The Effect of Trimethoprim on Macromolecular Synthesis in *Escherichia coli*

RIBOSOME MATURATION IN RC^{str} AND RC^{rel} STRAINS

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When *Escherichia coli* was inhibited with trimethoprim in media supplemented with nucleotide bases, glycine and methionine, both RC^{str} and RC^{rel} strains continued to accumulate RNA at rates very close to those in growing controls. The effects of trimethoprim on protein synthesis were studied by using as an experimental basis the rate of maturation of ribosomal particles from RNA-rich precursors. 1. In RC^{str} cultures given nucleotide bases but no amino acids, RNA accumulation was inhibited because of amino acid lack. However, maturation of ribosomes from their precursors was more severely inhibited than was the synthesis of rRNA. The restraints on protein synthesis were more severe at the level of translation than the transcription of operons specific for the formation of ribosomal proteins. The kinetic delay time in the passage of rRNA from RNA-rich intermediates to the final ribosome products was therefore increased some three- to four-fold. 2. In RC^{rel} cultures in the same conditions, trimethoprim inhibition stopped ribosomal particle synthesis, but rRNA-rich precursors accumulated. 3. If glycine + methionine were also added to inhibited RC^{str} cultures, RNA accumulation resumed at a high rate. However, ribosomal maturation was still considerably disturbed because of a disproportionate response of the cells in the formation of protein and RNA. 4. With RC^{rel} cultures, addition of the amino acids caused a large increase in the rate of ribosome maturation, though the degree of disproportionation between the rates of rRNA and ribosomal protein synthesis was now identical with that found in RC^{str} strains. 5. When inhibited RC^{rel} cultures were supplemented, there was still a severe inhibition of protein synthesis at the level of chain initiation, but inaccuracies in the process of polypeptide chain elongation were greatly decreased. This suggests that the effects of the RC^{rel} mutation on the fidelity of protein synthesis in bacteria are not directed at the point of chain initiation.

In previous studies on the effects of trimethoprim on RNA and protein synthesis in bacteria, it was proposed that the inhibition of RNA accumulation in RC^{str} strains was in some way connected with the inhibition of protein chain initiation (Shih *et al.*, 1966). However, more recently Moivic & Pizer (1971) and Dale & Greenberg (1972) suggested that the expression of RC^{str} control was due to a decrease in the intracellular concentrations of amino acids synthesized by pathways involving tetrahydrofolate, rather than a primary inhibition in the formation of *N*-formylmethionyl-tRNA^{fMet} for chain initiation of proteins. These findings were confirmed and extended by Smith & Midgley (1973*a, b*) who showed that the intracellular concentrations of glycine and methionine were together decreased sufficiently to permit the RC^{str} amino acid control of RNA synthesis to be expressed. The rate of formylation of methionyl-tRNA^{fMet} is severely decreased in trimethoprim-inhibited bacterial extracts *in vitro* (Eisenstadt & Lengyel, 1966). However, this effect

cannot be sufficient by itself *in vivo* to decrease protein biosynthetic rates below those at which the production of glycine and methionine by protein turnover can adequately supply the amino acid pools for new biosynthesis.

The probability that protein synthesis in nucleotide-base-supplemented trimethoprim-inhibited RC^{str} cultures is affected at several different stages has allowed us to devise methods to study each inhibitory step in isolation. Two examples were chosen for detailed study: (i) the relationships between the synthesis of rRNA and ribosomal proteins in trimethoprim-inhibited RC^{str} cultures; (ii) the effect of the RC^{rel} mutation on stable RNA and protein synthesis during trimethoprim inhibition.

Our results have shown that, in both RC^{str} and RC^{rel} strains of *E. coli*, trimethoprim inhibition decreases the rate of biosynthesis of ribosomal and other proteins more than the rate of biosynthesis of rRNA. We have also concluded that the RC^{rel} mutation is not primarily concerned with the process

of protein chain initiation, but is connected with the accuracy of polypeptide chain elongation (Hall & Gallant, 1971).

Materials and Methods

All the general techniques of bacterial growth, harvesting of cultures, the labelling, extraction and fractionation of RNA are referred to in the preceding paper (Smith & Midgley, 1973a). Radiochemicals were as described by Smith & Midgley (1973a), and L-[1-¹⁴C]leucine (61 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Ion-exchange column chromatography

Harvested pellets of bacterial cells were taken up in ice-cold buffer, containing 0.01 M-Tris-HCl 0.01 M-MgCl₂ and 0.05 M-KCl (pH 7.2). The suspension were broken in a pre-cooled French Press (American Instrument Co., Silver Spring, Md., U.S.A.) at a pressure of 100 kPa (15000 lb/in²). The extracts were treated with deoxyribonuclease I (Sigma Chemical Co., St. Louis, Mo., U.S.A., Grade DN-EP) at a concentration of 10 µg/ml at room temperature for 15 min. They were then loaded on to a column (1 cm × 10 cm) of DEAE-cellulose (Serva, Micro-Bio Laboratories, London W.11, U.K.) (capacity 0.91 mequiv/g). The nucleic acids and ribonucleoproteins were fractionated on a linear gradient of 0–1 M-NaCl in the above buffer, as described by McCarthy *et al.* (1962). In agreement with these workers, ribosomes eluted at 0.4 M-NaCl, tRNA and certain ribosome precursors ('neosomes') at 0.5 M-NaCl, and mRNA and very RNA-rich ribosome precursors ('eosomes') at 0.6 M-NaCl. DNA also eluted at 0.5 M-NaCl, but after the enzyme treatment, the fragments were eluted near the start of the gradient.

Antibiotics

Sources of rifampicin, trimethoprim and chloramphenicol were as described by Smith & Midgley (1973a). Puromycin was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Results

In this paper, the labelling of ribosomes and their precursors has been followed by the use of ion-exchange column chromatography on DEAE-cellulose (McCarthy *et al.*, 1962). Because ribosome precursors and the particles produced in chloramphenicol-inhibited cultures often do not display their true molecular characteristics on isolation and sedimentation through sucrose density gradients (Yoshida & Osawa, 1968), a method has been used that probably does not give rise to such artifacts. Whereas Yoshida & Osawa (1968) showed that the

RNA-rich 'chloramphenicol particles' could aggregate with general proteins in the course of cell breakage and fractionation, chromatography on DEAE-cellulose columns of this material, and the similar precursors accumulated in RC^{tr} mutants during amino acid deprivation (Turnock & Wild, 1965), has consistently shown that the particles are almost pure RNA. Indeed, from a chromatographic point of view, chloramphenicol particles and RC^{tr} particles behave like the first precursor stage in normal ribosomal maturation ('eosomes'; McCarthy *et al.*, 1962). It is thus likely that the fractionation of ribosomes and their precursors on DEAE-cellulose gives a truer picture of the distribution of precursor material than does the technique of sucrose-density-gradient sedimentation. Instability of ribosomes and their precursors is always a problem, during the prolonged times required for column elution (for example, McCarthy, 1962). However, provided care is taken to maintain a sufficient Mg²⁺ concentration in the buffers there is no evidence of breakdown (McCarthy *et al.*, 1962). We therefore believe that the technique of column chromatography on DEAE-cellulose is relatively free from the difficulties of interpretation implicit in other methods. Nevertheless, the clear separation of only three peaks on columns demonstrated the limitation of the column method in the fractionation of ribosomes and their precursors. Whereas the basis of fractionation in density gradients is largely by molecular weight, DEAE-cellulose columns separate the ribonucleoprotein particles according to the RNA protein (w/w) ratio typical for each fraction (McCarthy, 1962). This leads to cruder separations, though the peak eluting at 0.4 M salt corresponds to the 50S and 30S ribosomes, the peak at 0.5 M salt to tRNA and the 43S and 25S precursors to ribosomes, and the 0.6 M salt peak to mRNA and the 25S and 18S RNA-rich precursors (McCarthy *et al.*, 1962).

Kinetics of ribosome synthesis in steadily growing cultures of E. coli

Cultures of *E. coli* MRE600 (RC^{tr}) were grown in glucose-salts medium to $E_{650} = 0.4$. [5-³H]Uracil, specific radioactivity 10 mCi/mmol, (0.1 mCi/l) was added. Samples (100 ml) were taken at suitable intervals, and extracts were chromatographed on DEAE-cellulose, after removal of DNA by treatment with deoxyribonuclease (10 µg/ml for 15 min at 20°C). In each case the specific radioactivity of the ribosome peak eluting at 0.4 M-NaCl was measured and plotted against the time of labelling. Fig. 1 summarizes the results. In steadily growing RC^{tr} cultures of *E. coli*, there was a delay time in the labelling of ribosomes of approximately 2.5 min at 37°C. Essentially the same results were obtained from grow-

Fig. 1. Synthesis of ribosomes in *E. coli* MRE 600 (RC^{str}) growing steadily in glucose-salts medium (37°C)

DEAE-cellulose column chromatograms are shown for cell extracts labelled with $[5\text{-}^3\text{H}]\text{uracil}$ for: (a) 30s; (b) 5min; (c) 15min. For details see the text. The part of the chromatograms depicted is that region in which the ribosomes and their precursors were eluted. Fractions collected were 5ml. (d) shows the kinetics of ribosome labelling as a composite of (a)–(c) and other chromatograms not shown. The delay constant (shown by the dashed line) = 2.0–2.5min. $\circ$, E_{260} ; $\bullet$, acid-precipitable ^3H c.p.m./ml.

ing cultures of strain CP79 (RC^{el}). The delay time was the same as that found for *E. coli* by McCarthy *et al.* (1962).

Ribosome labelling in trimethoprim-inhibited RC^{str} mutants of *E. coli*

The cultures of strain MRE600 were grown to $E_{650} = 0.5$, in glucose-salts media. Adenine and guanosine were added to a final concentration of $50\mu\text{g/ml}$. After 5min, trimethoprim ($50\mu\text{g/ml}$) was added, and the cells stopped growing after a further 2 or 3min; 10min after the addition of trimethoprim, $[5\text{-}^3\text{H}]\text{uracil}$ (specific radioactivity 1 Ci/mmol) was added to a concentration of $0.5\mu\text{mol/ml}$. At suitable intervals, samples (100ml) were poured on to crushed ice, harvested and extracted as described above. After removal of DNA, the extracts were again subjected to DEAE-cellulose column chromatography. The results (Fig. 2) show that, compared with steadily growing cultures, there

appears to be a considerable delay in the maturation of ribosomal precursors rich in RNA to the final product eluted at 0.4M salt. Materials eluted at 0.5M - and 0.6M -NaCl (which, of course include tRNA and mRNA) are still a large proportion of the total labelled RNA even after 40min of labelling. Experiments described below show that these differences do not result from an undue accumulation of labelled tRNA, though some of the labelled material at 0.6M salt has the characteristics of mRNA. The kinetics of labelling the 0.4M salt peak of mature ribosomal material (Fig. 2d) show that its labelling is now delayed by about 8min. This is some 3–4 times larger than the delay time for steadily growing cultures (Fig. 1d). Some of this extra delay may stem from the fact that the rate of accumulation of stable RNA in trimethoprim-inhibited RC^{str} cultures is only about 10% of the rate in growing cells (Smith & Midgley, 1973a). If the rate of mRNA synthesis and turnover is a large proportion of the total flux of material entering RNA then it may take longer for

Fig. 2. Synthesis of ribosomes in *E. coli* MRE600 (RC^{str}) inhibited with trimethoprim and supplemented with purines. DEAE-cellulose column chromatograms are shown for cell extracts labelled with $[5\text{-}^3\text{H}]\text{uracil}$ for: (a) 4 min, (b) 10 min and (c) 40 min (for details see text); (d) depicts the kinetics of ribosome labelling as a composite of (a)–(c) and other chromatograms not shown. The delay constant (shown by the dashed line) was 8.5–9 min. $\circ$, E_{260} ; $\bullet$, acid-precipitable ^3H c.p.m./ml.

the nucleotide pools to become equilibrated with the exogenous labelled nucleotide base, causing an increased delay in the time required for the base to enter RNA and ribosomes at the specific radioactivity of the exogenously supplied material. However, the fact that labelled ribosome precursors accumulate in both the 0.5 M and 0.6 M salt regions shows that the maturation of ribosomes from their precursors also has been severely delayed. Further work indicates that this delay is caused by a deficiency in the biosynthesis of ribosomal proteins, probably the result of inhibition of mRNA translation more than inhibition of its transcription.

Ribosome labelling in trimethoprim-inhibited RC^{ret} mutants of *E. coli*

Cultures of *E. coli* CP79 (RC^{ret}) were grown in amino acid-supplemented glucose–salts media to $E_{650} = 0.4$. The cultures were then given unlabelled adenine and guanosine ($50 \mu\text{g/ml}$) followed by trimethoprim ($50 \mu\text{g/ml}$) as described previously for the RC^{str} strain MRE600. $[5\text{-}^3\text{H}]\text{uracil}$ (specific radioactivity 10 mCi/mmol) was added 10 min after trimethoprim, the labelled culture being sampled

periodically as described above. The extracts were fractionated on DEAE-cellulose (Fig. 3). In agreement with the findings of Turnock & Wild (1965), the RNA synthesized in *E. coli* RC^{ret} strains inhibited in protein synthesis was eluted from the column as a peak at 0.6 M salt, with a smaller peak at 0.5 M salt corresponding to tRNA and possibly some ribosome precursors. Thus most of the RNA produced was nearly protein-free (McCarthy, 1962). There was no evidence of any appreciable passage of material from the RNA-rich peak to the ribosome peak at 0.4 M salt, even after prolonged incubation of the inhibited cells.

Causes of the deficiency in ribosome maturation in trimethoprim-inhibited RC^{str} strains

The deficiency in the rate of ribosome maturation in trimethoprim-inhibited RC^{str} strains (Fig. 2) could arise from either a rate limitation at the level of transcription of the mRNA specific for ribosome protein synthesis, or a decrease in the rate of its translation or some deficiency in the actual process of ribosome maturation from an independent pool of protein. To resolve this problem, the residual synthesis of

Fig. 3. Ribosome labelling in a culture of *E. coli* CP 79 (RC^{rel}) inhibited with trimethoprim and supplemented with purines

DEAE-cellulose column chromatograms are shown for cell extracts labelled with $[5\text{-}^3\text{H}]\text{uracil}$ for (a) 10min and (b) 40min. $\circ$, E_{260} ; $\bullet$, acid-precipitable ^3H c.p.m./ml.

ribosomes was followed in trimethoprim-inhibited cultures given high concentrations of rifampicin to stop RNA synthesis at the point of chain initiation (Sippel & Hartmann, 1968). In such conditions, rifampicin-inhibited cells are still capable of synthesizing proteins while the pre-existing mRNA persists (Lancini & Sartori, 1968). This situation provides an ideal opportunity to study the residual synthesis of ribosomal proteins in the absence of further synthesis of rRNA, but in the presence of precursor molecules capable of accepting new proteins.

A culture of *E. coli* MRE600 (RC^{str}) was inhibited with trimethoprim, in the presence of exogenous adenine and guanosine ($30\mu\text{g/ml}$); 10min later, $[5\text{-}^3\text{H}]\text{uracil}$ (specific radioactivity 100mCi/mmol) was added, and the cultures were allowed to incorporate the isotope for $1\frac{1}{2}$ min. At this time, a hundredfold excess of unlabelled uracil was added. At 2min a sample (100ml) was poured into crushed ice, and rifampicin (0.2g/l) was added to the remainder. The rifampicin-inhibited cells were incubated for a further 30min and were also harvested. Cell extracts were then chromatographed on DEAE-cellulose (Fig. 4). Though after 2min of labelling very little acid-precipitable ^3H -labelled material was eluted with the ribosomes at 0.4M salt, a much larger proportion of the labelled material was found in the ribosome peak after 30min of labelling. At this time, the mRNA in the cells had decayed, giving negligible amounts of hybridized RNA in the usual tests for mRNA by DNA-RNA hybridization (J. E. M. Midgley, unpublished work). Assuming that the addition of an excess of unlabelled uracil just before the rifampicin effectively stopped further incorporation of label into RNA, over 40% of the rapidly labelled RNA formed before rifampicin inhibition was eventually coated with proteins, though not all was taken through to the 0.4M salt peak of ribosomes. This estimate is in reasonable agreement with the direct estimation of stable RNA content in rapidly labelled RNA made by DNA-RNA hybridization (Smith & Midgley, 1973a). The results suggest that, after the inhibition of RNA synthesis (by rifampicin) in trimethoprim-treated cells, ribosomal protein is either newly synthesized or made available from a pre-existing pool to convert much more of the RNA-precursors into complete ribosomes.

Further experiments were next carried out to distinguish between the various explanations of the delay in ribosome maturation in conditions of trimethoprim-inhibition. A culture of *E. coli* MRE600 was inhibited with trimethoprim in the presence of exogenous purines as before. After 10min, $[2\text{-}^{14}\text{C}]\text{uracil}$ (specific radioactivity 0.4mCi/mmol) was added, and the cultures were incubated for 20min. At this time, a sample (100ml) was taken, and the remainder (200ml) was inhibited with rifampicin (0.1g/l). The rifampicin-inhibited culture was then split into two equal parts. One acted as a control in which residual protein synthesis was permitted, but to the other, puromycin (0.1g/l) was added to stop the synthesis of further protein. Both cultures were again incubated for 20min. The cell extracts were again chromatographed on DEAE-cellulose (Fig. 5).

Though the specific radioactivity of the 0.4M salt ribosome peak more than doubled over a 20min period of incubation with rifampicin, it did not alter when puromycin was added to prevent further protein synthesis. This indicates that, over the period

Fig. 4. Fate of rapidly labelled RNA in trimethoprim-inhibited *E. coli* MRE 600 (RC^{str}) after the addition of rifampicin

(a) Chromatogram of cell extract labelled for 2min with $[5-^3\text{H}]\text{uracil}$. (b) As for (a), but given rifampicin (0.2g/l) and incubated for a further 30min. $\circ$, E_{260} ; $\bullet$, acid-precipitable ^3H c.p.m./ml.

of incubation, there was no pre-existing free ribosomal protein in an independent pool that could slowly mature the RNA-rich precursors into new particles. Further, there was evidence that the residual synthesis of ribosomal protein in rifampicin-inhibited cells stabilized much of the rRNA in RNA-rich precursors against a slow degradation to acid-soluble materials. If puromycin was added to inhibit protein synthesis after the addition of rifampicin, 67% of the labelled RNA was rendered acid-soluble after 20min. If no puromycin was added, only 35% of the

labelled RNA was lost, and more radioactivity was found in the 0.4M and 0.5M salt peaks corresponding to ribosomes and their immediate precursors. Since, in puromycin-inhibited cultures, mRNA is completely labilized (Friesen, 1966), the value of 35% unstable RNA in the labelled RNA of trimethoprim-inhibited cultures given 20min incubation with $[^{14}\text{C}]\text{uracil}$ is in good agreement with the direct hybridization measurements of Smith & Midgley (1973a), assuming that nearly all the RNA lost is in the messenger fraction. Further, rRNA in the form of 'chloramphenicol particles' is also unstable in the cells, and decays with a half-life at 37°C of about 20min (Midgley & Gray, 1971). Thus similar rRNA, prevented from maturing into more protein-rich ribosome precursors by puromycin, may have a similar instability. This suggests that, of the 67% labelled RNA lost after the addition of rifampicin and puromycin in 20min, about half was rRNA in a precursor form.

Obviously, the fact that ribosomal protein synthesis is detected in trimethoprim-inhibited cells at a rate lower even than the depressed rate for rRNA shows that at least some mRNA specific for these proteins is present for translation. Experiments were next designed to show whether the rate-limiting step on ribosomal protein synthesis was imposed by limitations on mRNA transcription or translation. *E. coli* MRE600 (RC^{str}) was grown in glucose-salts medium and inhibited with trimethoprim in the presence of purines as before. After 10min of inhibition, $[5-^3\text{H}]\text{uracil}$ (specific radioactivity 10mCi/mmol) was added. The culture was labelled for 10min, and then inhibited with rifampicin (0.1g/l). It was split into three portions (100ml each). After 2min in the presence of rifampicin the 20 common amino acids, each at a concentration of $20\mu\text{g/ml}$, were added to one portion. Another portion received the amino acids 5min after rifampicin, and the third acted as a control. Incubation of all portions was then continued for 30min. The cell extracts of the harvested cultures were chromatographed on DEAE-cellulose as before (Fig. 6).

The results show that the addition of amino acids, which relieve the limitations on protein synthesis imposed by lack of glycine and methionine (Smith & Midgley, 1973a), but do not relieve the limitation on chain initiation, leads to an approximate doubling of the specific radioactivity of the ribosomes eluting from DEAE-cellulose at 0.4M salt. As, in both cases, the amino acids were added sufficiently later after rifampicin to ensure that RNA synthesis had finished, the increase in the ribosomal radioactivity cannot be due to a momentary increase in the specific activity of RNA precursors during the completion of chains. It appears that the translation of the ribosome protein-specific mRNA species in trimethoprim-inhibited cells is more limited than is transcription,

Fig. 5. Formation of ribosomes in trimethoprim-inhibited RC^{str} *E. coli* after the addition of rifampicin and puromycin

(a) DEAE-cellulose chromatography of cell extract of trimethoprim-inhibited culture labelled with $[2-^{14}\text{C}]$ -uracil for 20 min. (b) As for (a) but given rifampicin and puromycin (0.1 g/l each) and incubated for a further 20 min. (c) As for (b) but given rifampicin only.

and that the addition of amino acids decreases the degree of the inhibition of translation by supplying glycine and methionine. Translation of the mRNA

is still severely limited at the point of protein chain initiation (Smith & Midgley, 1973a), so that the experiments still grossly underestimate the potential

Fig. 6. Effect of amino acids on ribosome maturation in trimethoprim-inhibited RC^{str} *E. coli* given rifampicin (a) DEAE-cellulose chromatography of cell extract from a trimethoprim-inhibited culture labelled with [5- 3H]uracil for 10min, then given rifampicin

of the cultures for producing ribosomal and other proteins.

The species of stable RNA formed in trimethoprim-inhibited RC^{str} cultures

One possible interpretation of the high peak of radioactivity found at 0.5M salt in DEAE-cellulose chromatograms of trimethoprim-inhibited cell extracts could be that the production of tRNA was unbalanced with respect to rRNA synthesis, rather than that ribosome precursors were accumulating. To answer this point, cultures of *E. coli* MRE600 were inhibited with trimethoprim in the presence of purines, and incubated with [2- ^{14}C]uracil (specific radioactivity 5mCi/mmol) for several hours. At intervals, samples (150ml) were removed and poured on to crushed ice. After cell breakage, the extracts were digested with pure deoxyribonuclease and deproteinized with phenol-cresol mixture (Pigott & Midgley, 1968). The RNA was then fractionated on sucrose density gradients (Fig. 7).

The proportions of 16S, 23S and 4S RNA found in trimethoprim-inhibited RC^{str} cells were the same as those normally encountered in steadily growing cells. However, compared with normally growing cultures, the persistence of the 17S precursor to 16S mature rRNA continued over a much longer period. This is probably a reflexion of increased delay in the maturation of ribosomal precursors, since a 17S-16S conversion is part of this process in the formation of 30S ribosomal particles (Attardi & Amaldi, 1970).

Ribosome synthesis in trimethoprim-inhibited RC^{str} cultures supplemented with glycine and methionine

In the previous paper (Smith & Midgley, 1973a), it was shown that the addition of glycine and methionine to RC^{str} cultures inhibited with trimethoprim and supplemented with nucleotide bases resulted in an enhancement in the rate of stable RNA accumulation. On the other hand, protein synthesis was only slightly affected. This suggested that there was still a very severe inhibition on protein synthesis now directed at the level of chain initiation, so that a gross imbalance still existed between RNA and protein formation. This should manifest itself in a similar imbalance between the synthesis of ribosomal protein and rRNA.

(0.1g/l) and incubated for a further 30min. (b) As for (a) except amino acids added 2min after rifampicin. (c) As for (a) except amino acids added 5min after rifampicin. $\circ$, E_{260} ; $\bullet$, acid-precipitable 3H c.p.m./ml.

Fig. 7. Sucrose-density-gradient sedimentation of total RNA from trimethoprim-inhibited RC^{str} *E. coli*. Labelled RNA was purified from cultures incubated with $[2-^{14}\text{C}]\text{uracil}$ for 4 min (a), 20 min (b) and 150 min (c) (for details see the text). $\circ$, E_{260} ; $\bullet$, acid-precipitable ^{14}C c.p.m./ml.

Fig. 8. Ribosome synthesis in trimethoprim-inhibited RC^{str} *E. coli* supplemented with glycine and methionine

DEAE-cellulose chromatography of extracts from trimethoprim-inhibited cultures given no amino acids and labelled with [5- 3 H]uracil for (a) 10 min and (b) 30 min. (c) As for (a) but then given glycine and methionine and incubated for a further 10 min (for details see text). (d) As for (c) but incubated with amino acids for 20 min. ○, E_{260} ; ●, acid-precipitable 3 H c.p.m./ml. (e) Time-course of ribosome labelling as a composite of (a)–(d) and results not depicted.

A culture of *E. coli* MRE600 (RC^{str}) was grown in glucose-salts medium to $E_{650} = 0.5$. Adenine + guanosine (50 μ g/ml) were added, followed immediately by trimethoprim (50 μ g/ml). After 15 min, [5- 3 H]uracil (specific radioactivity 10 mCi/mmol) was added and the culture was incubated for 10 min. At this time the culture was split into two portions (100 ml and 400 ml). To the larger portion was added glycine and methionine (50 μ g/ml each). A sample was immediately harvested (100 ml) from the larger portion, and both portions were incubated. Samples (100 ml) were taken from the supplemented culture at suitable intervals, and the control portion was harvested after a further 20 min incubation. The cells

were broken, extracts were prepared and then chromatographed on DEAE-cellulose (Fig. 8). The results show that the addition of the amino acids increased the rate of formation of rRNA and its maturation to ribosomes, though a continued discrepancy between the recovery of protein and RNA synthesis gave rise to the accumulation of ribosome precursors whose maturation was delayed through a lack of protein. Though protein synthesis overall was only increased by about a factor of two when glycine + methionine were added to trimethoprim-inhibited RC^{str} cells, the differential rate of ribosome labelling was more than a factor of six, when compared with the control. This indicates that

Fig. 9. Ribosome synthesis in trimethoprim-inhibited RC^{rel} *E. coli* supplemented with glycine and methionine

DEAE-cellulose chromatography of extracts from trimethoprim-inhibited cultures incubated with

ribosomal protein synthesis in trimethoprim-inhibited cultures responded much more to the addition of glycine+methionine than did protein synthesis generally.

Ribosome synthesis in trimethoprim-inhibited RC^{rel} cultures supplemented with glycine and methionine

Experiments similar to those described above were carried out with the RC^{rel} strain CP79. The results (Fig. 9) essentially show that the addition of the amino acids increased the rate of ribosome synthesis in RC^{rel} strains to give virtually the same discrepancies between ribosomal protein and rRNA synthesis as were present in the RC^{str} strain, in the corresponding conditions. This demonstrates that trimethoprim-inhibited, amino acid-supplemented RC^{str} and RC^{rel} strains behave similarly in that they can synthesize ribosomes equally well, even though both suffer from a deficiency in protein synthesis.

To check that the apparent increase in ribosome formation in RC^{rel} strains given glycine+methionine was not an artifact of the DEAE-cellulose columns, the stability of the RNA was checked by rapid sampling and acid precipitation. It was argued that, if glycine and methionine stabilized some of the rRNA in RC^{rel} cultures by maturing it into ribosomal particles, then the total labelled RNA formed in trimethoprim-inhibited bacteria should be more stable when the amino acids were added. Fig. 10 shows that this was the case. A culture of *E. coli* CP79 (RC^{rel}) was given exogenous purines and trimethoprim as before. After 10 min of inhibition, [5- 3H]uracil (specific radioactivity 10 mCi/mmol) was added. Immediately, the culture was divided into two. To one part was added glycine+methionine (50 μ g/ml), and the other acted as a control. At intervals, samples (1 ml) from both cultures were squirted into ice-cold 10% (w/v) trichloroacetic acid. After 10 min, rifampicin (0.2 g/l) was added to both cultures and rapid sampling was continued. Fig. 10 shows that, when the amino acids were added, a much higher proportion of the acid-precipitable radioactivity survived after the addition of rifampicin. This confirmed the finding by DEAE-cellulose column chromatography that the addition of glycine and methionine stabilized some of the rRNA in trimethoprim-inhibited RC^{rel} cultures, by markedly increasing the cellular potential for maturation of ribosome precursors.

[5- 3H]uracil for 30 min (a); (b) was treated like (a) but also given glycine+methionine. $\circ$, E_{260} ; $\bullet$, acid-precipitable 3H c.p.m./ml.

Fig. 10. Stability of RNA synthesized in trimethoprim-inhibited *RC^{rel}* *E. coli*

Discussion

When *RC^{str}* or *RC^{rel}* strains of *E. coli* are inhibited with trimethoprim in the presence of nucleotide bases, the state of the mutation in the RC function determines whether the accumulation of stable RNA is permitted, since the cells are deprived of the amino acids glycine and methionine (Smith & Midgley, 1973*a,b*). If these amino acids are also supplied, both *RC^{str}* and *RC^{rel}* strains accumulate stable RNA at the same rate, though protein synthesis is still severely inhibited at the level of chain initiation (Shih *et al.*, 1966; Phillips & Franklin, 1969; Smith & Midgley, 1973*a*). In this paper, we have studied the effects of the resulting imbalances in the rates of stable RNA and protein synthesis on the formation of ribosomes.

In cultures inhibited with trimethoprim, but given base supplements, imbalances also occur in RNA and protein synthesis, in that there are considerable delays in the maturation of rRNA into ribosomes. In *RC^{str}* cultures, the expression of the *RC^{str}* control leads to a severe decrease in the rate of rRNA formation (Lazzarini & Winslow, 1970; Travers *et al.*, 1970; Lazzarini & Dahlberg, 1971; Travers, 1971). However, in trimethoprim-inhibited cells, where this control is imposed upon stable RNA chain initiation through amino acid lack (Smith & Midgley, 1973*a,b*), protein synthesis is even more severely inhibited. Precursors to the ribosomes persist as labelled intermediates, with a kinetic delay in maturation about fourfold greater than found in steadily growing cells. This hold up in ribosome formation is also found in amino acid auxotrophs deprived of amino acids, where the kinetic delay in ribosome maturation is identical with the delay found for trimethoprim-inhibited *RC^{str}* cells (J. E. M. Midgley, unpublished work).

In *RC^{rel}* cultures, very little material enters the ribosome fraction, even though the rate of rRNA synthesis is as high as in a growing control (Turnock & Wild, 1965). However, Hall & Gallant (1971) suggested that, in *RC^{rel}* cultures deprived of amino acids, translation becomes defective, and inactive proteins and polypeptides are produced. This defect appears to include the synthesis of ribosomal proteins and of β -galactosidase, and its appearance in trimethoprim-inhibited *RC^{rel}* cultures supplemented with nucleotide bases reinforces the belief that such cultures are suffering primarily from a lack of amino acids.

When glycine and methionine are supplied, the

(a) Culture labelled for 10 min with $[5\text{-}^3\text{H}]\text{uracil}$, then given rifampicin (0.1 g/l). (b) As for (a) except that culture was supplemented with glycine + methionine before adding radioactive RNA precursor.

kinetics of ribosome maturation in RC^{str} and RC^{rel} cultures inhibited with trimethoprim are identical. In the case of the RC^{str} strain, the relaxation of the RC^{str} amino acid control of RNA synthesis leads to a renewed accumulation of RNA, but the continued imposition of an inhibition of protein synthesis leads to the accumulation of intermediates in ribosome maturation and an increased kinetic delay in ribosome completion. With the RC^{rel} strain, stable RNA accumulation is unaffected by trimethoprim, but the purported defective translation arising from amino acid deficiencies is now rectified by adding the two amino acids. This finding was true both for ribosomal protein formation and for the synthesis of β -galactosidase (Smith & Midgley, 1973a). Whereas deficiencies in the amino acid pools lead to inaccurate translation (Hall & Gallant, 1971) a deficiency in protein chain initiation (i.e. through the formation of *N*-formylmethionyl-tRNA^{fMet}) leads simply to inhibition of protein synthesis without giving rise to further translational problems. This gives further weight to the idea that mutations in the RC function are basically unconcerned with the special process of protein chain initiation.

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