Biochem. J. (1972) **128**, 1007–1020
Printed in Great Britain

The Control of Ribonucleic Acid Synthesis in Bacteria

THE SYNTHESIS AND STABILITY OF RIBONUCLEIC ACIDS IN RELAXED AND STRINGENT AMINO ACID AUXOTROPHS OF *ESCHERICHIA COLI*

By W. J. H. GRAY and J. E. M. MIDGLEY
Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(*Received* 28 *January* 1972)

The biosynthesis and stability of various RNA fractions was studied in RC^{str} and RC^{rel} multiple amino acid auxotrophs of *Escherichia coli*. In conditions of amino acid deprivation, RC^{str} mutants were labelled with exogenous nucleotide bases at less than 1% of the rate found in cultures growing normally in supplemented media. Studies by DNA–RNA hybridization and by other methods showed that, during a period of amino acid withdrawal, not more than 60–70% of the labelled RNA formed in RC^{str} mutants had the characteristics of mRNA. Evidence was obtained for some degradation of newly formed 16S and 23S rRNA species to heterogeneous material of lower molecular weight. This led to overestimations of the mRNA content of rapidly labelled RNA from such methods as simple examination of sucrose-density-gradient profiles. In RC^{rel} strains the absolute and relative rates of synthesis of the various RNA fractions were not greatly affected. However, the stability of about half of the mRNA fraction was increased in RC^{rel} strains during amino acid starvation, giving kinetics of mRNA labelling and turnover that were identical with those found in either RC^{str} or RC^{rel} strains inhibited by high concentrations of chloramphenicol. Coincidence hybridization techniques showed that the mRNA content of amino acid-starved RC^{str} auxotrophs was unchanged from that found in normally growing cells. In contrast, RC^{rel} strains deprived of amino acids increased their mRNA content about threefold. In such cultures the mRNA content of accumulating newly formed RNA was a constant 16% by wt.

The allelic state of the *RC* gene (Stent & Brenner, 1961) in *Escherichia coli* has a profound influence on the interrelationships between cellular protein and RNA biosynthesis. In RC^{str} organisms, which are also amino acid auxotrophs, the withdrawal of required amino acids leads simultaneously to an inhibition of protein and RNA accumulation (Fraenkel & Neidhardt, 1961; Kurland & Maaløe, 1962). With RC^{rel} strains, which are otherwise isogenic, this close coupling is lost, so that RNA synthesis can proceed at a high rate even when protein synthesis is severely inhibited (Borek *et al.*, 1955; Borek & Ryan, 1958; Fiil & Friesen, 1968). Various hypotheses have been put forward to explain the nature of the *RC*-gene mutation and its effect on RNA synthesis. The most recent explanations suggest either a general control of RNA synthesis through alterations in the intracellular concentrations of guanine nucleotides (Gallant & Cashel, 1967; Cashel, 1969; Gallant & Harada, 1969), or a more specific inhibition of the initiation of stable RNA chains in RC^{str} auxotrophs, arising from the accumulation of the unusual nucleotide guanosine tetraphosphate (ppGpp) in conditions of amino acid deprivation (Travers *et al.*, 1970). Alternatively, Sokawa *et al.* (1971) have suggested that the *RC* function is concerned with the organization of cellular protein-biosynthetic machinery.

The results of previous studies on the small amount of RNA labelled in amino acid-starved RC^{str} auxotrophs were interpreted in contradictory ways. On the one hand, some workers have argued that the RNA labelled in these conditions is almost entirely mRNA (Sarkar & Moldave, 1968; Lazzarini & Dahlberg, 1971). It has been proposed that the rate of synthesis and turnover of the mRNA fraction is only slightly diminished compared with that holding in the state of steady growth (Edlin *et al.*, 1968; Forchhammer & Kjeldgaard, 1968; Lavallé & de Hauwer, 1968; Nierlich, 1968; Lazzarini & Dahlberg, 1971). On the other hand, some studies have indicated that the rate of chain elongation of RNA in amino acid-starved auxotrophs is very much lower than in conditions of normal growth (Winslow & Lazzarini, 1969*a*,*b*). In addition, other work has implied that erstwhile stable RNA is produced in these conditions as a considerable proportion of the labelled material (Friesen, 1966; Stubbs & Hall, 1968). The arguments are complicated by the possibility that, during amino acid starvation of RC^{str} auxotrophs, labelled rRNA may be produced but cannot display its normal characteristics of metabolic stability,

secondary methylation of nucleotides and high molecular weight. This may arise from the lack of protein necessary to form mature ribosomal particles. In this situation many of the criteria usually sufficient to equate unstable RNA with mRNA are extremely difficult to satisfy.

With RC^{rel} strains, normal amounts of mRNA and stable RNA (rRNA+tRNA) are produced in conditions of amino acid deprivation (Fleissner & Borek, 1962; Dagley *et al.*, 1963; Turnock & Wild, 1964; Friesen, 1966). In many ways the strain behaves much as RC^{str} strains inhibited by high concentrations of chloramphenicol (Friesen, 1966). For example, the stability of the mRNA fraction is at least partly increased, so that it accumulates to an extent that is higher than normal (Turnock & Wild, 1965; Friesen, 1966; Avery *et al.*, 1969).

In the present work we have studied the synthesis and stability of the mRNA fraction in RC^{str} and RC^{rel} isogenic multiple amino acid auxotrophs of *E. coli*. We have also compared the kinetics of mRNA labelling and turnover in RC^{rel} auxotrophs, in similar conditions, with those found in RC^{str} strains inhibited by chloramphenicol (Midgley & Gray, 1971). Our findings suggest that mRNA synthesis and breakdown are identically affected by chloramphenicol and the RC^{rel} mutation. The stability of half the mRNA in starved RC^{rel} cultures may arise by similar mechanisms (Midgley & Gray, 1971). This suggests that mutation of the *RC* function may affect the ability of ribosomes to protect mRNA from degradation in a way that is operationally similar to the effects of chloramphenicol in RC^{str} strains.

Materials and Methods

Organisms

The organisms used were *Escherichia coli* CP78 (F^-, thr^-, leu^-, his^-, arg^-, mal^-, xyl^-, ara^-, gal^-, sm^s, RC^{str}) and CP79 (isogenic with strain CP78 except that the strain is RC^{rel}). They were obtained from Dr. D. G. Wild, Department of Microbiology, University of Oxford, Oxford, U.K. Both strains originally had a requirement for thiamine, but this was lost during repeated subculture.

Growth of bacterial cultures

The organisms were grown with forced aeration at 37°C in the glucose–salts medium of Roberts *et al.* (1957) supplemented with the required amino acids L-threonine, L-leucine, L-histidine and L-arginine. This contained (per litre) 2g of NH_4Cl, 3g of KH_2PO_4, 6g of Na_2HPO_4, 3g of NaCl, 0.12g of Na_2SO_4, 0.05g of $MgCl_2$, 2g of glucose and 0.05g of each of the amino acids. Cell doubling times observed at 37°C were usually about 50min (strain CP78) and 65–70min (strain CP79). Growth curves also showed different behaviour in the two strains: strain CP78 attained exponential growth very rapidly in batch media, whereas strain CP78 slowly increased its growth rate, which was stabilized some 2h after inoculation. Care was taken to ensure that all experiments were carried out with both strains in steady exponential growth. A standard procedure was adopted to obtain non-growing cultures in media lacking essential amino acids. Steadily growing cultures were harvested at E_{650} 0.5 by centrifugation at room temperature, and the cells were washed by resuspending in the same volume of glucose–salts medium lacking the amino acids ('starvation medium') prewarmed to 37°C. After resuspension, the cells were again harvested and resuspended in the original volume of prewarmed starvation medium. Aeration of the cultures was then continued. Harvesting of cultures, the incorporation of radioactive substances, the preparation of cell-free extracts and the purification of DNA and labelled RNA were carried out as described by Pigott & Midgley (1968).

DNA–RNA hybridization

E. coli DNA was denatured, immobilized on cellulose nitrate membrane filters and hybridized with RNA as described by Gillespie & Spiegelman (1965) and Pigott & Midgley (1968).

Determination of RNA and protein in whole cells

The method used was derived from that of Fraenkel & Neidhardt (1961). A sample (10ml) of culture was pipetted into 1ml of ice-cold 10% (w/v) trichloroacetic acid. After standing for 3h in ice, the suspension was centrifuged at 3000*g* for 15min in the 16×15ml swing-out rotor of a BTL bench centrifuge (Baird and Tatlock Ltd.). The supernatant fluid was decanted and the pellet was digested for 1h at room temperature with 0.5ml of 1M-NaOH. The mixture was shaken at intervals and, after digestion, the solution was transferred quantitatively to a graduated tube. The volume was made up to 5ml with water. The RNA content of the digest was determined by the colorimetric method of Schneider (1957), with ribose standards and protein was assayed by the method of Lowry, *et al.* (1951), with bovine serum albumin standards.

RNA was fractionated by sucrose-density-gradient sedimentation, as described by Avery & Midgley (1969).

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.

Radiochemicals

[2-^{14}C]Uracil (specific radioactivity 54.5mCi/mmol) and [5-^{3}H]uracil (specific radioactivity 1 or 28.2Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Effects of amino acid withdrawal on bacterial RNA synthesis in RC^{str} and RC^{rel} strains of E. coli

Cultures of *E. coli* CP78 and CP79, growing exponentially in glucose-salts media supplemented with the required amino acids, were divided into two portions at a turbidity E_{650} 0.5. One portion acted as a control, the other being harvested, washed and resuspended in starvation medium, as described in the Materials and Methods section. The cultures were incubated at 37°C and at intervals the RNA and protein contents of the starved and control cultures were measured colorimetrically. The results for RNA are depicted in Fig. 1(*a*) for strain CP78 (RC^{str}) and Fig. 1(*b*) for strain CP79 (RC^{rel}). The RNA content of strain CP78 (Fig. 1*a*) remained virtually constant during a period of amino acid withdrawal, showing that net synthesis had ceased. On the other hand, in strain CP79 the RNA content continued to increase at nearly the normal rate for some time after amino acid deprivation (Fig. 1*b*). In this strain the RNA content finally increased 2.5-fold after 3h starvation. In both strains amino acid withdrawal led to a cessation of net protein synthesis, though in both cases the rate of labelling of protein by [1-^{14}C]leucine was about 5% of the normal rate.

Examination of the kinetics of labelling the RNA in starved and control cultures of strains CP78 and CP79 gave rise to the curves shown in Figs. 2(*a*) and 2(*b*). In these experiments equivalent cultures of starved and growing cells were exposed to [2-^{14}C]-uracil, and the labelling of the nucleic acids was followed by trichloroacetic acid precipitation of 1ml samples. In amino acid-starved strain CP78 cultures, RNA labelling occurred at only about 1% of the rate found in the control culture. In each case the labelling of the DNA, as measured by the acid-precipitable radioactivity unaffected by prior incubation in 1M-KOH, accounted for about 10% of the total acid-precipitable material. With the RC^{rel} strain the labelling of RNA continued in starved cultures at about 80% of the rate found in the growing controls. Again, these kinetics verify the essential characteristics of RC^{str} and RC^{rel} mutants.

Synthesis of RNA during amino acid starvation

Strain CP78 (RC^{str}). (*a*) Determination of mRNA labelling: DNA–RNA hybridization. A steadily growing culture of *E. coli* CP78 was harvested, washed and resuspended in glucose-salts medium. After 25min incubation, [5-^{3}H]uracil (0.2μCi/ml; specific radioactivity 28.2mCi/mmol) was added to the culture. At intervals 1ml samples were precipitated with an equal volume of ice-cold 10% (w/v) trichloroacetic acid for measurement of the net uptake of radioactivity into RNA. Further samples of the culture were harvested and the RNA was extracted with phenol-cresol (50:7, w/v). The incorporation of radioactivity into the mRNA fraction was measured by DNA–RNA hybridization, DNA/RNA ratios of 5:1 (w/w) being used (Pigott & Midgley, 1968). A kinetic plot of the time-course of incorporation of radioactivity into total and mRNA is shown in Fig. 3.

As in exponentially growing cultures, radioactivity was incorporated from the medium directly into RNA without a measurable kinetic delay. During the first period of labelling (i.e., 30min after the start of

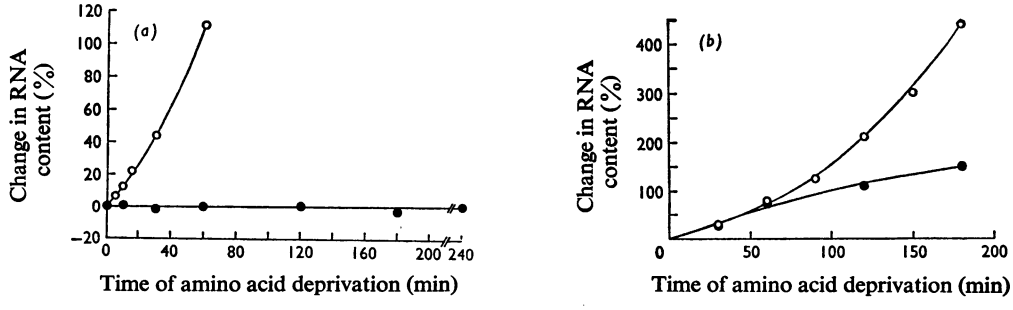


Fig. 1. *Changes in the RNA content of E. coli CP78 (RC^{str}) and CP79 (RC^{rel}) during amino acid starvation*

(*a*) Strain CP78; (*b*) strain CP79. o, Growing cultures; •, starved cultures. The amount of RNA in the cells at the onset of starvation was set arbitrarily at zero.

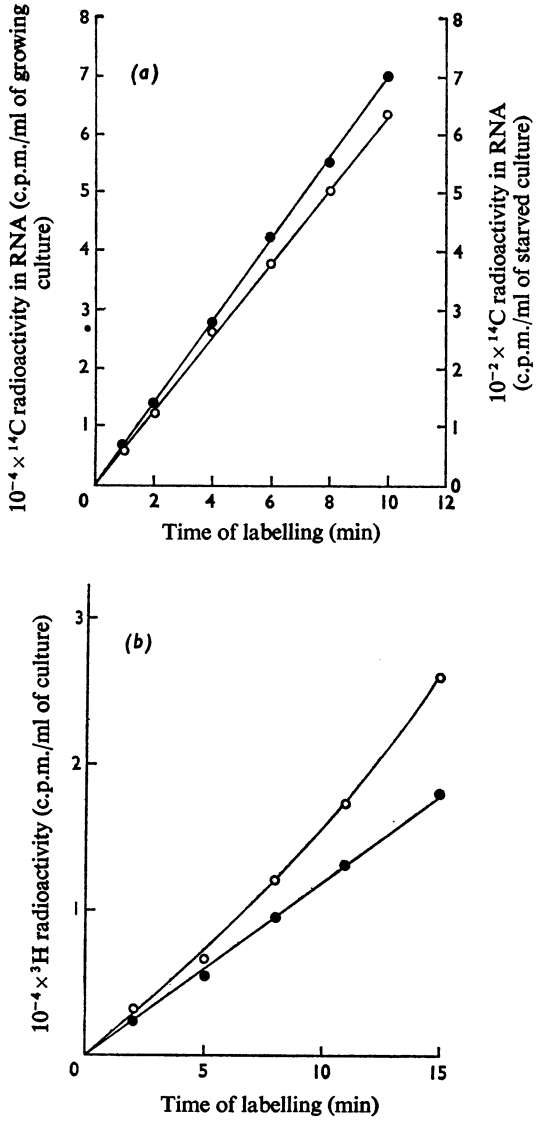


Fig. 2. *Labelling of RNA in amino acid-starved cultures of E. coli CP78 (RC^{str}) and CP79 (RC^{rel})*

(*a*) Strain CP78; (*b*) strain CP79. o, Growing cultures; •, starved cultures. The specific radioactivity of the [2-^{14}C]uracil used was 25mCi/mmol.

starvation), mRNA was at maximum 60% of the total labelled RNA, falling to somewhat lower proportions at later times. Thus the RNA synthesized in amino acid-starved cells included a considerably higher proportion of mRNA than in conditions of normal growth (Sarkar & Moldave, 1968; Lazzarini & Dahlberg, 1971). Further, the contribution of

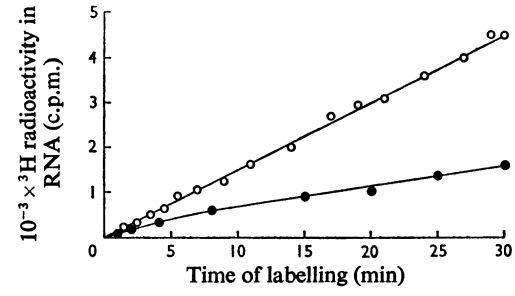


Fig. 3. *Labelling of mRNA in amino acid-starved cultures of E. coli CP78 (RC^{str})*

o, Total acid-precipitable RNA; •, hybridized RNA.

mRNA labelling to the total labelled RNA remained much higher than normal over long periods of incorporation (cf. Gray & Midgley, 1970). This showed that, if the mRNA fraction was still turning over in such conditions, the same was true of the bulk of the other RNA formed.

The estimation of the flux of radioactivity entering the mRNA as not greater than 60-70% of the total is considerably at variance with the findings by Lazzarini & Dahlberg (1971), who considered that mRNA synthesis accounted for at least 90% of the RNA labelling in these conditions. However, to verify the accuracy of estimations based on DNA–RNA hybridization a study was made of the RNA labelled after inhibition of initiation of RNA chains by the antibiotic rifampicin (Sippel & Hartmann, 1968; Wehrli *et al.*, 1968).

(*b*) Unstable RNA formed after rifampicin inhibition of amino acid-starved cultures. Although several workers have shown that the action of rifampicin on RNA chain initiations is virtually instantaneous in steadily growing cultures of *E. coli* (Pato & von Meyenberg, 1970; Rose *et al.*, 1970; Gray & Midgley, 1971), it is not clear that such rapid effects will be seen in cultures of strain CP78 (RC^{str}) deprived of essential amino acids. Preliminary experiments were carried out to assess the rapidity of rifampicin action in such cultures, by measuring the time needed for the antibiotic to suppress the induction of β-galactosidase (Pato & von Meyenberg, 1970; Gray & Midgley, 1971). Amino acid-starved cultures of RC^{str} auxotrophs are capable of inducing this enzyme (Morris & Kjeldgaard, 1968), though with diminished efficiency (Brunschede & Bremer, 1971).

A culture of *E. coli* CP78 was starved of exogenous amino acids for 30min. At this time samples were removed and tested for their ability to induce β-galactosidase just before or just after the addition

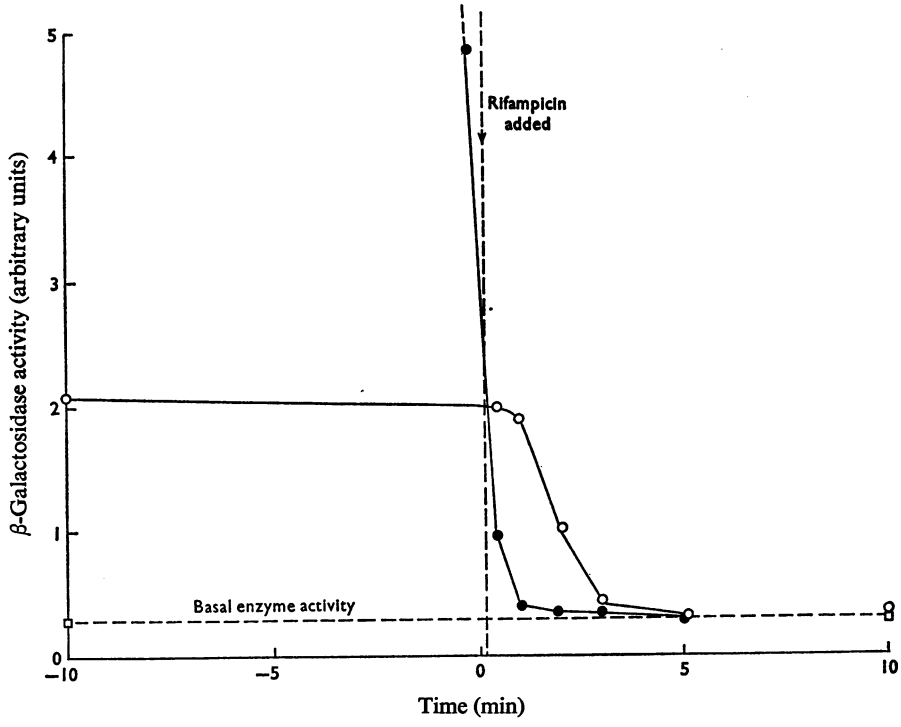


Fig. 4. *Inhibition of β-galactosidase induction by rifampicin in amino acid-starved cultures of E. coli CP78 (RC^{str}) in the presence or absence of the required amino acids*

●, Amino acid-supplemented cells; ○, non-supplemented cells. The technique of enzyme induction is fully described by Gray & Midgley (1971). For the amino acid-supplemented cultures, the addition of amino acids 10min before the addition of rifampicin gave a final enzyme activity of 21 arbitrary units.

of rifampicin (0.1mg/ml) (for experimental details see Gray & Midgley, 1971). The behaviour of the starved strain was contrasted with a control in which the amino acids were added together with inducer (isopropyl β-D-thiogalactoside). The results are shown in Fig. 4. The amino acid-starved cultures were somewhat slower to respond to rifampicin inhibition than those in which the multiple amino acid requirements were relieved when inducer was added. Whereas it took less than 20s for rifampicin to inhibit enzyme induction completely in amino acid-supplemented cells, starved cells required a period of about 3min before no induction was observed. Thus experiments in which amino acids are added to deprived cultures do not suffer appreciably from delays in the action of rifampicin on RNA chain initiation.

A culture of *E. coli* (CP78) was suspended in amino acid-free glucose–salts medium. At intervals samples were taken from the culture and rifampicin (0.1mg/ml) and the required amino acids (50μg/ml final concn. for each) were injected. After a further 15s [2-^{14}C]uracil (1μCi; specific radioactivity 54mCi/mmol) was added. The kinetics of incorporation of radioactivity into RNA was followed by trichloroacetic acid precipitation of 1ml samples. The results are shown in Fig. 5. Throughout the period of amino acid withdrawal, not more than 33% of the radioactivity in the RNA formed after rifampicin inhibition (by the completion of chains) was unstable and decayed to non-precipitable products. Any labelled mRNA formed in the presence of the required amino acids would be translated normally, and would be expected to display its usual instability. Indeed, measurements of the half-life of the mRNA in these experiments, by a study of the kinetics of residual protein labelling by L-[1-^{14}C]leucine (Gray & Midgley, 1971), gave a value of 4.5min, as found for steadily growing cultures (J. E. M. Midgley & M. Bell, unpublished work).

It has been assumed by other workers (e.g. Schwartz *et al.*, 1970; Pato & von Meyenberg, 1970) that the proportion of labelled RNA that is unstable after inhibition of chain initiation by rifampicin is a

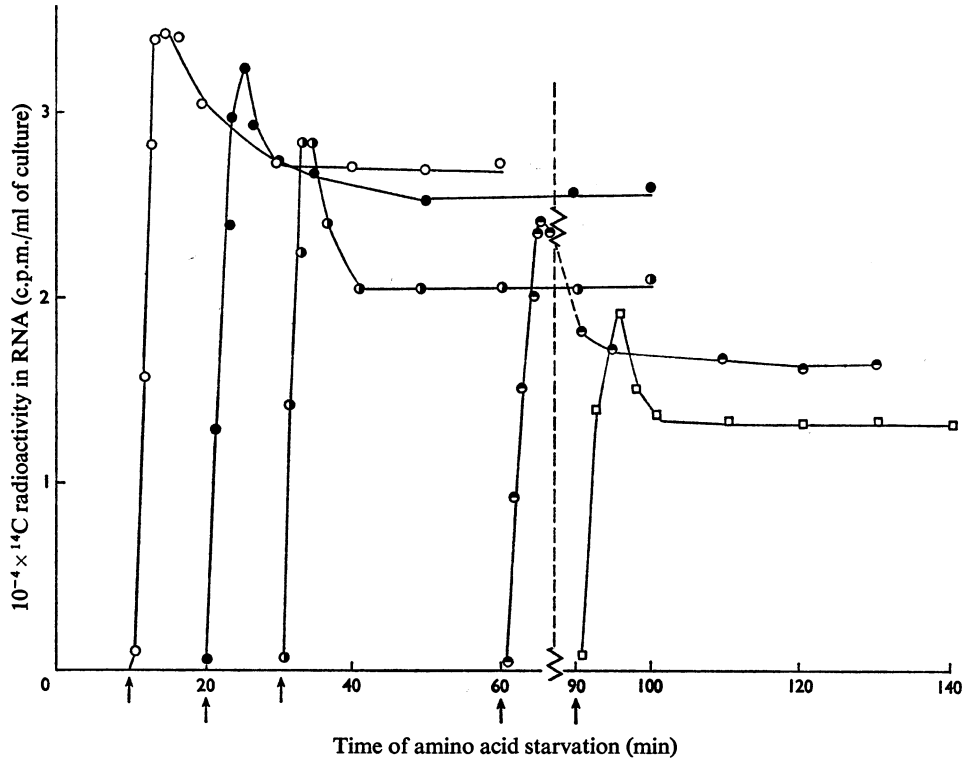


Fig. 5. *Kinetics of residual RNA labelling in rifampicin-inhibited amino acid-supplemented cultures of E. coli CP78 (RC^{str}) after various periods of prior amino acid starvation*

Samples (20ml) were removed from a flask of non-growing amino acid-starved organisms at the times indicated by the arrows. Rifampicin was added to the samples, followed by the required amino acids (for details see the text). The contribution of labelling in DNA was corrected for by incubating samples (1ml) of the cultures for 18h in 1M-KOH, followed by acid precipitation and counting of radioactivity (Gray & Midgley, 1971). The results in the figure thus represent labelling of RNA only. RNA synthesis is indicated after the following times of starvation: ○, 10min; ●, 20min; ◑, 30min; ◓, 60min; □, 90min.

direct measure of the flux of radioactivity entering mRNA in steady states of initiation and polymerization. This is incorrect, as serious discrepancies arise from the probability that the average molecular weight of large stable RNA species such as rRNA is very much greater than the average molecular weight of mRNA molecules (Munro & Korner, 1964; Samarina, 1964). Assuming constancy of parameters such as chain-polymerization rates, the ratio of radioactive fluxes into any two RNA fractions in steady states indicates the ratio of the polymerases in action at any moment on the two cistron groups. On the addition of rifampicin, RNA chain initiation is stopped, and the total amount of RNA formed by completion of unfinished chains now depends on the product of the number of transcribing polymerases and the average number of nucleotide residues polymerized on a given cistron. As rRNA is probably produced by the transcription of a tandem cistron with one rifampicin-sensitive initiation point, which produces one molecule each of 16S and 23S rRNA (Pato & von Meyenberg, 1970; Doolittle & Pace, 1971), an average length of some 2500 nucleotide residues will be traversed by each polymerase after rifampicin inhibition. On the other hand, polymerases transcribing a cistron of 1000 residues will polymerize on average 500 residues of any mRNA chain (assuming in each case a uniform distribution of enzymes along the cistrons). Therefore if the ratio (mol.wt. of the 16S+23S rRNA cistron)/(root mean square of the mol.wt. of mRNA cistrons) is as much as 5:1, the contribution to residual RNA labelling by the rRNA fraction will be exaggerated by this factor. On the other hand, the relatively low molecular

Table 1. *Determination of the fraction of radioactivity entering mRNA in amino acid-deprived E. coli CP78 (RC^{str}) from studies of RNA labelling after inhibition by rifampicin*

Assumed ratio (mol.wt. of 16S+23S+5S rRNA)/(av. mol. wt. of mRNA)	True fraction of radioactivity (c.p.m.) entering mRNA as corrected by column 1 ratio (% of total RNA labelling)	Fraction calc. by correction for lack of tRNA labelling and for different U+C content of mRNA (% of total RNA labelling)
1:1	33	27
2:1	50	44
3:1	60	53
4:1	67	60
5:1	71	64
6:1	75	67
10:1	83	75

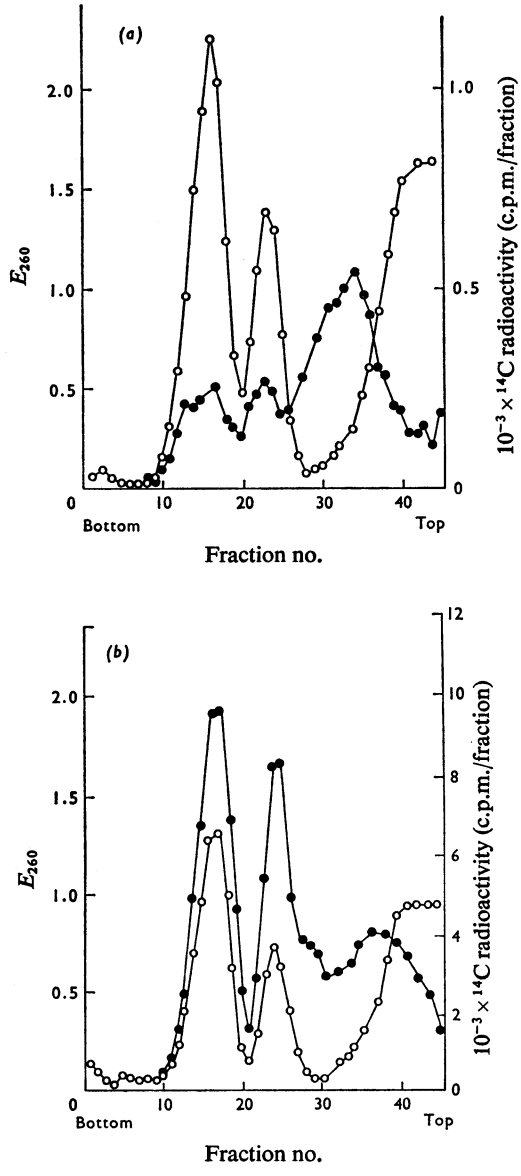
Corrections for lack of tRNA labelling were made by assuming that in uninhibited amino acid-starved RC^{str} cells 20% of stable RNA formed is tRNA (Raue & Gruber, 1971). The U+C contents of stable RNA and mRNA in *E. coli* are 43 and 49mol/100mol respectively (Midgley, 1962; Midgley & McCarthy, 1962). The percentages of labelled RNA labilized after rifampicin inhibition (see Fig. 5) were 32, 33, 35, 32 and 35 (taken from five samples at various stages of amino acid deprivation). Mol.wt. of rRNA = 1.7×10^6.

weight of the cistrons responsible for tRNA synthesis (Altman, 1971; Vickers & Midgley, 1971) will affect the calculations for total stable RNA in the other direction. Thus a minor correction upwards will be necessary for the contribution of tRNA, since, in any event, it is only a small fraction of stable RNA (Maaløe & Kjeldgaard, 1966). Thus, as in all experiments with amino acid-starved cultures of *E. coli* CP78, only 33% of the RNA formed after rifampicin inhibition was unstable when the required amino acids were added back, and the true contribution of mRNA labelling in uninhibited cultures could be calculated, assuming various (mol.wt. of 16S+23S rRNA)/(root mean square of the mol.wt. of mRNA) ratios (Table 1).

Assuming these ratios to be as low as 1:6 (i.e. the root mean square mol. wt. of mRNA is 2.8×10^5), not more than 75% of the flux of radioactivity entering total RNA in starved organisms is due to mRNA labelling. If corrections are applied for the relative lack of tRNA labelling after antibiotic inhibition (Vickers & Midgley, 1971) and for the slightly greater uridine+cytidine content of mRNA (Midgley & McCarthy, 1962), a reasonable upper limit for the mRNA flux is some 65% of the total. Since the work described below suggests that a little of the rRNA formed in such conditions may also be unstable, and may not be capable of forming ribosomal particles after rifampicin inhibition, even this value may be too high. Thus we consider that the results from direct DNA–RNA hybridizations and from a study of unstable RNA labelled after rifampicin inhibition of amino acid-starved cultures are in good agreement.

(*c*) Sucrose-density-gradient analysis of RNA formed in amino acid-deprived cultures. A culture (300ml) of *E. coli* CP78 was starved of its required amino acids by suspension in glucose–salts medium for 30min. At this time the culture was inhibited by the addition of rifampicin (0.1mg/ml). After 20s [2-^{14}C]uracil (5μCi; specific radioactivity 20mCi/mmol) was added. Immediately the culture was split into one 100ml sample and one 200ml sample. To the larger sample, the required amino acids (50μg/ml final concn. for each) were added. After 2min a 100ml sample was removed from the larger volume and the cells were harvested and the labelled RNA was extracted. After 10min the remaining samples were harvested and extracted. The RNA was then subjected to sucrose-density-gradient sedimentation (Avery & Midgley, 1969) (Fig. 6). With the rifampicin-inhibited unsupplemented culture it is apparent that much of the labelled material produced after rifampicin addition was distributed between the 16S rRNA marker and the top of the gradient. Small amounts of 16S and 23S rRNA were also formed. In essentials this finding confirms the work of Lazzarini & Winslow (1970), who used polyacrylamide-gel electrophoresis to fractionate the RNA. At first glance this suggests that a very high proportion of the total labelled RNA is mRNA (i.e. it does not co-sediment with the u.v.-absorbing marker peaks of 16S and 23S rRNA). However, examination of Figs. 6(*b*) and 6(*c*) show (i) much better resolution and appearance of peaks in the 16S and 23S region, (ii) a higher relative contribution of radioactivity to material co-sedimenting with the unlabelled markers, (iii) a confirmation that, after 10min inhibition by rifampicin, the stable labelled RNA remaining was virtually all 16S and 23S rRNA (see Fig. 5) and (iv) that the radioactivity incorporated into amino acid-supplemented cultures over 10min was an order of magnitude greater than was the case with unsupplemented cultures. Indeed, although the ratio (sp. radioactivity of RNA in amino acid-supplemented cultures)/(sp. radioactivity in unsupplemented cultures) was 9.5:1, the corresponding specific-radioactivity ratio for the 16S and 23S rRNA peaks in the fractionated density gradient was about 20:1. These experiments simply represent the residual labelling of the same uncompleted RNA chains in the presence

or absence of amino acids; therefore discrepancies between the specific-radioactivity ratios of the total RNA and the purified rRNA fractions must indicate that, in unsupplemented cultures, a considerable proportion of the labelled material sedimenting between 16S rRNA and the top of the gradient (Fig. 6*a*) was degraded (or incomplete) rRNA. On the other hand, in amino acid-supplemented cultures the rRNA showed a better appearance on the gradients, and survived undegraded after the loss of mRNA.



Even then, it could not be assumed that all labelled rRNA molecules remained stable after antibiotic inhibition even in conditions of amino acid supplementation, though from the results of DNA–RNA hybridization experiments this could only be a small fraction of the rRNA. Finally, the experiments indicate that the transcription of the 16S+23S rRNA tandem cistron may not be normal in such conditions, as the specific-radioactivity ratio of 23S and 16S rRNA formed as stable molecules after rifampicin inhibition and amino acid supplementation (Fig. 6*c*) is only about 1.3:1 (23S/16S). Pato & von Meyenberg (1970) and Doolittle & Pace (1971) have shown that residual transcription of the tandem cistrons for rRNA after rifampicin inhibition gives rise to 23S/16S rRNA specific-radioactivity ratios of 3.3:1 in steadily growing cultures. This discrepancy suggests either that some labelled 23S rRNA was preferentially degraded in amino acid-supplemented cultures (Fig. 6*c*) or that the tandem cistron was not

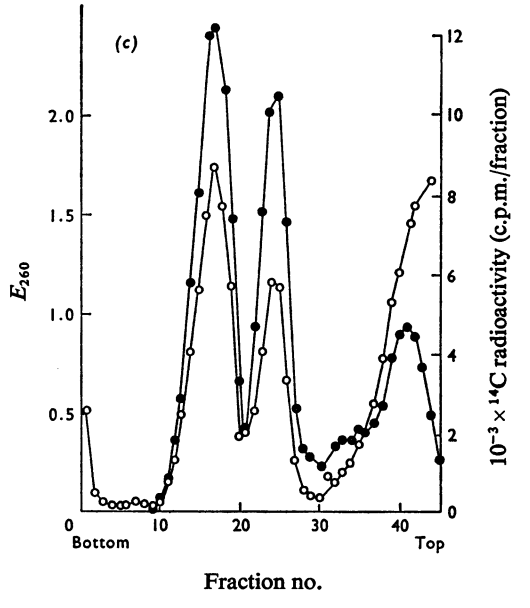


Fig. 6. *Sucrose-density-gradient sedimentation of total RNA from rifampicin-inhibited amino acid-starved cultures of E. coli CP78 (RC^{str})*

(*a*) Rifampicin-inhibited amino acid-starved cultures, incubated with [2-^{14}C]uracil for 10min; (*b*) rifampicin-inhibited amino acid-starved cultures, supplemented with the required amino acids, and labelled with [2-^{14}C]uracil for 2min; (*c*) as (*b*), except that the RNA was labelled for 10min. ○, E_{260}; ●, ^{14}C radioactivity.

completely traversed by all polymerases bound to it. In either event this would lead to an exaggeration of residual 16S rRNA labelling, and an overestimation of the contribution of unstable mRNA, since these events may not occur in amino acid-starved but not rifampicin-inhibited cultures.

(*d*) Determination of the amount of mRNA in amino acid-starved cultures. Because of the extremely low rate of incorporation of exogenous precursors (e.g. [^{14}C]uracil) into the RNA of starved RC^{str} cultures, and the uncertainties about the possible instability of other RNA fractions, it was difficult to measure mRNA contents and thus compare the results with those found for normally growing cells.

The technique of 'coincidence hybridization' (Midgley & Gray, 1971) was used to obtain accurate values for mRNA contents in starved cultures of strain CP78. Cultures were grown over several generations in supplemented glucose–salts media. At E_{650} 0.03, [5-^{3}H]uracil or [2-^{14}C]uracil was added to allow continuous incorporation of radioactivity into the cells over a long period. When the turbidity of the cultures reached E_{650} 0.5, the whole of the ^{14}C-labelled and one-quarter of the ^{3}H-labelled cultures were harvested and the RNA was extracted. The remainder of the ^{3}H-labelled culture was harvested, washed and resuspended in amino acid-free glucose–salts medium containing [5-^{3}H]uracil at the same specific radioactivity as before. At intervals during starvation portions were removed from the culture and the labelled RNA was extracted. Mixtures were then made of equal weights of randomly ^{14}C-labelled RNA and randomly ^{3}H-labelled RNA. These were then hybridized with DNA at low DNA/RNA ratios (5:1) to bind the mRNA. After corrections for small spurious binding of RNA to the filters by ribonuclease treatment (Gillespie & Spiegelman, 1965) and for a small contribution by labelled rRNA (Midgley & Gray, 1971), the ^{14}C/^{3}H radioactivity ratio in the hybrids was compared with the ratio in the input RNA. As argued by Midgley & Gray (1971), this technique is capable of detecting very small changes in relative amounts of mRNA produced in various conditions, even when as little as 2% of the total RNA input is in the messenger fraction. The results are shown in Table 2. Assuming that mRNA constitutes 2.2% of the total labelled RNA in normally growing cultures of *E. coli* (Gray & Midgley, 1970), then the mRNA content is unchanged in strain CP78 deprived of amino acids. These

Table 2. *Amounts of mRNA in amino acid-starved cultures of E. coli CP78 (RC^{str})*

The DNA/RNA weight ratio used in hybridization was 5:1.

Duration of starvation (min)	Radioactivity hybridized (c.p.m.)		^{3}H/^{14}C radioactivity ratio	% of RNA as mRNA
	^{3}H	^{14}C		
0	3085	1458	2.12	2.2
	4663	2072	2.25	
	4243	1951	2.17	
	4839	2446	2.00	
30	5350	2171	2.46	2.3
	6038	3022	2.00	
	5693	2529	2.25	
	4380	2066	2.12	
60	4608	2034	2.26	2.1
	7741	3919	1.97	
	2331	1212	1.92	
	6573	3380	1.94	
90	5144	2212	2.32	2.1
	5491	2440	2.25	
	4644	2112	2.20	
	5087	2494	2.04	
120	5336	1714	3.11	2.8
	5937	1937	3.06	
	6372	2430	2.62	
	6743	2264	3.00	
	5472	2042	2.68	

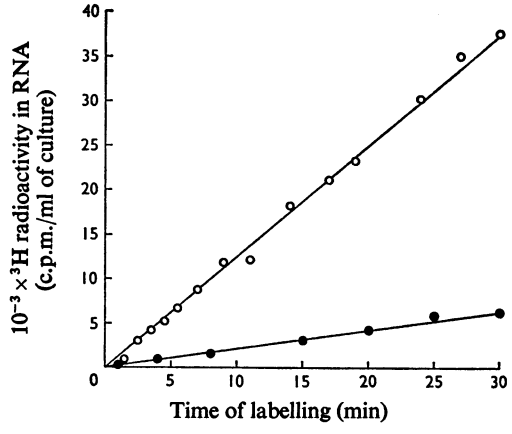


Fig. 7. *Labelling of total acid-precipitable RNA and hybridizable RNA in amino acid-starved cultures of E. coli CP79 (RC^{rel})*

o, Total acid-precipitable RNA; ●, hybridized mRNA. The DNA/RNA weight ratio used for hybridization was 5:1.

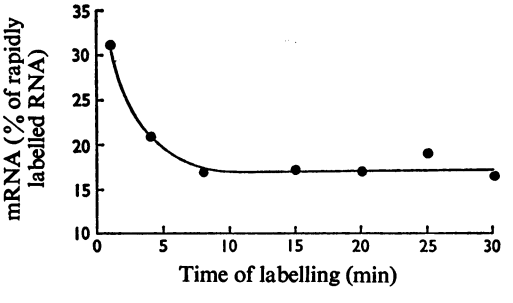


Fig. 8. *Percentage of rapidly labelled RNA as mRNA in amino acid-starved cultures of E. coli CP79 (RC^{rel}), measured against the time of labelling*

results confirm the findings obtained by Friesen (1966), which were based on less accurate direct determinations.

Strain CP79 (RC^{rel}). (*a*) Determination of mRNA labelling: DNA–RNA hybridization. A culture of strain CP79 that had been starved of amino acids for 20min was supplemented with unlabelled uracil (5μg/ml), followed 5min later by [5-^{3}H]uracil (0.5μCi/ml; specific radioactivity 1Ci/mmol). The uptake of radioactivity into total and mRNA was measured as described for strain CP78 (RC^{str}). The results are shown in Fig. 7.

As in strain CP78, radioactivity was incorporated from the medium directly into the RNA of starved cells without a measurable kinetic delay. In contrast with strain CP78, the fraction of radioactivity entering mRNA at early times was 32%, as was found for steadily growing cultures (Gray & Midgley, 1970). Fig. 8 shows the fraction of accumulated labelled RNA as mRNA during the experiment. Over a period of approx. 7min the fraction of labelled RNA as mRNA fell from 32 to 17% and remained close to this value over the duration of the experiment. These results imply that about half the mRNA labelled was unstable, whereas the rest was relatively stable and accumulated in the cells. The kinetics of incorporation of radioactivity into total and mRNA fractions in amino acid-deprived strain CP79 were indistinguishable from those obtained with steadily growing *E. coli* CP78 (RC^{str}), MRE600 (RC^{str}) and CP79 (RC^{rel}) cultures inhibited with chloramphenicol (0.2mg/ml) (Midgley & Gray, 1971; W. J. H. Gray, unpublished work). The measurement of the proportion of radioactive mRNA in the total RNA formed after amino acid withdrawal was confirmation of the results obtained by Friesen (1966) and Avery *et al.* (1969).

In a further experiment, portions of a culture of strain CP79, starved of required amino acids, were labelled for a short period at various times after the onset of starvation. Each portion was supplemented with [5-^{3}H]uracil (1μCi/ml; specific radioactivity 1Ci/mmol) and was allowed to incorporate radioactivity for 20s at 37°C. The proportion of mRNA in the labelled cultures was estimated by DNA–RNA hybridization. The results (Table 3) show that the fraction of total rapidly labelled RNA as mRNA was constant at 34% throughout the period of starvation. As was the case for RC^{str} or RC^{rel} cultures inhibited with chloramphenicol, the formation of mRNAwas normal, but about half the fraction made at any time quickly decayed and half was stable (Midgley & Gray, 1971).

(*b*) Determination of mRNA contents in amino acid-deprived RC^{rel} cultures. The fact that half the mRNA formed at any time in deprived RC^{rel} cultures is relatively stable implies that it will accumulate in the cells to extents that are considerably higher than normal. Thus randomly ^{14}C- and ^{3}H-labelled cultures of strain CP79 were obtained, and the RNA species were extracted. Randomly ^{3}H-labelled RNA was also obtained from strain CP79 during suspension in amino acid-free glucose–salts medium. As described for strain CP78, mixtures of ^{14}C-labelled RNA and ^{3}H-labelled RNA were made and hybridized with DNA at DNA/RNA ratios 5:1. The $^{14}C/^3H$ radioactivity ratios of the hybrids were compared with those of the input RNA samples as described above. The results are shown in Table 4. They show that the mRNA content of amino acid-starved strain CP79 cultures increased steadily over a period of 2h to a final value of some 8% of the total cellular RNA (Friesen, 1966). These results agree with those calculated from the knowledge that

Table 3. *Fraction of mRNA in rapidly labelled RNA during amino acid starvation of E. coli CP79 (RC^{rel})*

The DNA/RNA weight ratio used in hybridization was 5:1. The values in column 5 were obtained by knowing that the efficiency of RNA hybridization was 80% and that, with the DNA/RNA weight ratio used, 2.5% of the hybridized RNA was rRNA (Pigott & Midgley, 1968).

Period of prior starvation (min)	Radioactivity in RNA after 20s pulse (c.p.m.)		% of labelled RNA hybridized	Corrected % of RNA hybridized
	Hybridized	Non-hybridized		
60	2891	6873	29.6	34.7
120	1238	3183	28.5	33.3
180	888	2172	28.9	33.8

Table 4. *Amounts of mRNA in amino acid-starved cultures of E. coli CP79 (RC^{rel})*

The DNA/RNA weight ratio used in hybridization was 5:1.

Duration of starvation (min)	Radioactivity hybridized (c.p.m.)		$^3H/^{14}C$ radioactivity ratio	% of RNA as mRNA in starved culture
	^3H	^{14}C		
0	2801	3938	0.71	2.5
	3156	4445	0.71	
	2535	3170	0.80	
	3313	4348	0.76	
30	3536	3663	0.97	3.2
	3767	3901	0.86	
	3396	3941	0.86	
60	3931	3269	1.20	4.3
	3963	3335	1.19	
	3900	3100	1.26	
90	6533	3188	2.05	7.0
	5914	2877	2.05	
	6588	3200	2.05	
	5412	2884	1.87	
120	5972	2869	2.08	7.3
	6196	2993	2.08	
	6209	2959	2.08	

stabilized mRNA constituted 16% of the RNA formed in conditions of amino acid deprivation (Fig. 8) and that the total RNA increased 2.5-fold after 3h starvation (Fig. 1*b*). From these values the mRNA content should be $100[(16/100)\times(1.5/2.5)]\times 100=10\%$ of the total cellular RNA. As with the chloramphenicol-inhibited cultures (Midgley & Gray, 1971), no difficulties in interpretation of the kinetics of labelling mRNA were caused by the technique of DNA–RNA hybridization.

Discussion

Though the results in this paper have indicated that the synthesis of the stable RNA species (rRNA + tRNA) is preferentially inhibited in RC^{str} amino acid auxotrophs suspended in media lacking the required supplements, they do not go so far as to support the belief that the synthesis of stable RNA is almost entirely suppressed (cf. Sarkar & Moldave, 1968; Lazzarini & Dahlberg, 1971). By two independent methods, invoking the special characteristics of mRNA in its hybridization with DNA and in its metabolic instability, estimations of the flux of radioactive precursors into RNA in starved cells show that in these conditions not more than 60–70% of the labelled RNA is mRNA. This proportion is higher than found by some workers for mRNA in normally growing RC^{str} or RC^{rel} strains (Midgley & McCarthy, 1962; Friesen, 1966; Pigott & Midgley, 1968; Fry & Artman, 1969; Gray & Midgley, 1970), but is comparable with values for normal growth obtained by

others (Kennell, 1968; Salser *et al.*, 1968; Winslow & Lazzarini, 1969*a*,*b*; Pato & von Meyenberg, 1970; Schwartz *et al.*, 1970; Lazzarini & Dahlberg, 1971). Travers (1971) has suggested that in amino acid-starved RC^{str} strains the unusual nucleotide (ppGpp) that accumulates in the cells (Gallant & Cashel, 1967; Gallant & Harada, 1969) is a specific inhibitor of the initiation of chains of stable RNA. Thus, since it is proposed that mRNA synthesis and turnover is relatively unaffected by the accumulation of this compound, the mRNA fraction will constitute the dominant species in any specimen of rapidly labelled RNA.

In contrast, Gallant & Harada (1969) obtained evidence that in amino acid-starved RC^{str} strains the concentration of intracellular GTP falls considerably from its normal values, since ppGpp is an inhibitor of the enzyme IMP dehydrogenase (Gallant *et al.*, 1970), an essential enzyme in the pathway of new guanine nucleotide synthesis. This suggests that a primary effect of amino acid deprivation in RC^{str} organisms lies in the ability of ppGpp to interfere with nucleic acid biosynthesis, by depriving the cells of sufficient GTP to permit high rates of RNA chain polymerization. An alternative explanation of changes in the mRNA/stable RNA ratio labelling can be made on these lines.

It has been proposed that RNA chain initiation is more easily inhibited by low concentrations of ATP or GTP than is the subsequent process of chain polymerization (Anthony *et al.*, 1969; Wu & Goldthwait, 1969*a*,*b*). However, Thomas *et al.* (1970) and Varney *et al.* (1970) suggested that, in multiple purine-requiring mutants of *E. coli*, the lack of one of the purine nucleoside triphosphates could lead simultaneously to the inhibition of initiation of RNA chains that begin with this residue and to inhibition of polymerization in chains begun by the other. They further showed that net RNA synthesis was much more dependent on the ability of the cells to synthesize GTP than on their ability to synthesize ATP. In conditions of purine deprivation, where guanine nucleotide concentrations were especially lowered, the bulk of any RNA formed was mRNA (Varney *et al.*, 1970).

Midgley & Gray (1971) confirmed the proposals made by Maaløe & Kjeldgaard (1966) that, in media permitting bacteria to grow at a low rate (e.g. glucose–salts), there are DNA-dependent RNA polymerase molecules in the cells in excess over the requirements for the observed steady-state rate of synthesis of RNA. In all media, however, the cistrons responsible for rRNA and tRNA synthesis are more crowded with polymerases than are other cistrons for mRNA (Mueller & Bremer, 1968). Thus any situation that can lead to a decrease in the rate of RNA chain initiation and polymerization (e.g. by lack of GTP) will give rise to a preferential crowding of the extra enzymes on those cistrons that do not form RNA chains initiated by GTP. Jorgensen *et al.* (1969) have found that about half of the 5′-termini of nascent RNA in *E. coli* are initiated as pppA---. If such chains are primarily concerned with mRNA synthesis, then a preferential crowding of polymerases will occur automatically on mRNA cistrons. Changes in the proportion of labelled RNA species formed in amino acid-deprived RC^{str} cultures could arise from factors in addition to the known specific inhibition of stable RNA chain initiation by increasing concentrations of ppGpp (Travers *et al.*, 1970). Further work (Gray *et al.*, 1972) has indicated that, after prolonged amino acid deprivation of RC^{str} multiple amino acid auxotrophs, a general decrease in the rate of RNA chain polymerization occurs, presumably caused by a lowering of the intracellular concentration of GTP (Gallant & Harada, 1969).

In RC^{rel} strains the rate of mRNA degradation is decreased (Friesen, 1966) and this fraction accumulates. In disagreement with Friesen's (1966) findings, half the newly formed mRNA in deprived RC^{rel} organisms is very labile, and turns over at rates that are at least as rapid as found in normal growth. The rapid turnover of such a large proportion of the mRNA in these conditions is comparable with the behaviour of mRNA in chloramphenicol-inhibited RC^{str} strains of *E. coli* (Midgley & Gray, 1971). This similarity in behaviour indicates that, operationally, the effect of the RC^{rel} mutation on the stability of mRNA formed during amino acid withdrawal is similar to that caused by chloramphenicol inhibition. Suggestions have been made (Sussman & Gilvarg, 1969) that protein stability is affected by the state of the *RC* gene in amino acid-starved RC^{rel} auxotrophs. Hall & Gallant (1971) and Sokawa *et al.* (1971) have also shown that in these conditions RC^{rel} auxotrophs are much less able than RC^{str} auxotrophs to synthesize normal proteins, indicating a defect in the protein-biosynthetic machinery. Since it is known that chloramphenicol also interferes with the formation of peptide bonds, by association with the 50S ribosomal subunit (Vasquez, 1964), it is possible that the effect of the antibiotic and the RC^{rel} allele on mRNA stability in certain conditions arises from alterations in the structure of the ribosomes.

From our findings we suggest that the stability of some of the mRNA in amino acid-starved RC^{rel} organisms arises from its protection by immobilized ribosomes that, either by inaccurate translation of mRNA strands (Hall & Gallant, 1971) or by translocation without translation (Gurgo *et al.*, 1969; Midgley & Gray, 1971), have become unable to release themselves normally from polyribosomal complexes. Unstable mRNA could be the result of a random attachment of lethal ribosomes bearing the polar nuclease described by Kuwano *et al.* (1970), which then degrades the RNA strands as far as the

region in which other ribosomes are held (Midgley & Gray, 1971). This would again suggest that, in amino acid-starved cultures of RC^{rel} organisms, mainly distal regions of mRNA would be preserved. This proposal is not in agreement with the findings of others (e.g. Morse & Guertin, 1971) that, for certain polycistronic mRNA molecules, the chains are especially labile in distal regions, if translation is artificially interrupted by 'nonsense' mutations. However, many operons *in vivo* may be monocistronic, so that problems arising from this effect may be of less significance in the whole messenger fraction.

W. J. H. G. thanks the Medical Research Council for a postgraduate studentship. J. E. M. M. is Senior Research Associate 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.

References

Altman, S. (1971) *Nature New Biol. (London)* **229**, 19
Anthony, D. D., Wu, C. W. & Goldthwait, D. A. (1969) *Biochemistry* **8**, 246
Avery, R. J. & Midgley, J. E. M. (1969) *Biochem. J.* **115**, 383
Avery, R. J., Midgley, J. E. M. & Pigott, G. H. (1969) *Biochem. J.* **115**, 395
Borek, E. & Ryan, A. (1958) *J. Bacteriol.* **75**, 72
Borek, E., Ryan, A. & Rockenback, J. (1955) *J. Bacteriol.* **69**, 460
Brunschede, H. & Bremer, H. (1971) *J. Mol. Biol.* **58**, 35
Cashel, M. (1969) *J. Biol. Chem.* **244**, 3133
Dagley, S., Turnock, G. & Wild, D. G. (1963) *Biochem. J.* **88**, 555
Doolittle, W. F. & Pace, N. R. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 1786
Edlin, G., Stent, G. S., Baker, R. F. & Yanofsky, C. (1968) *J. Mol. Biol.* **37**, 257
Fiil, N. & Friesen, J. D. (1968) *J. Bacteriol.* **95**, 729
Fleissner, E. & Borek, E. (1962) *Proc. Nat. Acad. Sci. U.S.* **48**, 1199
Forchhammer, J. & Kjeldgaard, N. O. (1968) *J. Mol. Biol.* **37**, 245
Fraenkel, D. G. & Neidhardt, F. C. (1961) *Biochim. Biophys. Acta* **53**, 96
Friesen, J. D. (1966) *J. Mol. Biol.* **20**, 559
Friesen, J. D. (1968) *J. Mol. Biol.* **32**, 183
Fry, M. & Artman, M. (1969) *Biochem. J.* **115**, 295
Gallant, J. A. & Cashel, M. (1967) *J. Mol. Biol.* **25**, 545
Gallant, J. A. & Harada, B. (1969) *J. Biol. Chem.* **244**, 3125
Gallant, J. A., Ehrlich, H., Hall, B. & Laffler, T. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 397
Gillespie, D. & Spiegelman, S. (1965) *J. Mol. Biol.* **12**, 829
Gray, W. J. H. & Midgley, J. E. M. (1970) *Biochem. J.* **120**, 279
Gray, W. J. H. & Midgley, J. E. M. (1971) *Biochem. J.* **122**, 161
Gray, W. J. H., Vickers, T. G. & Midgley, J. E. M. (1972) *Biochem. J.* **128**, 102
Gurgo, C., Apirion, D. & Schlessinger, D. (1969) *J. Mol. Biol.* **45**, 205
Hall, B. G. & Gallant, J. A. (1971) *J. Mol. Biol.* **61**, 271
Jorgensen, S. E., Buch, L. B. & Nierlich, D. P. (1969) *Science* **164**, 1067
Kennell, D. (1968) *J. Mol. Biol.* **34**, 85
Kurland, C. G. & Maaløe, O. (1962) *J. Mol. Biol.* **4**, 193
Kuwano, M., Schlessinger, D. & Apirion, D. (1970) *Nature (London)* **226**, 514
Lavallé, R. & de Hauwer, G. (1968) *J. Mol. Biol.* **37**, 269
Lazzarini, R. A. & Dahlberg, A. E. (1971) *J. Biol. Chem.* **246**, 420
Lazzarini, R. A. & Winslow, R. M. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 383
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Maaløe, O. & Kjeldgaard, N. O. (1966) *Control of Macromolecular Biosynthesis*, p. 97, W. A. Benjamin, New York
Midgley, J. E. M. (1962) *Biochim. Biophys. Acta* **61**, 513
Midgley, J. E. M. & Gray, W. J. H. (1971) *Biochem. J.* **122**, 149
Midgley, J. E. M. & McCarthy, B. J. (1962) *Biochim. Biophys. Acta* **61**, 696
Morris, D. W. & Kjeldgaard, N. O. (1968) *J. Mol. Biol.* **31**, 145
Morse, D. E. & Guertin, M. (1971) *Nature New Biol. (London)* **232**, 165
Mueller, K. & Bremer, H. (1968) *J. Mol. Biol.* **38**, 329
Munro, A. J. & Korner, A. (1964) *Nature (London)* **201**, 1194
Nierlich, D. P. (1968) *Proc. Nat. Acad. Sci. U.S.* **60**, 1345
Pato, M. L. & von Meyenberg, K. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 497
Pigott, G. H. & Midgley, J. E. M. (1968) *Biochem. J.* **110**, 251
Raue, H. A. & Gruber, M. (1971) *Biochim. Biophys. Acta* **232**, 314
Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. J. (1957) *Carnegie Inst. Wash. Publ.* **607**, 5
Rose, J. K., Mosteller, R. D. & Yanofsky, C. (1970) *J. Mol. Biol.* **51**, 541
Salser, W., Janin, J. & Levinthal, C. (1968) *J. Mol. Biol.* **31**, 237
Samarina, O. P. (1964) *Biochim. Biophys. Acta* **91**, 688
Sarkar, S. & Moldave, K. (1968) *J. Mol. Biol.* **33**, 213
Schneider, W. C. (1957) *Methods Enzymol.* **3**, 680
Schwartz, T., Craig, E. & Kennell, D. (1970) *J. Mol. Biol.* **54**, 299
Sippel, A. & Hartmann, G. (1968) *Biochim. Biophys. Acta* **157**, 218
Sokawa, Y., Sokawa, J. & Kaziro, Y. (1971) *Nature New Biol. (London)* **234**, 7
Stent, G. S. & Brenner, S. (1961) *Proc. Nat. Acad. Sci. U.S.* **47**, 2005
Stubbs, J. & Hall, B. D. (1968) *J. Mol. Biol.* **37**, 289
Sussman, A. J. & Gilvarg, C. (1969) *J. Biol. Chem.* **244**, 6304
Thomas, G. A., Varney, N. F. & Burton, K. (1970) *Biochem. J.* **120**, 117

Travers, A. (1971) *Nature New Biol.* (*London*) **229**, 69
Travers, A., Kamen, R. I. & Cashel, M. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 415
Turnock, G. & Wild, D. G. (1964) *Biochem. J.* **90**, 32P
Turnock, G. & Wild, D. G. (1965) *Biochem. J.* **95**, 597
Varney, N. F., Thomas, G. A. & Burton, K. (1970) *Biochem. J.* **120**, 125
Vasquez, D. (1964) *Nature* (*London*) **203**, 257
Vickers, T. G. & Midgley, J. E. M. (1971) *Nature New Biol.* (*London*) **233**, 210
Wehrli, W., Buesch, J., Knusel, F. & Staehelin, M. (1968) *Biochim. Biophys. Acta* **157**, 215
Winslow, R. M. & Lazzarini, R. A. (1969*a*) *J. Biol. Chem.* **244**, 1128
Winslow, R. M. & Lazzarini, R. A. (1969*b*) *J. Biol Chem.* **244**, 3387
Wu, C. W. & Goldthwait, D. A. (1969*a*) *Biochemistry* **8**, 4450
Wu, C. W. & Goldthwait, D. A. (1969*b*) *Biochemistry* **8**, 4458