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

REGULATION OF RIBONUCLEIC ACID SYNTHESIS BY 'MAGIC SPOT' NUCLEOTIDES

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During the inhibition of RC^{str} , but not RC^{re1} mutants of *Escherichia coli* by trimethoprim the unusual nucleotides MSI (guanosine tetraphosphate, ppGpp) and MSII rapidly accumulated. The production of these nucleotides was not dependent on the addition of nucleotide base supplements to RC^{str} cultures before trimethoprim, and the MSI nucleotide concentrations in non-supplemented or purine-supplemented cultures were comparable with the concentrations obtained when the cells were inhibited with L-valine (1 g/l). Rifampicin rapidly decreased MSI and MSII nucleotide concentrations in trimethoprim-inhibited cultures to the basal values. Several situations were noted, in which MS nucleotide concentrations in trimethoprim-inhibited RC^{str} cells could be drastically lowered without giving rise to an immediate resumption of stable RNA accumulation. If RC^{str} mutants were first inhibited with trimethoprim and then given purines 15 min later, MS nucleotide concentrations fell rapidly, because of a temporarily enhanced rate of accumulation of stable RNA. However, after a further 5 min, RNA accumulation stopped, though MS nucleotide concentrations remained low. Also, if either glycine or methionine were added to trimethoprim-inhibited cultures supplemented with purines, RNA accumulation did not resume, though MS nucleotide concentrations rapidly declined. With both amino acids present, there was both a decline in MS nucleotide concentration and a resumption in stable RNA synthesis. These findings suggest that MSI nucleotide concentrations in trimethoprim-inhibited bacteria are not the sole factors in the control of stable RNA synthesis. It is possible that, during the period when the RC^{str} cells contained high concentrations of MS nucleotides, some factor important in the MSI-mediated control of stable RNA synthesis was irreversibly inactivated. However, as antibiotics (e.g. chloramphenicol) both abolished high MS nucleotide concentrations and permitted a rapid resumption of stable RNA accumulation in the same conditions, it is more likely that an additional control mechanism has come into play.

In bacteria, the accumulation of stable RNA (rRNA+tRNA) is under the control of a regulatory process that involves the intracellular supply of amino acids. Most bacterial strains are 'stringent', that is, they do not accumulate stable RNA when amino acids are withheld (Edlin & Broda, 1968). One-step mutations in the *RC* genetic locus (Stent & Brenner, 1961) can lead to the decoupling of RNA and protein synthesis, so that, in these 'relaxed' mutants, amino acid deprivation does not lead to a significant decrease in the rate of accumulation of stable RNA (Ryan & Borek, 1971). The control mechanism for RNA accumulation in RC^{str} strains must be concerned with the aminoacylation of tRNA, because temperature-sensitive aminoacyl-tRNA synthetase mutants do not synthesize large quantities of stable RNA at non-permissive temperatures, even if the free amino acid is available in the pools

(Eidlic & Neidhardt, 1965; Neidhardt, 1966; Ezekiel & Elkins, 1968; Kaplan, 1969; de Boer *et al.*, 1971).

Recently, a model has been put forward to explain the relationships between stable RNA synthesis and amino acid supply in RC^{str} bacterial strains. The model suggests that, when amino acids are in short supply in RC^{str} cells, the synthesis of aminoacyl-tRNA is decreased and an 'idling' reaction takes place in the process of polypeptide chain elongation on the ribosomes (Cashel & Gallant, 1969; de Boer *et al.*, 1971). This gives rise to greatly increased concentrations of an unusual guanine nucleotide (MSI, ppGpp) (Cashel, 1969; Cashel & Gallant, 1969; Cashel & Kalbacher, 1970) that is believed to be of importance in the regulation of the rate of initiation of stable RNA chains (Travers *et al.*, 1970; Travers, 1971). ppGpp and the accompanying nucleotide MSII have been synthesized *in vitro*, by using a bacterial

ribosome preparation from RC^{str} strains, ATP and GTP and all ancillary protein synthesizing factors, but no amino acids (Haseltine *et al.*, 1972). RC^{rel} strains are presumed to have a defect in polypeptide chain elongation that prevents the 'idling' reaction occurring in the absence of amino acids and thus prevents MS nucleotide production (Cashel & Gallant, 1969; Hall & Gallant, 1971).

It was originally believed (Shih *et al.*, 1966) that trimethoprim elicited the RC^{str} or RC^{rel} response of stable RNA accumulation in bacteria by preventing the initiation of protein chains. This suggested that the loss of *N*-formylmethionyl-tRNA^{fMet} required for this process in the cells was sufficient by itself to prevent stable RNA accumulation. However, more recent re-examinations of this phenomenon have shown that the primary response of RC^{str} strains to trimethoprim is through a deprivation of the amino acids glycine and methionine, and only secondarily through a decrease in the rate of biosynthesis of *N*-formylmethionyl-tRNA^{fMet} (Moivic & Pizer, 1971; Dale & Greenberg, 1972; Midgley & Smith, 1973, Smith & Midgley, 1973). Further, the fact that RC^{str} and RC^{rel} strains synthesize protein and stable RNA at identical rates during trimethoprim inhibition if glycine, methionine and nucleotide bases are supplied exogenously suggests that, if protein synthesis is presumably inhibited only at the point of chain initiation, the RC control does not respond to this particular deficiency.

In the present paper, we have studied the circumstances in which the unusual nucleotides ppGpp and MSII accumulate in trimethoprim-inhibited RC^{str} strains of *Escherichia coli*. In agreement with previous findings (Moivic & Pizer, 1971; Dale & Greenberg, 1972) we have obtained evidence that amino acids such as glycine and methionine are in sufficiently short supply in trimethoprim-inhibited cultures to permit the expression of the RC^{str} amino acid control of stable RNA synthesis and the accumulation of MS nucleotides (Cashel & Gallant, 1969). If all amino acids, with the exception of *N*-formylmethionine acylated to tRNA^{fMet}, are in plentiful supply, MS nucleotide concentrations remain low and stable RNA accumulation occurs. In no circumstances do ppGpp and MSII nucleotides accumulate in RC^{rel} strains simply as a result of trimethoprim inhibition. This work has been reported briefly elsewhere (Midgley, 1972).

Materials and Methods

General techniques in bacterial growth and choice of mutants have been described by Smith & Midgley (1973). All cultures to be labelled heavily with [³²P]P_i were grown in media similar to those described by Gray & Midgley (1970) except that the concentrations of the sodium and potassium phosphates

were decreased by a factor of 1500, Tris was added to 0.15M and KCl to 1g/l. The pH was adjusted to 7.2 by HCl. The [³²P]phosphoric acid (specific radioactivity 3Ci/μmol) was neutralized just before use by adding an equal volume of NaOH solution that had been previously diluted to give a pH of 11. Cultures were labelled for 30min before commencing experiments, to ensure that the nucleotide pools were equilibrated with ³²P (Cashel, 1969). Throughout the labelling period, checks were made continually to ensure that the bacteria were growing steadily.

Labelled bacteria were pipetted into formic acid as described by Cashel (1969), to keep ppGpp and MSII nucleotides in the intracellular pools. AnalaR formic acid (0.1ml, 98%, v/v) was placed in each of several conical glass centrifuge tubes (11cm×1.5cm) cooled in ice, and 0.2ml of the labelled bacterial cultures was pipetted into the ice-cold tube and contents. After 20min, the tubes were centrifuged in the cold at 3000rev./min for 10min in the BTL Bench Centrifuge (Baird and Tatlock). The top two-thirds of the supernatant fluids were carefully removed by a Pasteur pipette, attention being paid to avoid contamination by the pellets. The supernatant was stored at -20°C in the deep-freeze, where the nucleotides were stable for at least 1 week.

Polyethyleneimine(PEI)-cellulose-coated t.l.c. glass plates (Schleicher & Schuell, Dassel, West Germany, 20cm×20cm, G-1440-PEI) were washed with distilled water. The plates were placed upright in 25cm×25cm×8cm chromatography jars, with a 2cm layer of water in the bottom. Water was allowed to reach the top of the plates, and they were then removed and air-dried flat. Washed plates were then used, with the same orientation, for the separation of ³²P-labelled nucleotides in the formic acid extracts. A portion (10μl) of the labelled extracts was spotted on to the plate from each sample, and the spots were air-dried. The plates were developed in 1.5M-KH₂PO₄, pH 3.4, as described by Cashel (1969).

The development time at 18°C was between 150 and 170min. The *R_F* values of the various nucleotides were identical with those given by Cashel (1969). The developed plates were again air-dried and radioautographed, with 35cm×43cm X-ray film (RB-54, Kodak Ltd., London W.C.1, U.K.). Radioactive ink markers were placed on the corners of the plates to aid in later alignment of plates and developed X-ray film.

Exposure time for radioautography was 36–48h. Radioactive spots corresponding to ppGpp, MSII, other nucleotides and P_i were scraped off the plates into scintillation vials containing 5ml of NE 221 Gel Scintillator (Nuclear Enterprises Ltd., Edinburgh, U.K.) diluted by a factor of three with toluene scintillant [6g of 2,5-diphenyloxazole and 0.05g of 1,4-bis(phenyloxazol-2-yl)benzene/l of sulphur-free toluene]. The vials were counted for radioactivity

in the Beckman LS-200 liquid-scintillation counter (Beckman Instruments Inc., Glenrothes, U.K.).

Rifampicin was a gift from Lepetit Pharmaceuticals Ltd., Maidenhead, Berks., U.K. Trimethoprim lactate was a gift from Wellcome Research Laboratories, Dartford, Kent, U.K. Chloramphenicol was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. [2- 14 C]Uracil (specific radioactivity 54mCi/mmol, and [32 P]phosphoric acid (specific radioactivity 3Ci/ μ mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Cultures of *E. coli* MRE600 (RC^{str}) and CP79 (RC^{rel}) were grown in Tris-low phosphate glucose-salts media with appropriate supplements, as described in the Materials and Methods section. [32 P] P_i (specific radioactivity 3Ci/ μ mol) was supplied to a final amount of 0.1mCi/ml. Samples of 0.2ml were used for t.l.c. chromatography on PEI-cellulose, one sample being taken 1 min before the start of each experiment, as a control from growing cells.

Development of MS nucleotides in valine-inhibited cultures

When L-valine was added to growing cultures of *E. coli* MRE600 (RC^{str}) bacterial RNA accumulation was temporarily halted (e.g. Cashel 1969; Cashel & Gallant, 1969). Valine interferes with the endogenous biosynthesis of isoleucine, so that a deficiency in this amino acid occurs. The MS nucleotide concentrations rose rapidly to more than tenfold greater than those in steadily growing cells (Fig. 1). In the RC^{rel} strain, MS nucleotide concentrations were not affected by valine. These results are in agreement with those of others (Cashel, 1969; Cashel & Gallant, 1969), though the shut-off of RNA accumulation was not as complete as described by these authors.

MS nucleotide synthesis in trimethoprim-inhibited cultures

When bacteria were inhibited by trimethoprim, the point at which macromolecular synthesis was inhibited depended very much on the way in which the growth medium was supplemented. If trimethoprim was added without further supplementation, bacterial RNA synthesis in RC^{str} or RC^{rel} strains became limited through a lack of purines and pyrimidines. However, early in the period of inhibition, the major regulating function for RNA synthesis was probably acting through a lack of glycine and methionine (Smith & Midgley, 1973). When nucleotide bases were supplied with trimethoprim, the inhibition of

Fig. 1. Kinetics of RNA labelling in valine-inhibited RC^{str} strains of *E. coli* and development of MS nucleotides in valine-inhibited RC^{rel} and RC^{str} strains of *E. coli*

(a) Gross labelling of the valine-inhibited culture was corrected for DNA labelling as described by Gray *et al.* (1972). L-Valine (1000 μ g/ml) was added as indicated by the arrow. (b) Concentrations of ●, GTP (RC^{str} strain); ▲, GTP (RC^{rel} strain); ○, ppGpp (RC^{str} strain); △, ppGpp (RC^{rel} strain).

RNA accumulation was effected entirely through the RC control in RC^{str} cells, and arose through the deprivation of the above amino acids. On the other hand, if glycine, methionine and nucleotide bases were added with the trimethoprim, there was no decrease in the rate of stable RNA synthesis, though protein synthesis was still severely inhibited at the level of chain initiation (Smith & Midgley, 1973). According to the findings of Cashel (1969) and Cashel & Gallant (1969), trimethoprim-inhibited RC^{str} cultures should accumulate MS nucleotides in all conditions where amino acid deprivation is the prime limiting factor for RNA synthesis, but no such change in MS nucleotide concentrations should occur in RC^{rel} cells.

Growing cultures of strain MRE600 (RC^{str}) and CP79 (RC^{rel}) were labelled with [^{32}P]P_i, and were split into two parts. To one was added trimethoprim (50 µg/ml) alone, and to the other was added trimethoprim (50 µg/ml) and nucleotide bases (20 µg/ml each). In both cases, RC^{str} cells accumulated MS nucleotides after trimethoprim was added, but RC^{rel} cells did not (Fig. 2). The concentrations of MS nucleotides finally obtained were of the same order as in RC^{str} cultures given L-valine. Thus, even when the deficiency in the endogenous synthesis of nucleotide bases had become sufficiently severe to dictate the rate of RNA chain growth, the lack of amino acids was still great enough to produce large quantities of MS nucleotides. Thus, at the onset of trimethoprim inhibition, amino

acid lack preceded, and was maintained through, a later deficiency in nucleotide bases. In purine-requiring RC^{str} mutants of *E. coli*, deprived of purines but not amino acids, MS nucleotide concentrations did not rise, even though RNA accumulation was prevented by purine lack (Gallant & Harada, 1969; Gallant *et al.*, 1970).

Though high concentrations of MS nucleotides were found in trimethoprim-inhibited RC^{str} cultures given no purines and pyrimidines, they remained capable of incorporating exogenous base supplements from the medium. A culture of strain MRE600 (RC^{str}), grown in glucose-salts medium, was inhibited with trimethoprim and was then incubated for 15 min. [2- ^{14}C]Uracil (specific radioactivity 20 mCi/mmol) was added, and the rate of entry of radioactivity into trichloroacetic-acid-precipitable material was followed by rapid sampling. After 10 min, the culture was split into two, and to one part adenine and guanosine (30 µg/ml each) were added. Incubation of both parts was continued, with further sampling (Fig. 3). A similar experiment was carried out for strain CP79 (RC^{rel}) (Fig. 3b).

In agreement with Smith & Midgley (1973) the addition of purines to trimethoprim-inhibited RC^{str} strains resulted in a burst of rapid RNA labelling at a rate close to that of a growing control. This period of labelling lasted for some 5 min, after which the rate fell to one typical of normal trimethoprim inhibition. With the RC^{rel} strain, the addition of purines gave an immediate and continuous resump-

Fig. 2. MS nucleotide accumulation in trimethoprim-inhibited RC^{str} and RC^{rel} strains

(a) RC^{str} cultures inhibited with trimethoprim but given no purines; (b) RC^{str} cultures inhibited with trimethoprim and supplemented with purines; (c) RC^{rel} cultures inhibited with trimethoprim but given no purines; (d) RC^{rel} cultures inhibited with trimethoprim and supplemented with purines. ●, GTP concentration; ○, ppGpp concentration.

Fig. 3. Kinetics of RNA labelling in trimethoprim-inhibited RC^{str} strains of *E. coli* when supplemented later with purines

(a) RC^{str} strain; (b) RC^{rel} strain. ●, RNA labelling after purine supplementation (indicated by the arrow); ○, RNA labelling, no supplementation.

tion in RNA labelling at a high rate. Analysis of the labelled RNA made during the short burst of rapid labelling in RC^{str} cells, by sucrose-density-gradient sedimentation (Fig. 4), showed that the synthesis of stable RNA species played its full part in this phenomenon. This probably represented a true accumulation of stable RNA, as trimethoprim-inhibited non-supplemented RC^{str} cultures accumulated less material in a given time than nucleotide base-supplemented cultures (Smith & Midgley, 1973).

From these findings there is some uncertainty as to the fate of MS nucleotides in trimethoprim-inhibited RC^{str} cells, when nucleotide bases are

added exogenously to relieve a purine deficiency arising from tetrahydrofolate lack. Because of a decrease in the rate of elongation of nascent RNA chains in trimethoprim-inhibited cultures (Smith & Midgley, 1973) the DNA cistrons appear to become crowded with DNA-dependent RNA polymerases. The severity of the trimethoprim-imposed base deficiency is therefore great enough to decrease chain elongation even more than the increased concentration of MS nucleotides decreases chain initiation. In trimethoprim-inhibited RC^{str} cultures given the necessary bases, it is not clear whether MS nucleotides will be consumed by a rapid, though transient, resumption in RNA synthesis, or whether the continued amino acid lack will maintain high concentrations of MS nucleotides through the 'idling' mechanism in protein synthesis.

In a typical experiment with strain MRE600 (RC^{str}), the MS nucleotide concentration in a non-supplemented trimethoprim-inhibited strain was first raised. After a suitable time, the nucleotide bases ($30\mu\text{g/ml}$ each) were added. As shown in Fig. 5, the high concentrations of MS nucleotides typical of trimethoprim-inhibited RC^{str} cells were decreased to basal values within 7–8 min. During a further 20 min of incubation, MS nucleotide concentrations did not rise again. This effect is surprising, as the short burst of rapid labelling occurred for a period of some 5 min only, and the imposition of an inhibition of RNA accumulation through amino acid lack afterwards would be expected to re-elevate MS nucleotide concentrations. Thus the inability of RC^{str} cells to maintain the accumulation of stable RNA over a longer period in the absence of MS nucleotides must rest on some other control mechanism.

As expected, the addition of glycine and methionine to trimethoprim-inhibited, purine-supplemented RC^{str} cells rapidly decreased MS nucleotide concentrations to basal values (Fig. 6), as a high rate of accumulation of stable RNA resumed in these conditions (Midgley & Smith, 1973; Smith & Midgley, 1973). However, the addition of either methionine or glycine to trimethoprim-inhibited RC^{str} cultures also decreased the concentration of MS nucleotides to basal values (Fig. 3) though, in these cases, stable RNA accumulation did not resume (R. J. Smith & J. E. M. Midgley, unpublished work). The lack of stable RNA accumulation in the absence of MS nucleotides is unlikely to rest on the rate of supply of nucleotide bases for intracellular nucleotide synthesis. For example, the GTP concentrations, initially decreased by the addition of trimethoprim, rise rapidly to their former values when exogenous bases are supplied (Fig. 5). Further checks were made to show that the availability of exogenous base supplements for intracellular nucleic acid synthesis is a general phenomenon in trimethoprim-inhibited

Fig. 4. Sucrose-density-gradient sedimentation of labelled RNA formed during the additions of purine to trimethoprim-inhibited RC^{str} *E. coli*

(a) Non-supplemented, trimethoprim-inhibited culture labelled for 3 min with $[2-^{14}\text{C}]\text{uracil}$. (b) As for (a) except that purines were added with the labelled uracil (for details see text). $\circ$, E_{260} ; $\bullet$, acid-precipitable ^{14}C radioactivity.

Fig. 5. Effect of purines on MS nucleotide concentrations in non-supplemented trimethoprim-inhibited RC^{str} *E. coli*

Concentrations of: $\bullet$, GTP, purines added; $\circ$, ppGpp, purines added; Δ , ppGpp, no purines added. Purines were added as indicated by the dashed line and arrow.

RC^{str} cells, whether or not there is also a deficiency in glycine and methionine.

Because DNA synthesis is blocked by a lack of thymidylic acid, addition of this precursor to trimethoprim-inhibited cultures should cause a resumption in DNA formation. However, if the lack of glycine and methionine also severely decreased the rate of uptake of exogenous adenine, guanine and uracil, the addition of thymidylic acid should not result in a normal rate of DNA synthesis, as other precursors would now be rate-limiting. In the presence of glycine and methionine accumulation of stable RNA resumes (Smith & Midgley, 1973) and there should be no

Fig. 6. Effect of glycine and methionine on MS nucleotide concentrations in purine-supplemented, MS -inhibited RC^{str} *E. coli*

Concentrations of: $\bullet$, GTP, no additions; $\circ$, ppGpp, (before and after the addition of glycine+methionine); Δ , ppGpp after the addition of glycine only; $\blacktriangle$, ppGpp, no amino acids added. The dashed line indicates addition of amino acids.

limitations on the rate of base uptake, and normal rates of DNA synthesis should then occur in the presence of exogenous thymidylic acid.

A culture of *E. coli* MRE600 (RC^{str}) was inhibited with trimethoprim ($50 \mu\text{g/ml}$) in the presence

Table 1. *Synthesis of DNA in trimethoprim-inhibited RC^{str} E. coli cultures*

For details see the text.

Conditions	Time of sampling (min)	Amount of DNA/ml of culture (% of control at 0min)
Steady growth	0	100
Glucose-salts	60	245
Trimethoprim-inhibited, unsupplemented	0	100
	60	117
+Thymidylic acid at 0min	60	108
Trimethoprim-inhibited, purine-supplemented	0	100
	60	128
+Thymidylic acid at 0min	60	230
+Thymidylic acid+glycine	60	240
+Methionine at 0min		
+Thymine+deoxyadenosine at 0min	60	215
+Glycine+methionine at 0min	60	122

of exogenous adenine, uracil and guanosine (30 µg/ml), then 10min later a sample (40ml) was poured on to 2ml of cold 5M-HClO₄. The culture was now split into five parts. One acted as a control, and to the others was added (i) glycine+methionine (100 µg/ml each), (ii) thymidylic acid (100 µg/ml), (iii) glycine+methionine+thymidylic acid (100 µg/ml each) and (iv) thymine+deoxyadenosine (100 µg/ml each). These parts were incubated at 37°C for 1h, then 40ml samples were taken as before, and all were assayed (in triplicate) for DNA by the diphenylamine test of Burton (1956).

Table 1 shows the results obtained. Whereas the amount of DNA in non-supplemented cells rose by only about 10–25% in 1 h, the amounts in thymidylic acid- or thymine+deoxyadenosine-supplemented cultures rose by 110–130%. No differences were noted in the rate of DNA synthesis if glycine and methionine were also added to relieve the amino acid deficiency imposed by trimethoprim inhibition. If bases other than thymine were omitted from the medium, there was no increase in DNA. Thus the essential supply of nucleotide bases for DNA synthesis during trimethoprim inhibition of bacteria is primarily from external sources, and is unaffected by amino acid lack.

Action of rifampicin on MS nucleotide concentrations in trimethoprim-inhibited bacteria

Ehrlich *et al.* (1971) showed that in an RC^{str} temperature-sensitive aminoacyl-tRNA synthetase mutant of *E. coli*, rifampicin did not abolish the accumulation of MS nucleotides at non-permissive temperatures. However, Wong & Nazar (1970) and Ehrlich *et al.* (1971) showed that in normal RC^{str} amino acid auxotrophs deprived of required amino acids, rifampicin quickly abolished MS nucleotide accumulation. Ehrlich *et al.* (1971)

Fig. 7. *Effect of rifampicin on MS nucleotide concentrations in trimethoprim-inhibited bacteria*

Rifampicin was added as indicated by the dashed line and arrow; ○, ppGpp concentration, rifampicin added; ●, ppGpp concentration, no rifampicin.

explained this phenomenon by proposing that upon the addition of rifampicin, unstable mRNA was labilized and thus protein synthesis rates declined quickly, in the absence of any new RNA synthesis. The amino acid pools originally decreased by deprivation were now replenished by protein turnover with increasing efficiency. Thus the 'idling' reaction on existing polyribosomes would be replaced by correct polypeptide chain elongation, and MS nucleotide concentrations would decline.

In trimethoprim-inhibited RC^{str} cultures, the high concentrations of MS nucleotides were also rapidly decreased by rifampicin (Fig. 7). The probable

explanation of this result can be made on the lines suggested by Ehrlich *et al.* (1971), namely that, as the rate of protein synthesis declined, glycine and methionine were made available from protein turnover in the cells, and the 'idling' reaction in polypeptide chain elongation ceased.

Discussion

A trimethoprim-induced lack of tetrahydrofolate in bacterial cells has complex and widespread implications for the synthesis of macromolecules (Midgley & Smith, 1973; Smith & Midgley, 1973). However, if a deficiency in amino acids is the first result of a tetrahydrofolate lack, the amino acid-controlled RC^{str} function should inhibit the synthesis of stable RNA and allow the accumulation of MS nucleotides (Cashel, 1969; Cashel & Gallant, 1969). High concentrations of MS nucleotides are very quickly formed in trimethoprim-inhibited RC^{str} , but not RC^{rel} strains of *E. coli*, and this accumulation occurs whether or not nucleotide bases are supplied to prevent an eventual exhaustion of the intracellular nucleotide pools. When glycine and methionine are added, stable RNA synthesis resumes in RC^{str} cells at a high rate, and the MS nucleotides again become only a small component of the guanine nucleotide pools. This finding further confirms that the principal response in bacterial cells to trimethoprim is a depletion of the amino acid pools causing the classical symptoms of deficiency.

The fact that protein synthesis does not resume at a normal rate in trimethoprim-inhibited cells when the required amino acids are added, indicates that chain initiation is strongly inhibited, at the level of formylation of methionyl-tRNA^{fMet} (Lengyel, 1967). However, MS nucleotide concentrations are immediately decreased in RC^{str} cells upon addition of amino acids. This means that lack of chain initiation in protein synthesis does not set up any equivalent 'idling' reaction in polypeptide chain elongation that gives rise to MS nucleotides (Haseltine *et al.*, 1972). As Anderson *et al.* (1967a, b) and Economou & Nakamoto (1967) showed that unformylated methionyl-tRNA^{fMet} is only poorly bound to ribosomes, thus inhibiting the rate of formation of the mRNA-30S ribosome complex for protein synthesis (Wahba *et al.*, 1968), we would expect that the rate of ribosomal attachment to mRNA in trimethoprim-inhibited bacteria is much decreased. Indeed, trimethoprim-inhibited cells contain fewer polyribosomes than normally growing cells (Phillips & Franklin, 1969). Therefore, the ability of trimethoprim-inhibited RC^{str} bacteria to produce MS nucleotides when protein synthesis is inhibited at chain initiation is probably connected with a lack of polyribosome formation. This is quite different from situations where the lack of other amino acids causes deficiencies in the formation of aminoacyl-tRNA

species that normally enter the A binding site on the ribosomes (Bretscher, 1966; Bretscher & Marcker, 1966), since this will lead to an inhibition of polypeptide chain elongation on already constituted polyribosomes.

Most of the results have supported the current concepts of MS nucleotides as controlling elements in stable RNA synthesis in bacteria (Travers, 1971). However, some findings are difficult to reconcile easily with the present theory, though they may have special explanations that are not concerned with general theories of control. In several cases, loss of MS nucleotides occurred in RC^{str} cells without giving rise to a resumption in stable RNA accumulation, even though all precursors necessary for RNA synthesis were apparently present. It could be argued that the prolonged inhibition of cells by trimethoprim had adversely affected some component of the stable RNA chain initiation system, so that removal of MS nucleotides did not give the expected result. However, strong evidence against this explanation comes from the finding that chloramphenicol and other antibiotics, or glycine+ methionine, allowed the successful resumption of RNA accumulation (Smith & Midgley, 1973) even in cells inhibited for a long period with trimethoprim.

A further possibility could be that, in certain circumstances, the supply of exogenous nucleotides for RNA synthesis is limiting, owing to the combination of the inhibition of endogenous synthesis by trimethoprim, and a decrease in the rate of uptake of exogenous nucleotide bases caused by the restrictions typical of amino acid lack (Edlin & Neuhaud, 1967). In this particular case, MS nucleotide concentrations in RC^{str} cells would not be the prime factors responsible for the control of stable RNA formation, but rather the problem of nucleotide supply. As guanine nucleotide concentrations may be especially important in the regulation of RNA synthesis (Gallant & Harada, 1969), any decrease could lead to a preferential inhibition of the rate of initiation of stable RNA chains (Jorgensen *et al.*, 1969; Thomas *et al.*, 1970; Varney *et al.*, 1970). However, strong evidence against this explanation has come from measurements of the rate of DNA synthesis in trimethoprim-inhibited, thymidylic acid-supplemented RC^{str} cultures, where it was shown that amino acid lack imposed by trimethoprim had no undue effects on the rate of general base uptake. This is unlike the situation reported for specific amino acid withdrawal (Edlin & Neuhaud, 1967). Further, the GTP pool in trimethoprim-inhibited RC^{str} cultures is expanded by the addition of exogenous guanosine, even though the cells display the symptoms of amino acid lack, but stable RNA synthesis only transiently resumes and does not become a predominant fraction of rapidly labelled RNA.

Another explanation of these findings could be that

two control systems are active in the regulation of stable RNA chain initiation. As suggested by Fiil *et al.* (1972), one of these could be a positive protein control factor that determines the maximum possible expression of rRNA transcription in a given medium (i.e., a 'coarse' control). Within the limits set by this overall regulatory process, the 'fine' control of stable RNA transcription could then be set by MS I nucleotide. With this model, it would seem that in trimethoprim-inhibited cultures, the alternative 'coarse' factor is rendered inoperative temporarily, so that, even when there are large fluctuations in the cellular concentrations of MS nucleotides, stable RNA synthesis cannot be resumed. Upon the addition of glycine+methionine, or antibiotics, the 'coarse' regulating factor rapidly becomes operative, and a high rate of stable RNA accumulation ensues.

Finally, it is possible that the rRNA and tRNA formed in certain conditions is no longer stable. In this case, decreases in MS nucleotide concentrations might allow the resumption of a high rate of stable RNA synthesis, without its concomitant accumulation. Because of the lack of methionine in trimethoprim-inhibited organisms (Smith & Midgley, 1973), secondary methylation of tRNA may be strongly inhibited, and the resulting material may be less resistant to attack by degradative nucleases. The stability of rRNA could suffer both from a lack of protection by ribosomal proteins as well as a deficiency in methylation (Midgley & Smith, 1973). However, we have no compelling evidence to suggest high rates of stable RNA turnover, and the proportion of messenger to rRNA+tRNA remains fairly constant in rapidly labelled fractions of trimethoprim-inhibited RC^{str} cultures.

Our findings strongly suggest that MS nucleotides are not the only important regulatory agents for the synthesis of stable RNA species in bacteria (Fiil *et al.*, 1972). Other, perhaps more comprehensive, regulatory mechanisms must be present, and these appear also to be affected by trimethoprim inhibition.

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