in *rel*⁺ and *rel*⁻ strains of *E. coli* during 'stepdown'

Cultures of strain CP78 (*rel*⁺) and CP79 (*rel*⁻) were 'stepped down' from glucose/salts/amino acids to lactate/salts/amino acids medium by the centrifugation technique (see Materials and Methods section). At 10 min after resuspension in lactate medium, the cultures were labelled with [5-³H]uracil, (sp. radioactivity 25 mCi/mmol, concn. 0.05 µCi/ml). Samples (100 ml) were taken at intervals and poured into centrifuge buckets containing crushed ice. The samples were then centrifuged at 2°C (10 min at 40000g) and were stored at -20°C as frozen cells. These were then taken up into 5 ml of cold buffer containing 0.01 M-

Fig. 1. Labelling of RNA in *rel*⁺ and *rel*⁻ strains of *E. coli* throughout a transition from glucose to lactate medium

(a) Strain CP78 (*rel*⁺); (b) strain CP79 (*rel*⁻). ●, Culture turbidity (*E*₆₅₀); ○, acid-precipitable ³H radioactivity (c.p.m.) in RNA/ml of culture.

Fig. 2. Kinetic analysis of RNA labelling during stepdown of *rel⁻* strain CP79: ion-exchange column chromatography of labelled ribosomes and their precursors on DEAE-cellulose

Labelling was for (a) 2 min, (b) 12 min, (c) 20 min and (d) 40 min. $\circ$, E_{260} ; $\bullet$, ^3H radioactivity (c.p.m./5 ml fraction). (e) and (f) Time-course of ribosome labelling as a composite of (a) to (d) and other chromatograms not depicted. (e) refers to CP79 (*rel⁻*) and (f) to CP78 (*rel⁺*).

Tris, 0.01 M-MgCl₂, 0.05 M-KCl, pH 7.2, and were broken in a precooled French pressure cell (American Instruments Co., Silver Spring, MD, U.S.A.) at 110 MPa (16000 lb/in²). The extracts were then treated with deoxyribonuclease [Sigma (London)

Chemical Co., Kingston-upon-Thames, Surrey, U.K.] and chromatographed on DEAE-cellulose as described earlier (McCarthy *et al.*, 1962; Midgley & Smith, 1973; Midgley, 1974). Fig. 2 shows representative chromatograms of ribosomes and their precursor

sors (together with tRNA and mRNA) during the labelling of a stepped-down culture of strain CP79 (*rel*⁻). The distributions of label in the ribosomes and precursors in the sister strain CP78 (*rel*⁺) were identical with those in CP79 at all equivalent times.

From the Figures and the time-course of labelling mature ribosomes (Figs. 2*e* and 2*f*) it seems that a kinetic delay in ribosome labelling of about 12–15 min is typical of the stepped-down cultures, whatever the state of the *rel* gene. The results are closely comparable with the kinetics of labelling of ribosomes in CP78 (*rel*⁺) cultures simply starved of a few amino acids (Midgley, 1974) although, if anything, the kinetic delay in ribosome labelling is larger during stepdown from glucose to lactate medium. However, there is no similarity between stepped-down and amino acid-starved *rel*⁻ cultures as regards ribosome labelling (Midgley, 1974). During amino acid starvation, the synthesis of stable RNA in *rel*⁻ strains is unchanged (Borek *et al.*, 1955; Ryan & Borek, 1971) but inadequate protein synthesis ensures that only RNA-rich ribosome precursors accumulate (Turnock & Wild, 1965; Midgley, 1974). During stepdown the maturation of newly formed rRNA into ribosomal particles is slowed, but only to the same extent as in the corresponding *rel*⁺ strain. It could be that a high rate of 'stable' RNA turnover in the *rel*⁻ strain is obscuring a high gross rate of synthesis in the stepdown period, but it seems more than coincidental that the relative amounts of materials in the three labelled peaks eluted from DEAE-cellulose between 0.4M- and 0.6M-NaCl should be equivalent at all times in both strains (Fig. 2). The results more strongly suggest that any inaccurate translation that affects ribosome maturation in amino acid-starved *rel*⁻ strains (Hall & Gallant, 1971; 1972; Midgley, 1974) is in stepdown no longer able to prevent particle synthesis to any greater extent than in the *rel*⁺ strain. However, just as in amino acid-starved *rel*⁺ cells, there is a large discrepancy between the synthesis of stable RNA and ribosome-specific proteins, allowing much of the newly formed rRNA an opportunity to decay.

Ribosome protein synthesis during stepdown

At 10 min after the start of stepdown, *rel*⁺ and *rel*⁻ cultures were given [U-¹⁴C]valine (sp. radioactivity 25 mCi/mmol, concn. 0.1 µCi/ml). The amino acid was incorporated into cellular protein over a further 40 min. The cultures were then harvested, extracts were prepared, and DEAE-cellulose chromatography was carried out. To separate labelled proteins co-eluting with the ribosome peak at 0.4M-NaCl from another large peak of protein at 0.3M-NaCl, the elution gradient was stretched to give a linear salt gradient of 0–0.6M-NaCl in 400 ml of buffer (Midgley & Smith, 1973). The percentage of total ¹⁴C-labelled

protein that were co-eluted with the ribosome peak at 0.4M-NaCl was measured (Table 1). In both strains, about 4–5% of the labelled protein synthesized in the stepdown period was associated stably with ribosomes. This compares well with the value of 3.5% in the *rel*⁺ strain subjected to simple amino acid withdrawal in glucose/salts medium, or to inhibition by trimethoprim (Midgley, 1974). However, in the *rel*⁻ strain, this value is much greater than the 0.5% of newly formed protein stably associated in ribosomes in amino acid-starved *rel*⁻ cells (Midgley, 1974). In conjunction with the results for the kinetics of ribosome maturation in Fig. 2, this again suggests that inaccurate translation is no more prevalent in the *rel*⁻ than in the *rel*⁺ strain in these conditions. As is the case for amino acid-starved *rel*⁺ cells, the proportion of newly formed protein that could be classified as ribosome-specific is much smaller under conditions of stepdown than in the controls growing in glucose or lactate medium (Midgley, 1974). This again demonstrates a shortfall in the synthesis of this protein fraction relative to rRNA.

Synthesis of other proteins in stepdown

It was considered possible that the evidence for a lack of mistranslation in stepped-down *rel*⁻ cells, based on studies of ribosome maturation, might not be true of the synthesis of other proteins. Accordingly, the ability of the cultures to induce the enzyme β-galactosidase in the stepdown period was compared with their ability to synthesize and mature new ribosomes. A bacterial culture (200 ml) was transferred from glucose to lactate medium as described above. After 15 min at 37°C, [5-³H]uracil (sp. radioactivity 50 mCi/mmol, concn. 0.1 µCi/ml) was added. The

Table 1. Formation of ribosome-specific protein in *rel*⁺ and *rel*⁻ cells during stepdown and steady growth

The amino acid used for labelling cellular protein was L-[U-¹⁴C]valine. Determinations of ribosome-specific radioactive proteins were carried out by DEAE-cellulose column chromatography [for details, see McCarthy *et al.* (1962), Midgley & Smith (1973) and Midgley (1974)]. All results are means of three estimations.

Conditions	¹⁴ C-labelled protein associated with ribosomes and their precursors (%)	
	CP78 (<i>rel</i> ⁺)	CP79 (<i>rel</i> ⁻)
Steady growth		
(glucose/salts)	12.8	14.1
(lactate/salts)	8.6	9.4
'Stepdown' from		
glucose to lactate	4.4	4.0
broth to lactate	3.5	3.7

Fig. 3. Distribution of [^3H]uracil in the ribosomes and precursors of rel^+ and rel^- strains of *E. coli* labelled for 30 min during a stepdown period compared with the induction of β -galactosidase

(a) and (b) Distribution of ^3H radioactivity in ribosomes and precursors of CP78 (rel^+) and CP79 (rel^-): $\circ$, E_{260} ; $\bullet$, ^3H radioactivity (c.p.m./5ml fraction). (c) and (d) Time-course of induction of β -galactosidase during the stepdown period in CP78 and CP79.

culture was immediately split into two 100ml portions. One was incubated for a further 30min, and was then harvested in the cold, and extracts were made. The ribonucleoproteins and RNA were chromatographed on DEAE-cellulose as before. The other portion was given 1.6ml of 0.04M-isopropyl β -D-thiogalactoside immediately after division, to induce β -galactosidase. At several suitable times, up to 30 min after induction, samples (3ml) were removed and were assayed for enzyme activity (Smith & Midgley, 1973a). The rate of enzyme synthesis was then compared with the specific radioactivity of the mature ribosomes formed over the same period. The same experiment was carried out on the other strain, by using the same conditions for bacterial growth, stepdown, enzyme induction and ribosome labelling. Fig. 3 shows the time-course of β -galactosidase syn-

thesis and the distribution of ^3H radioactivity in ribosomes and their precursors over a 30min labelling period. The ratio of the specific radioactivity of the ribosomes (c.p.m./ E_{260} unit) in the column chromatogram to the slope of the induction curve for β -galactosidase was (i) for the rel^+ strain (CP78), 1.04×10^6 , and (ii) for the rel^- strain (CP79), 1.09×10^6 . Thus not only were the labelling patterns of the ribosomes plus precursors identical in the two strains during stepdown (Figs. 3a and 3b) but the rate of synthesis of active β -galactosidase relative to the maturation of new ribosomes was also identical. Evidence of normal protein synthesis, already indicated by similarities in the labelling of ribosomes in stepped-down rel^+ and rel^- strains, is also found in the synthesis of β -galactosidase and presumably applies to proteins generally.

Fig. 4. Thermolability of β -galactosidase in strain CP79 (rel^-) formed in various growth conditions

○, Enzyme induced during steady growth; ●, enzyme induced during stepdown from glucose to lactate medium; △, enzyme induced during starvation for carbon source and arginine. The conditions of enzyme induction and preparation of cell extracts for assay were as described by Hall & Gallant (1971). For details of induction in each condition, see the text.

Thermolability of β -galactosidase produced during stepdown

Hall & Gallant (1972) showed that, as a consequence of the rel^- mutation, enzymes induced during amino acid starvation were inaccurately synthesized and were therefore more thermolabile at a critical temperature than were the same enzymes induced in steadily growing organisms. To test whether inaccurate translation was also a feature of stepdown in the rel^- strain CP79, the enzyme β -galactosidase was induced in the following conditions: (i) in cultures growing steadily in lactate medium, (ii) in cultures first grown in lactate medium and then transferred to a medium lacking both carbon source and arginine, and (iii) in cultures growing in glucose medium which were then transferred to lactate medium. In culture (i) the enzyme was first induced with isopropyl β -D-thiogalactoside for 10 min, in culture (ii) for 60 min, after a preliminary incubation for 10 min to remove residual carbon source and arginine, and in culture (iii) for 30 min, after a preliminary incubation for 10 min in lactate medium to establish stepdown. The cultures were then harvested, concentrated to a suitable cell density and were then sonicated to prepare cell-free extracts (Hall & Gallant, 1972). The extracts were heated at 56.5°C over a period of 30 min, samples being taken at intervals for assay of the remaining enzyme activity. The results are shown in Fig. 4. Quantitatively they indicate a slower rate of loss of enzyme activity than shown by Hall & Gallant (1972), but qualitatively it is clear that β -galactosidase formed by the amino acid-

starved rel^- mutant was more than twice as thermolabile as the enzyme formed either during steady growth or during stepdown. In strain CP78 (rel^+), the induced enzyme was in all cases as stable to heat as the enzyme formed in rel^- strains during steady growth. This finding fortifies the conclusion that inaccurate translation is not as obvious in rel^- strains during stepdown.

Proportions of mRNA in rapidly and randomly labelled RNA of rel^+ and rel^- strains during stepdown

To determine the relative rate of synthesis of mRNA and stable RNA in stepped-down cultures, rapidly labelled RNA fractions were purified from cells at various stages in the transitional period. A culture (300 ml) of strain CP78 (rel^+) and CP79 (rel^-) growing in glucose medium was divided into two parts (50 ml and 250 ml). To the smaller, [$5\text{-}^3\text{H}$]uracil (sp. radioactivity 28 Ci/mmol, concn. 1 $\mu\text{Ci/ml}$) was added. After 1 min at 37°C, the culture was poured on to crushed ice and the RNA was purified as described previously (Pigott & Midgley, 1968). The larger portion was stepped-down into lactate medium, and, at various times, 50 ml samples were removed and also labelled with [^3H]uracil for 1 min. All the RNA specimens were given the treatments necessary to withstand the rigours of hybridization with DNA at elevated temperatures (Pigott & Midgley, 1968). A further specimen of rapidly labelled RNA was made by injecting 200 ml of the same strain growing steadily in glucose medium with [$2\text{-}^{14}\text{C}$]uracil (sp. radioactivity 54 mCi/mmol, concn. 0.1 $\mu\text{Ci/ml}$) and labelling for 1 min. This specimen was the standard, with which each ^3H -labelled hybrid was compared. Suitable mixtures of the ^{14}C -labelled standard and each of the ^3H -labelled specimens were simultaneously hybridized on DNA-bearing membrane filters by using an overall DNA/RNA ratio (w/w) of 4:1, and a DNA concentration of 0.2 mg/ml of hybridization fluid (Midgley & Gray, 1971; Smith & Midgley, 1973a). The $^3\text{H}/^{14}\text{C}$ ratio in the hybrids was compared with that of the input. This ratio in the RNA labelled in cultures steadily growing in glucose medium was the same for the hybrid and the input RNA, thus fulfilling the requirements for a control. In all stepped-down specimens of RNA, the $^3\text{H}/^{14}\text{C}$ ratio was higher in the hybrid than in the input, indicating a higher proportion of ^3H -labelled mRNA capable of hybridization (Midgley & Gray, 1971; Midgley & Smith, 1974). By this method of 'coincident hybridization' (Midgley & Gray, 1971) the percentage of the mRNA in the rapidly labelled fractions was calculated. Table 2 shows the results. If the percentage of mRNA in the rapidly labelled RNA of the control was taken as 35% (Midgley & McCarthy, 1962) then the mRNA in stepdown in rel^+ cultures initially constituted 60–70% of this

Table 2. *Changes in the percentage of mRNA in the rapidly labelled RNA of rel⁺ and rel⁻ cultures through a period of stepdown*

The values below refer to the percentage of mRNA in total RNA labelled for 1 min at 37°C at various stages in a growth transition from glucose to lactate medium. Determinations of mRNA were carried out by the 'coincident-hybridization' technique (Midgley & Gray, 1971). The ¹⁴C standard used was a specimen of rapidly labelled RNA isolated from a culture of strain CP78 growing steadily in glucose/salts medium.

Time of labelling	Rapidly labelled RNA as mRNA (%)	
	CP78 (rel ⁺)	CP79 (rel ⁻)
Preshift (steady growth on glucose medium)	35	34
Stepdown to lactate medium		
(a) 10 min	60	49
(b) 20 min	64	47
(c) 40 min	56	38
(d) 60 min	49	44
Steady growth in lactate medium	41	42

fraction (Nierlich, 1972; Midgley, 1974) and declined to 40% when slower growth in lactate medium began. In the *rel⁻* strain, the percentage of mRNA in the rapidly labelled fraction in the control growing on glucose was the same as in the *rel⁺* strain, but in stepdown the ratio of mRNA/stable RNA did not rise as high. This phenomenon has already been described (Lazzarini & Winslow, 1970). These workers also pointed out that the concentration of ppGpp in *rel⁻* strains during stepdown was not as great as in the corresponding *rel⁺* strains. Therefore although both strains respond to stepdown by diminishing gross rates of synthesis of stable RNA species, the *rel⁺* strain appeared to decrease stable RNA synthesis some 3-4-fold, relative to mRNA, whereas the *rel⁻* strain only decreased stable RNA synthesis by a relative factor of 2 (Lazzarini & Winslow, 1970).

The proportion of mRNA in randomly labelled cultures of *E. coli rel⁺* and *rel⁻* strains was also measured through the stepdown period. In this case the cultures were labelled throughout initial growth in glucose medium with [5-³H]uracil, choosing a suitable concentration to give a steady uptake of isotope over the experiment. Cultures were stepped-down into lactate medium containing the same concentration of [³H]uracil of the same specific radioactivity. Samples were taken at suitable times, before, during and after stepdown, and the RNA was purified. A control specimen of ¹⁴C-labelled RNA was prepared by growing a culture on glucose in the presence of [2-¹⁴C]-

Table 3. *Changes in the percentage of mRNA in randomly labelled RNA of rel⁺ and rel⁻ cultures through a period of stepdown*

Determinations of the the percentage of mRNA were made in quadruplicate for each sample. The technique of 'coincident hybridization' was used with a randomly ¹⁴C-labelled sample of RNA from a glucose-grown culture of strain CP78 (*rel⁺*) as the standard. All measurements were corrected for contributions to the hybridization by stable RNA. Non-specific binding of labelled RNA to the filters was abolished by incubation with purified pancreatic ribonuclease (10 µg/ml) at room temperature for 10 min before washing the hybrids (Midgley & Smith, 1974).

Time of sampling	Total RNA as mRNA (%)	
	CP78 (rel ⁺)	CP79 (rel ⁻)
Preshift (steady growth on glucose medium)	2.3	2.5
Stepdown		
(a) 10 min	2.6	2.4
(b) 20 min	2.1	2.4
(c) 40 min	2.1	1.9
(d) 60 min	2.0	2.2
Steady growth in new lactate medium	2.1	2.2

uracil over several generations. Hybridization of suitable mixtures of ³H- and ¹⁴C-labelled RNA species was carried out as described earlier (Midgley & Gray, 1971), and the ³H/¹⁴C ratios of input and hybrid were compared. The results are shown in Table 3. In neither the *rel⁺* nor the *rel⁻* strain was there any evidence of changes in the absolute amount of mRNA in the cells throughout the period of stepdown and later recovery. If the chemical lifetime of the mRNA fraction is the same in stepdown as it is in steady growth, this indicates that gross rates of mRNA synthesis are relatively little changed in the cells throughout the whole period.

Decay of RNA-rich ribosome precursors and mRNA during stepdown

Cultures of strain CP78 (*rel⁺*) and CP79 (*rel⁻*) were injected with [2-¹⁴C]uracil (sp. radioactivity 10 mCi/mmol, concn. 9.1 µCi/ml). After 10 min a sample (50 ml) was harvested in the cold. To the remainder of the culture, rifampicin (0.5 g/litre) and puromycin (50 mg/litre) were added to stop RNA chain initiation and protein synthesis. Further samples were removed from the culture. Extracts were chromatographed on DEAE-cellulose and the decay of radioactivity associated with the peak of RNA-rich ribosome precursors and mRNA at 0.6M-NaCl was followed (Midgley, 1974). The results are shown in Fig. 5.

Fig. 5. Stability of ribosomes and their precursors in stepped-down *rel*⁺ and *rel*⁻ strains after inhibition with rifampicin and puromycin

(a) Strain CP78 (*rel*⁺); (b) strain CP79 (*rel*⁻). For details of separation of ribosomes and their RNA-rich precursors on DEAE-cellulose, see the text and McCarthy *et al.* (1962). ●, Radioactivity in ribosomes; ○, radioactivity in RNA-rich ribosome precursors.

The half-life of the RNA in this peak was 5.5 min for strain CP78 and 4.5 min for strain CP79. This is a composite of decay for both the mRNA and the RNA-rich ribosome precursor fractions (Midgley, 1974). However, these values are similar to those obtained for the decay of mRNA plus rRNA in amino acid-starved *rel*⁺ cells (Midgley, 1974). In stepdown rRNA turnover must be considerable, since the kinetic delay in ribosome labelling is even

Fig. 6. Functional lifetime of mRNA in stepped-down *rel*⁺ and *rel*⁻ strains

For details of the experiment, see the text. ○, CP78 (*rel*⁺); ●, CP79 (*rel*⁻). The vertical bars indicate uncertainty expressed to 95% limits.

larger. With such a discrepancy between the lifetime of the naked rRNA fraction and the slow rate of ribosome maturation, it is not surprising that little of the rRNA synthesized eventually enters the mature state.

The functional half-life of the mRNA was also measured in stepped-down *rel*⁺ and *rel*⁻ strains, so as to put a lower limit on the chemical lifetime of this fraction. Bacterial cultures in stepdown were injected simultaneously with rifampicin (0.5 g/litre) and [¹⁴C]valine (sp. radioactivity 270 mCi/mmol, concn. 0.05 μ Ci/ml). The kinetics of incorporation of [¹⁴C]valine into the total cell protein was measured as described previously (Gray & Midgley, 1971). The functional half-life of the mRNA was then determined (Gray & Midgley, 1971). The results are shown in Fig. 6. In both *rel*⁺ and *rel*⁻ strains, the functional half-life of mRNA during stepdown was 3 min, whereas in growing cultures the half-life was 2–2.5 min (Midgley & Smith, 1974). If the chemical lifetime of the mRNA was as small as this, the half-life of the rRNA fraction in the peak eluted from DEAE-cellulose at 0.6M-NaCl (Fig. 2) must be slightly

larger than that determined from Fig. 5. Nevertheless, this does not affect the implication that turnover of RNA-rich ribosome precursors in stepdown is large.

Discussion

In the transition period of diauxic lag or stepdown in bacteria, the accumulation of stable RNA is regulated independently of the state of the *rel* gene (Borek *et al.*, 1955; Stent & Brenner, 1961; Edlin & Broda, 1968; Harshman & Yamazaki, 1971; Ryan & Borek, 1971). In the more-defined conditions of amino acid starvation without further medium change, the *rel* gene has an important role in the observed interdependence of stable RNA accumulation and protein synthesis (Lazzarini & Winslow, 1970; Winslow, 1971; Sokawa *et al.*, 1971). Work by Dennis & Nomura (1974, 1975) showed that the synthesis of ribosome-specific proteins is also under the control of the *rel* gene under such conditions. However, the relatively high rate of ribosome-specific protein synthesis in amino acid-starved *rel*⁻ strains (Dennis & Nomura, 1974, 1975) is not reflected in a high rate of ribosomal maturation, as evidenced by column chromatography on DEAE-cellulose (Turnock & Wild, 1965; Midgley & Smith, 1973; Midgley, 1974). It appears that the inaccurate translation that is known to occur in amino acid-starved *rel*⁻ cells (Hall & Gallant, 1971, 1972) is sufficient to interfere with the normal rate of true ribosome maturation, though there is evidence to suggest that eventually some of the newly formed ribosome-specific proteins are used in the formation of ribosomes (Midgley, 1974).

The present study shows that, with respect to the synthesis of proteins and stable RNA, stepdown in either *rel*⁺ or *rel*⁻ cells displays most of the features typical of a general amino acid deprivation. In both strains, there is a considerable shortfall in the synthesis of ribosome-specific proteins relative to rRNA, and most of the RNA-rich ribosome precursors are turned over without appearing in the ribosome fraction. Further, *rel*⁻ strains do not display in stepdown the mistranslation shown in a defined amino acid starvation (Lavalle & de Hauwer, 1968; Hall & Gallant, 1971, 1972). A minor difference exists in that *rel*⁻ strains do not decrease the rate of synthesis of stable RNA relative to mRNA to the same extent as in the *rel*⁺ cells (Lazzarini & Winslow, 1970), but the deficiency in the synthesis of ribosomal protein seems to be sufficient in each case to effectively prevent rRNA accumulation. This provides a rationale as to why RNA does not accumulate under these conditions, and one which is independent of the role of ppGpp and the *rel*-gene product in the regulation of gross rates of stable RNA synthesis (Midgley, 1974). A reasonable estimate of the relative importance of increased ppGpp concentrations and of the shortfall

in ribosomal protein synthesis has been given for amino acid-starved *rel*⁺ strains of *E. coli* (Midgley, 1974) and seems to apply here.

Although it was not the primary aim of the present study to establish the metabolic events that control the glucose/lactate stepdown phenomenon, the similarities of the response to medium change and to selected amino acid withdrawal make it necessary to place stepdown in the context of a possible amino acid deficiency (cf. Midgley & Smith, 1973; Midgley, 1974). When cultures of *E. coli* grown in glucose medium are incubated with lactate, they remain fully capable of metabolizing the new carbon source and consume oxygen at similar rates (Burton, 1971). There are therefore no obvious difficulties in providing metabolites for the supply of energy through nucleoside triphosphates. In addition, the synthesis of the nucleotides themselves is more than adequate for the rate of RNA synthesis actually observed during stepdown. If high concentrations of chloramphenicol are added to *rel*⁺ or *rel*⁻ cultures lagging after transfer to lactate, the accumulation of stable RNA is considerably accelerated (J. E. M. Midgley, unpublished work). Thus given the availability both of energy and of substrates for RNA synthesis, an explanation of the lack of RNA accumulation must rest on other criteria.

The formation of high concentrations of ppGpp in bacterial cells is indicative of a lack of protein synthesis, arising from the 'idling' of the translational machinery on the ribosomes (Cashel, 1969; Cashel & Gallant, 1969). The likelihood that the increase in ppGpp concentrations in *rel*⁺ and *rel*⁻ strains during stepdown is indeed due to amino acid deficiency is reinforced by the similarities in ribosome maturation shown by *rel*⁺ cultures in stepdown and in amino acid deprivation (Midgley, 1974). In additional respects, such as (a) the relative rates of synthesis of mRNA and stable RNAs, (b) the maintenance of mRNA concentrations by synthesis and turnover, and (c) the induction of new enzymes such as β -galactosidase, the *rel*⁺ and *rel*⁻ strains behave virtually identically with amino acid-starved *rel*⁺ cultures (Midgley, 1974). That other metabolic disturbances have arisen as a result of stepdown is undeniable, but it would seem that those that are rate-limiting in determining stable-RNA synthesis rates over the bulk of the lag period principally concern a lack of amino acids.

Additional evidence that this is the case has come from the finding that if the *rel*⁺ or *rel*⁻ strains are shifted from glucose, supplemented with the four amino acids for which they are auxotrophic, to an unsupplemented lactate medium, and then the 16 non-essential amino acids are returned to the new medium, RNA accumulation is stimulated markedly in the *rel*⁻ strain, although protein synthesis is not (J. E. M. Midgley, unpublished work). With *rel*⁺ cultures, on the other hand, increases in RNA synthesis do not occur until all 20 amino acids are

returned, when protein synthesis also restarts. This evidence indicates that (a) the strains probably maintain their auxotrophy throughout stepdown and (b) that suppression of the *rel*⁻ response to selective amino acid withdrawal is due to additional insufficiencies in amino acid supply. Possibly such a more general amino acid lack sufficiently activates the lowered amount of 'stringent factor' typical of *rel*⁻ mutants (Block & Haseltine, 1973) to give ppGpp at a rate high enough to mimic the *rel*⁺ response.

Finally, it is worth noting that even during much more severe stepdowns (e.g. broth/lactate) exactly equivalent findings are made on ribosome maturation delays and on mRNA metabolism (J. E. M. Midgley, unpublished work). This may mean that, even in extreme cases where energy supply and nucleotide synthesis may be limited in a severe shift, amino acid lack could be the rate-limiting block on the synthesis of stable RNA throughout the lag period. This has obvious advantages to the cell in protecting the nucleoside triphosphate pools from undue drainage, and thus obviating further damage to the energy status of the cell in a temporarily adverse environment.

An interesting question that arises from the results is 'how can the *rel*⁻ strain display two very different behaviour patterns in both RNA synthesis and the fidelity of translation, depending on whether the supply of a few or possibly very many amino acids is insufficient?' A clue to this problem has come from the observations that residual *rel*-gene activity may remain in *rel*⁻ mutants (Block & Haseltine, 1973) and that in cells subject to stepdown the rate of ppGpp turnover is greatly decreased (Winslow, 1971; Lazzarini *et al.*, 1971; Harshman & Yamazaki, 1971; Gallant *et al.*, 1972). It has been proposed that the product of the *spoT* gene (Laffler & Gallant, 1974a), believed to be responsible for the further metabolism of ppGpp, is inhibited during stepdown but not during simple amino acid withdrawal (Laffler & Gallant, 1974b). When this nucleotide accumulates, it seems to have a direct inhibitory effect on the initiation and elongation of polypeptide chains (Arai *et al.*, 1972; Legault *et al.*, 1972; Yoshida *et al.*, 1972). Elevated concentrations of ppGpp in stepped-down *rel*⁻ cells may therefore mitigate the effects on translation normally seen when cultures are simply starved of a few amino acids by selective withdrawal. The overall rate of peptide-bond formation may be directly diminished, and inaccurate translation minimized. Such a model would propose that the phenomenon of inaccurate translation in *rel*⁻ strains simply arises through a lack of ppGpp. As a result, the pressure of those ribosomes that are translating regions of the mRNA for which amino acids are plentiful, on those that have encountered a codon for which amino acyl-tRNA is not immediately available, could not be prevented. This could lead to the premature termina-

tion of peptide chains, as well as to mistranslation to form incorrect proteins (Hall & Gallant, 1971, 1972).

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