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THE EFFECT OF AMINO ACID STARVATION ON RIBONUCLEIC ACID
ACCUMULATION AND METABOLISM OF GUANOSINE
TETRAPHOSPHATE IN ESCHERICHIA COLI

by

Sabih-ur-Rahman Khan, B.Sc. (Hons), M.Sc.

A thesis submitted to the Faculty of Graduate
studies in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario
November 28, 1973

The undersigned hereby recommend to the Faculty of Graduate Studies acceptance of the thesis, entitled, "The effect of amino acid starvation on ribonucleic acid accumulation and metabolism of guanosine tetraphosphate in Escherichia coli" submitted by Sabih-ur-Rahman Khan, B.Sc. (Hons), M.Sc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

....
Chairman, Department of Biology

....
Supervisor

....
External Examiner

This thesis is dedicated to the cherished memory
of my father, Dr. Ibad-ur-Rahman Khan, and to my
mother.

ABSTRACT

When aminoacylation of tRNA is restricted in bacteria, inhibition of RNA as well as protein accumulation ensues which is accompanied by a rapid accumulation of guanosine 5'-diphosphate, 3'-diphosphate (ppGpp). Other stress conditions which cause inhibition of RNA accumulation also elicit ppGpp accumulation. The excellent kinetic correlation between ppGpp accumulation and inhibition of RNA accumulation has led to the hypothesis that ppGpp is an inhibitor of stable RNA synthesis. However, an absence of ppGpp accumulation was reported when RNA accumulation was curtailed by trimethoprim which causes the depletion of N-formyl-methionyl-tRNA, the initiator of polypeptide chains in bacteria. The reason for this apparent exception to the hypothesis has been investigated. It was found that the addition of trimethoprim to an Escherichia coli culture evoked a typical stringent response: inhibition of protein and RNA accumulation was accompanied by ppGpp accumulation in stringent but not in relaxed cells. Addition of chloramphenicol caused a rapid disappearance of the accumulated ppGpp and relieved the inhibition of RNA accumulation. Oxytetracycline which is known to inhibit ppGpp synthesis directly also caused a rapid disappearance of ppGpp indicating that trimethoprim causes ppGpp accumulation by stimulating its synthesis rather than by retarding its degradation. The slow decay of the accumulated ppGpp upon the addition of

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rifampicin is consistent with the known requirement for mRNA in the synthesis of ppGpp. Thus, these results are consistent with the hypothesis mentioned above.

A previous report that the basal levels of ppGpp vary inversely with the RNA content in a strain of Escherichia coli has implicated ppGpp in the regulation of RNA accumulation during balanced growth. The generality of this relationship has been examined in a wild-type strain of Escherichia coli over a wider range of growth rates. No inverse relationship between the basal ppGpp levels and the RNA content per unit DNA was found. Thus, it is concluded that the basal level of ppGpp itself has no direct relation to the cellular capacity to accumulate RNA during balanced growth. It was also found that ppGpp accumulated extracellularly to varying degrees depending upon the carbon source used. An additional interesting observation was that the RNA/DNA ratio had an extrapolated value of zero at zero growth rate. This verifies the constant efficiency theory that protein synthesis per unit stable RNA is constant at any growth rate.

The correlation between ppGpp and RNA accumulation has been previously established under relatively rich growth conditions such as glucose-minimal medium. In order to extend the generality of this relationship, the effect of amino acid starvation was investigated at various growth rates created by different carbon sources

or limitation in the intracellular supply of thiamine. A nutritionally poor environment yielded a less stringent control of RNA accumulation and a lesser extent of ppGpp accumulation. Slow growth created by a low incubation temperature did not significantly affect the amino acid control of RNA accumulation.

Although the results of all in vivo studies are consistent with the postulated inhibitory role of ppGpp, direct evidence is lacking. Proposals for future investigation in this respect are presented.

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LIST OF ABBREVIATIONS

<u>rel</u> ⁺	stringent genotype
<u>rel</u> ⁻	relaxed genotype
ppGpp	guanosine tetraphosphate (guanosine 5'-diphosphate, 3'-diphosphate)
pppGpp	guanosine pentaphosphate (guanosine 5'-triphosphate, 3'-diphosphate)
<u>thi</u>	thiamine
<u>arg</u>	arginine
<u>his</u>	histidine
<u>leu</u>	leucine
<u>thr</u>	threonine
<u>met</u>	methionine
F ⁻	female <u>Escherichia coli</u> cell
RNA	ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA
mRNA	messenger RNA
DNA	deoxyribonucleic acid
ATP	adenosine-5'-triphosphate
GTP	guanosine-5'-triphosphate
UTP	uridine-5'-triphosphate
TCA	trichloroacetic acid
TMM	tris minimal medium
TMA	tris minimal agar
Tris	tris (hydroxymethyl) aminomethane
v/v	volume by volume
w/v	weight by volume

hr	hours
min	minutes
sec	seconds
µg	micrograms
mg	milligrams
g	grams
ml	milliliters
µl	microliters
mCi	millicuries
µCi	microcuries
nm	nanometers
A ₅₀₀	absorbance at 500 nanometers
rpm	revolutions per minute
cpm	counts per minute
conc.	concentrated
MW	molecular weight
S	Svedberg unit

INTRODUCTION

Ribonucleic acid (RNA) is functionally and chemically classified into three different types: transfer RNA (tRNA), ribosomal RNA (rRNA), and messenger RNA (mRNA). Transfer RNA is found free in the cytoplasm and is the smallest nucleic acid molecule with a sedimentation constant of about 4 S, corresponding to a molecular weight of 25,000 and to a chain length of 70-80 nucleotides. It is a single-stranded molecule with extensive self-complementarity. It is unique in that it contains a large number of unusual bases not found in the other nucleic acids. At least 30-40 different tRNA species have been isolated but the total number of species is probably greater than this. Transfer RNA is stable during balanced growth. Ribosomal RNA is found as a nucleoprotein complex, the ribosome which contains 67-70% RNA and 30-33% protein. The bacterial 70 S ribosome is composed of a 30 S subunit (MW: 9×10^5) and a 50 S subunit (MW: 1.8×10^6). The 30 S ribosomes contain 16 S RNA (MW: 0.55×10^6) and approximately 20 kinds of proteins and the 50 S ribosomes contain 23 S RNA (MW: 1.1×10^6) and a 5 S RNA (MW: 40,000) and approximately 30 kinds of proteins. Ribosomal RNA synthesized during balanced growth is known to be metabolically very stable. Messenger RNA carries the information relating to the structure of a given protein from DNA. Messenger RNA is a single-stranded molecule

and contains only the common RNA bases adenine, guanine, uracil and cytosine. Most cellular mRNA's fall in the molecular-weight range of about 10^5 - 10^6 . Messenger RNA is the most elusive of the RNA types because of its extremely short half-life in bacteria.

Determination of the relative rates of synthesis of ribosomal, transfer and messenger RNA's during balanced growth indicates that in glucose-grown cells of Escherichia coli, approximately 40% of the total rate is comprised of ribosomal and transfer RNA species whereas mRNA accounts for the remaining 60% (Lazzarini and Dahlberg, 1971). However, due to the instability of mRNA, the bulk of the RNA accumulated at any instant is stable RNA (rRNA and tRNA) which comprises about 98% of the total cellular RNA, while mRNA only constitutes the remaining 2-3%. Therefore, determinations of the RNA content of the cell by the orcinol colorimetric assay or by following the incorporation of radioactive tracers measures largely stable RNA.

Since all of the stable RNA species (rRNA, tRNA) participate in protein synthesis, it is not surprising that the RNA content of the cell varies with the growth rate and, consequently, with the rate of protein synthesis (Maaløe and Kjeldgaard, 1966). Thus, the faster the growth rate of the cell the higher would be its RNA content and the slower the growth rate of the cell the lower would be its RNA content. The regulatory

relationship between protein and RNA synthesis was first suggested by an observation of Sands and Roberts (1952). They showed that when a culture of a strain of Escherichia coli that was auxotrophic for tryptophan and histidine was deprived of either amino acid, not only did protein accumulation cease, but there was also a substantial reduction in the rate of RNA accumulation. This observation was later confirmed and extended by the work of others (Gale and Folkes, 1953; Gros and Gros, 1956; Pardee and Prestidge, 1956). Although, auxotrophs have been conveniently used to demonstrate this relationship, this phenomenon is demonstrable in prototrophs as well. When a prototrophic strain growing in an amino acid-rich medium is shifted to an amino acid-free medium, the cells suffer from a transient state of amino acid starvation before the synthesis of the amino acid biosynthetic enzymes is fully derepressed. During this period both protein and RNA accumulation are transiently curtailed.

A more recent technique to demonstrate the amino acid control of RNA synthesis involves the use of amino acid analogues, such as β -2-thienylalanine and 5-methyltryptophan, which interfere with the biosynthesis of the corresponding natural amino acid causing amino acid starvation. For example, β -2-thienylalanine, a structural analogue of phenylalanine, inhibits the biosynthesis of phenylalanine by acting as a false feedback

inhibitor of the enzyme 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) synthetase in Escherichia coli (Ezekiel, 1965). Similarly, K-12 strains of Escherichia coli can be starved for isoleucine by the addition of valine which acts as a feedback inhibitor of the first enzyme (threonine deaminase) of the isoleucine biosynthetic pathway (Leavitt and Umbarger, 1962). One of the advantages of the use of amino acid analogues is that prototrophic bacteria can also be conveniently starved for an amino acid.

Mutants have been obtained in which amino acids no longer exert any control over RNA accumulation (Borek, Rockenbach, and Ryan, 1956; Fiil and Friesen, 1968). Such mutants are referred to as having a "relaxed" control of RNA synthesis, in contrast to the "stringent" control exercised by their parent strains. This property of stringent or relaxed control of RNA synthesis maps as a single genetic locus, called the rel locus or RC (RNA control) locus which is located at 53 min on the genome of Escherichia coli K-12 (Stent and Brenner, 1961; Alföldi et al., 1962). The stringent genotype is indicated by the symbol rel⁺ and the relaxed genotype by the symbol rel⁻. Thus, the stringent response may be defined as the severe restriction in the rate of RNA accumulation observed during amino acid starvation while the relaxed response is the continued accumulation of RNA observed during amino acid starvation. The phenomenon of amino acid control of RNA synthesis is also

found in Bacillus subtilis and Salmonella typhimurium and relaxed mutants have been isolated in these organisms as well (Gallant and Margason, 1972; Swanton and Edlin, 1972; Martin, 1968). Using merodiploids of the genotypes rel⁺/F'rel⁻ and rel⁻/F'rel⁺, the rel⁺ allele has been shown to be dominant over the recessive rel⁻ allele (Fiil, 1969) indicating that the rel gene expresses its control via a cytoplasmic factor.

Three models describing the control of RNA accumulation during amino acid deprivation can be visualized as outlined below:

- (A) Restricted synthesis with coordinate control. In this model the rate of synthesis of all three classes of RNA is severely reduced during amino acid starvation.
- (B) Degradative control. The severe reduction in RNA accumulation during amino acid starvation is due to the degradation of stable (ribosomal and transfer) RNA. Such a turnover of stable RNA has been shown to occur in Escherichia coli deprived of uracil, phosphorus or nitrogen (Lazzarini et al., 1969; Julien et al., 1967; Mandelstam and Halvorson, 1960).
- (C) Non-coordinate control. The synthesis of rRNA and tRNA is more preferentially curtailed as compared to mRNA during amino acid deprivation.

Although some workers have suggested that RNA synthesis is coordinately controlled (Model A) during

amino acid starvation (Friesen, 1966; Stubbs and Hall, 1968; Donini, 1972), the consensus of opinion is in favour of a non-coordinate regulation of RNA synthesis by amino acids (Model C). Lazzarini and Dahlberg (1971) measured the instantaneous rates of RNA synthesis and the composition of the RNA pulse-labelled in the presence or absence of a required amino acid in Escherichia coli. They found that ribosomal RNA synthesis is reduced to less than 10% whereas mRNA synthesis is reduced to approximately 40% that observed in growing cells. The overall rate of RNA synthesis during amino acid deprivation is reduced to about 30% (Lazzarini and Dahlberg, 1971). Therefore, stable (ribosomal and transfer) RNA synthesis is more preferentially inhibited. Furthermore, the synthesis of several types of mRNA's is known to proceed at a rate close to normal during amino acid starvation (Edlin et al., 1968; Lavallo and DeHauwer, 1968; Morris and Kjeldgaard, 1968; Forchhammer and Kjeldgaard, 1968; Sarkar and Moldave, 1968; Primakoff and Berg, 1970). The non-coordinate model also appears to provide a more suitable adaptative mechanism for bacteria. For example, a derepression of the amino acid biosynthetic enzymes would occur when Escherichia coli cells are subjected to a nutritional step-down from an amino acid-rich to an amino acid-poor medium. Therefore, synthesis of mRNA for these enzymes must proceed actively even though RNA accumulation is sharply restricted.

The mere absence of a ~~required~~ amino acid is not a sufficient condition to evoke the stringent response but rather it is the inability to aminoacylate tRNA (Neidhardt, 1966). This was demonstrated by the use of cells harbouring a temperature-sensitive aminoacyl-tRNA synthetase. Restriction of aminoacylation at the non-permissive temperature caused inhibition of RNA accumulation (Fangman and Neidhardt, 1964; Eidlic and Neidhardt, 1965). Use of such mutants has indicated that a full complement of the twenty aminoacyl-tRNA's are required for continued RNA accumulation. These results have led to the consideration that either the aminoacylation of tRNA or some step beyond it is involved in the regulation of RNA synthesis. The absence of N-formyl-methionyl-tRNA is also known to evoke the stringent response (Shih et al., 1966). Another characteristic of the stringent response is that the addition of antibiotic inhibitors of protein synthesis, such as chloramphenicol and oxytetracycline, to amino acid-starved rel⁺ cells abolishes the stringent response (Aronson and Spiegelman, 1961; Kurland and Maaløe, 1962; Ezekiel and Elkins, 1968).

Various models have been proposed in the last decade to explain the regulation of RNA synthesis by amino acids (reviewed by Edlin and Broda, 1968 and Ryan and Borek, 1971). Briefly, these theories postulate, alternatively, (i) that uncharged tRNA prevents RNA synthesis by inhibiting the RNA polymerase (Stent and Brenner, 1961;

Kurland and Maaløe, 1962; Tissieres et al., 1963),

(ii) that the control of RNA synthesis is mediated by the polyamines (Raina and Cohen, 1966; Raina, Jansen and Cohen, 1967), (iii) that RNA synthesis is regulated by the supply of its nucleotide precursors (Gallant and Cashel, 1967; Cashel and Gallant, 1968; Edlin and Neuhaard, 1967), (iv) that amino acid starvation disaggregates polysomes to free ribosomes and releases an inhibitor of RNA synthesis (Morris and DeMoss, 1966), and (v) that a special protein must be made to permit RNA accumulation (Aronson and Spiegelman, 1961). However, neither of these models has withstood the critical test of experimental verification (see Edlin and Broda, 1968).

A key observation was made by Cashel and Gallant in 1969 when they reported that two unusual nucleotides rapidly accumulated in Escherichia coli during the stringent response. These unusual magic spots, originally christened MS I and MS II, have since been identified respectively as guanosine 5'-diphosphate, 3-diphosphate (ppGpp) and guanosine 5'-triphosphate, 3'-diphosphate (pppGpp) (Cashel and Kalbacher, 1970; Haseltine et al., 1972; Sy and Lipmann, 1973; Que et al., 1973). Some rel⁺ strains do not accumulate pppGpp whereas all rel⁺ strains tested accumulate ppGpp. This accumulation of ppGpp constitutes the second hallmark of the stringent response. Guanosine tetraphosphate (ppGpp) is being actively considered as an inhibitor of stable RNA

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synthesis for the reasons summarized below:

First, the level of ppGpp increases markedly in rel⁺ cells when RNA accumulation is severely restricted as a result of amino acid starvation (Cashel and Gallant, 1969; Cashel, 1969) or restricted aminoacylation of tRNA (Cashel, 1969). The accumulation of ppGpp precedes the shut-off of RNA accumulation during amino acid starvation of rel⁺ cells and, on readdition of the deprived amino acid, the rapid disappearance of the accumulated ppGpp (half-life of 20-30 sec) precedes the resumption of RNA accumulation (Cashel, 1969). Secondly, the addition of chloramphenicol abolishes the stringent response by causing a rapid disappearance of the accumulated ppGpp and a resumption of RNA accumulation (Cashel, 1969; Lund and Kjeldgaard, 1972A). The third reason for implicating ppGpp as a regulatory nucleotide is that mutations in the rel gene (relaxed mutations) abolish the cells' ability to carry out the stringent response and accumulate ppGpp during amino acid deprivation (Cashel, 1969). Such relaxed mutants are nevertheless able to accumulate ppGpp when RNA accumulation is curtailed in response to other changes in cell conditions such as carbon-source starvation (Lazzarini et al., 1971; Harshman and Yamazaki, 1971) and plasmolysis in hypertonic salt solutions (Harshman and Yamazaki, 1972). It must be concluded, therefore, that the rel gene product itself does not catalyze ppGpp biosynthesis but rather transmits the signal (of amino

acid starvation) to the ppGpp synthetic machinery.

Finally, the existence of a versatile low molecular weight inhibitor, such as ppGpp, would seem to account for various other phenomena associated with the stringent response such as reduced rates of biosynthesis of nucleotides (Cashel and Gallant, 1968), glycolytic esters (Irr and Gallant, 1969) and lipids (Sokawa, Nakao and Kaziro, 1968; Golden and Powell, 1972; Merlie and Pizer, 1973) and restricted uptake of various exogenous metabolites (Edlin and Neuhaard, 1967; Nierlich, 1968; Sokawa and Kaziro, 1969). In fact, ppGpp has been shown in vitro to inhibit enzymes (inosine monophosphate dehydrogenase and adenylosuccinate synthetase) in de novo purine biosynthesis (Gallant, Irr and Cashel, 1971) and purine transport in isolated membrane vesicles (Hochstadt-Ozer and Cashel, 1972).

Recently, the requirements for ppGpp synthesis in vitro have been described (Haseltine et al., 1972; Haseltine and Block, 1973; Pedersen et al., 1973). It is now known that ppGpp is synthesized on the mRNA-ribosome complex in the presence of uncharged tRNA and its synthesis requires a protein factor called rel⁺ factor which is washed off ribosomes by high salt concentrations. This necessary factor is found only associated with ribosomes isolated from rel⁺ cells but is absent from ribosomes isolated from rel⁻ cells. The synthesis of ppGpp requires as substrates GDP or GTP and ATP. Although

the requirements for ppGpp synthesis are now known, its proposed regulatory role in the preferential inhibition of stable RNA synthesis during the stringent response has not yet been directly proven.

An apparent exception to the postulated role of ppGpp was reported by Chen et al. (1972). They claimed that the severe inhibition of RNA accumulation caused by trimethoprim addition in rel⁺ strains was not accompanied by the accumulation of ppGpp. Trimethoprim is a specific inhibitor of the enzyme dihydrofolate reductase (Burchall and Hitchings, 1966) and it prevents the formation of N-formyl-methionyl-tRNA indirectly by inhibiting the regeneration of its formyl donor, tetrahydrofolate. Since this report by Chen et al. (1972) would have seriously affected the postulated role of ppGpp during the stringent response, a detailed analysis of the trimethoprim-imposed stringent response was conducted in the present investigation. It will be shown in this thesis that (i) the addition of trimethoprim causes not only a severe inhibition of RNA accumulation in rel⁺ strains but also an immediate and rapid accumulation of ppGpp and (ii) the trimethoprim-imposed stringent response is identical to the stringent response elicited by amino acid starvation.

Lazzarini et al. (1971) found an inverse relationship between the basal levels of ppGpp during balanced growth and the RNA content per unit DNA, suggesting a

more general role for ppGpp in the regulation of stable RNA synthesis. Because of the relative importance of such an observation, this relationship has been re-examined. It will be shown in this thesis that (iii) no apparent inverse relationship exists between the ppGpp levels during balanced growth and the RNA/DNA ratios of the cell.

A study of the stringent phenotype under various growth conditions indicates that the chemical environment of the cell significantly affects the degree of stringency of the cell. The degree of stringency can be correlated to the level of ppGpp accumulation.

The role of ppGpp as a putative effector molecule modulating stable RNA synthesis is discussed in the light of the evidence presented in this thesis.

MATERIALS AND METHODS

Bacterial Strains

The following Escherichia coli K-12 strains were used:

- 1) The isogenic pair CP 78 (rel⁺, arg, his, thr, leu, thi, F⁻) and CP 79 (rel⁻, arg, his, thr, leu, thi, F⁻) (Fiil and Friesen, 1968) was obtained from Dr. J.D. Friesen, York University, Toronto and also from Dr. Barbara Bachmann, Coli Genetic Stock Centre, Yale University, New Haven, Conn., through the courtesy of Dr. Peter Lengyel.
- 2) CP 78 Thi^L, isolated from CP 78, is the same as CP 78 except that it is leaky for the thiamine requirement.
- 3) CP 78-Thi⁺, is a thiamine revertant of CP 78.
- 4) NF 161 (rel⁺, arg, met, F⁻) and NF 162 (rel⁻, arg, met, F⁻) are also isogenic except for the rel locus and were obtained from Dr. Michael Cashel, National Institutes of Health, Bethesda, Md.
- 5) Prototrophic strain 2012 (rel⁺, F⁻) (Alternate Designations: E.M. Lederberg K-12, 3100; ATCC 14948) is a standard wild-type K-12 strain and was obtained from the National Research Council of Canada Culture Collection, Ottawa.
- 6) A rel⁺ prototroph was provided by Dr. Ira Pastan, National Institutes of Health, Bethesda, Md. This strain has been designated HY 1 by us and it was used

extensively throughout this work as a wild-type K-12 strain.

7) CSH 62 (rel⁻, thi) (Alternate Designations: CA 8000; Hfr H) was obtained from the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Isolation of CP 78 Thi^L from CP 78 Thi⁻

As described under Results, strain CP 78 was found to be heterogeneous with respect to the thiamine requirement. Considerable growth was observed when CP 78 was grown overnight in thiamine-free TMM containing all other requirements. The culture was diluted twenty-fold in fresh medium and incubation was continued in the absence of thiamine until the cell density had reached an $A_{500} = 0.4-0.5$. 0.1 ml amounts of the appropriately diluted exponential culture were plated on Tris-minimal agar (TMA) plates containing all supplements including thiamine (0.4% glucose, 50 $\mu\text{g/ml}$ each of arginine, leucine, threonine, histidine, isoleucine and 10 $\mu\text{g/ml}$ thiamine). Fifteen colonies were selected at random and their requirement for thiamine was checked individually by monitoring their growth in liquid culture in the absence of thiamine for 24 hours. Thirteen of the 15 colonies examined showed either very little growth or no growth at all in the absence of thiamine whereas two colonies were found capable of sustained growth with a doubling time of 330 min on.

glucose. One of the two colonies showing a leaky requirement for thiamine was propagated and used in experiments involving growth in the absence of thiamine.

Isolation of CP 78 Thi⁺ from CP 78 Thi⁻

The procedure for the isolation of a thiamine revertant of strain CP 78 was identical to that used to obtain CP 78 Thi^L, except that the culture grown in the absence of thiamine was plated on TMA-plates containing all supplements except thiamine. Normally, colonies do not appear on such plates before 48 hours of incubation at 37°C. However, one or two colonies appeared on one plate within 24 hours. These were considered as prospective thiamine revertants. One of the colonies was further characterized by examining each individual auxotrophic marker of CP 78 and it was found to have the same requirements as CP 78 except that it grows with a doubling time of 70 min in the absence of thiamine (60 min in its presence).

Media and Culture Conditions

Unless indicated otherwise in the text, the growth medium used throughout was Tris-maleate minimal medium (TMM) which contains (grams/liter), tris (hydroxymethyl) aminomethane, 6.05; maleic acid, 5.8; NaCl, 2.5; KCl, 2.0; NH₄Cl, 1.0; MgCl₂·6H₂O, 1.0; Na₂ SO₄, 0.142 (final pH adjusted to 7.3). Prior to use, 0.1 ml sterile 0.1M

Na_2HPO_4 was added per 10 ml of basal medium to obtain a final phosphate concentration of 10^{-3} M. Certain specific experiments with trimethoprim were also done in Tris-buffered minimal medium A (Chen, Weissman and Lengyel, 1972) containing (grams/liter), tris-(hydroxymethyl) aminomethane, 6.05; KH_2PO_4 , 3.48; KCl, 1.33; $(\text{NH}_4)_2\text{SO}_4$, 1.0; sodium citrate. $5\text{H}_2\text{O}$, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 (pH adjusted to 7.4 with HCl). Experiments in minimal medium A involved growing the cells overnight in the high-phosphate medium (3.48g/liter KH_2PO_4), collection of a portion of the overnight culture by filtration, washing with the same medium except containing reduced phosphate (0.68g/liter KH_2PO_4), resuspension at a low cell density in the same low-phosphate medium and renewed incubation at 37°C .

For experiments with trimethoprim, both media were supplemented with 100 $\mu\text{g}/\text{ml}$ of each of the 19 common amino acids and 20 $\mu\text{g}/\text{ml}$ of tyrosine, 50 $\mu\text{g}/\text{ml}$ each of thymine, uracil, adenine and guanine in addition to glucose (0.4%). When required, thiamine-HCl was also added at 10 $\mu\text{g}/\text{ml}$. In all other experiments where trimethoprim was not used, bacteria were cultured in TMM containing 100 $\mu\text{g}/\text{ml}$ of each amino acid required by the strain for growth. Whenever necessary, thiamine was also added at 10 $\mu\text{g}/\text{ml}$. In the case of leucine-requiring strains (CP 78 and CP 79), isoleucine (100 $\mu\text{g}/\text{ml}$) was added along with the strain requirements to counteract

possible leucine inhibition (Alföldi et al., 1963; Alföldi and Kerekes, 1964). Various carbon sources were used: glucose (0.4%) plus 20 amino acids (100 µg/ml each); glucose (0.4%); sodium succinate hexahydrate (0.9%); sodium lactate (0.5%); sodium pyruvate (0.5%); sodium acetate trihydrate (1.0%); alanine (0.4%); or aspartic acid (0.5%). The final concentrations of the carbon sources used were such that the terminal growth was approximately equivalent to that on glucose. Unless otherwise indicated, bacteria were grown at 37°C with gyrotory agitation in a water bath shaker (Model G76; New Brunswick Scientific Co., Inc.) and growth was followed by measuring absorbance at 500 nm (A_{500}) with a Bausch and Lomb Spectronic 20 spectrophotometer using a 13X100 mm round cuvette.

The doubling times of the cultures were always determined from the linear region of the logarithmic plot of bacterial growth according to the relationship

$$\frac{\log (A_{500})_2 - \log (A_{500})_1}{\Delta t} = \frac{\log 2}{T}$$

, where

$(A_{500})_1$ is the absorbance at 500 nm of the culture at time t_1 ;

$(A_{500})_2$ is the absorbance at 500 nm of the culture at time t_2 ;

Δt is the time taken for the A_{500} to increase exponentially from t_1 to t_2 ; and T is the doubling time of the culture.

Antibiotics and Chemicals

Trimethoprim (2,4-diamino-5 (3',4',5'-trimethoxybenzyl) pyrimidine) obtained from Calbiochem and Dr. G.H.

Hitchings, Burroughs Wellcome Co. was solubilized in basal TMM and used at a final concentration of 50 µg/ml. L-

Chloramphenicol was obtained from Sigma Chemical Co. and it was also solubilized in basal TMM and generally used at a final concentration of 200 µg/ml. Oxytetracycline

hydrochloride was obtained from Sigma Chemical Co. and it was solubilized in H₂O and used at a final concentration

of 250 µg/ml. Rifampicin was obtained from Mann Research Laboratories and because of its poor solubility in aqueous solutions, it was solubilized in 20% dimethylsulfoxide

and used at a final concentration of 200 µg/ml. The final concentration of dimethylsulfoxide was 2% (v/v). Uniformly

labelled L-arginine-C¹⁴ (S.A. 210 mCi/m mole) was obtained from International Chemical and Nuclear Corporation;

carrier-free ³²P-orthophosphoric acid from Atomic Energy of Canada, Ltd.; β-2-thienylalanine from Nutritional

Biochemicals Corporation; and amino acids from either

Sigma Chemical Co. or Nutritional Biochemicals Corporation.

All other chemicals were reagent grade from either

Fisher Chemical Co. or British Drug Houses.

Radioactive Assay of RNA Accumulation

RNA accumulation was measured by following ³²P-

orthophosphate incorporation according to the procedure of Roodyn and Mandel (1960) as modified by Watson and Yamazaki (1973). ³²P-Orthophosphate was added to a portion of a growing culture at a final concentration of 5 μ Ci/ml and a final specific activity of 5 Ci/mole. The label was allowed to equilibrate with the culture for at least one-doubling prior to manipulation and sampling. Two 50 μ l portions of labelled bacterial cultures were removed at intervals and spread on Whatman 3MM filter paper disks (2.3 cm diameter) which had been numbered with a lead pencil and suspended on pins. One disk was used to assay total nucleic acid and the second disk, to assay DNA.

(i) Assay of total nucleic acids. After allowing them to air dry for 60 sec., the disks with pins were collected in a beaker of ice-cold 10% trichloroacetic acid (TCA). After standing in ice for at least 30 min to ensure complete fixation of the bacterial cells to the disks, the pins were removed and the disks were first washed three times with ice-cold 5% (w/v) TCA in a Büchner funnel under slight vacuum to remove labelled intracellular nucleotides and excess unincorporated ³²P-orthophosphate. The disks were then washed three times with an ether-ethanol mixture (1:1, v/v) to remove the TCA and any labelled cellular lipids. The disks were spread on aluminum foil and dried in an oven at 120°C for 15 min. The radioactivity of the disks was counted in 5 ml toluene-based scintillation fluid,

containing, 4 g Omnifluor (New England Nuclear Corporation, Boston, Mass.) per litre of reagent grade toluene, with a Packard Model 3003 Tri-Carb scintillation spectrometer. This radioactivity gives a measure of total nucleic acid (RNA plus DNA).

(ii) Assay of DNA. After allowing 60 sec to air dry, the disks with pins were collected in a beaker of 1N NaOH. The beaker was covered with para-film and incubated overnight at 37°C to hydrolyze RNA. The NaOH solution was then poured off and replaced by ice-cold 10% TCA. After at least 30 min in ice, the disks were washed, dried and counted as described above. This radioactivity is due to the alkali-insoluble DNA. The radioactivity incorporated into RNA was calculated by subtracting this radioactivity from the radioactivity in total nucleic acids.

Radioactive Assay of Protein Accumulation

Protein accumulation was generally determined by following ^{14}C -arginine incorporation, essentially as described by Mans and Novelli (1961). When arginine was present as a supplement in the medium at 100 $\mu\text{g}/\text{ml}$, ^{14}C -arginine was added to the culture at 1 $\mu\text{Ci}/\text{ml}$. In all other cases, one volume of culture was mixed with one-tenth the volume of a protein-labelling mixture which consisted of ^{14}C -arginine (5 $\mu\text{Ci}/\text{ml}$) and L-arginine (100 $\mu\text{g}/\text{ml}$). At various times, 50 or 100 μl portions of the labelled cultures were withdrawn and spread on Whatman

3MM filter paper disks (2.3 cm diameter) suspended on pins. After being air-dried for 60 sec, the disks with pins were immersed in ice-cold 10% TCA for at least 30 min. The pins were then removed and the disks placed in 5% TCA at 80°C for 30 min, then washed three times with cold 5% TCA to ensure removal of any unincorporated ^{14}C -amino acid(s) and small peptides. The disks were finally washed three times with an ether-ethanol mixture (1:1), dried at 120°C for 15 min and counted as described above. The hot-TCA insoluble radioactivity was taken to represent accumulated labelled cellular proteins (Bollum, 1968; Mans and Novelli, 1961).

Colorimetric Estimation of RNA and DNA.

To determine the RNA and DNA content of the cells, the procedure of Lazzarini, Cashel and Gallant (1971) was used. Triplicate samples (10 ml) were removed from a culture during exponential growth and mixed with 1.0 ml of 50% TCA at 0°C. After at least 30 min at 0°C the cells were collected on glass fiber filters (25 mm diameter, type A from Gelman Instrument Co.) and washed with 45 ml of ice-cold 5% TCA. The filters containing the entrapped cells were folded and placed in the bottom of a 13 x 100 mm test tube, covered with 2.0 ml of 5% TCA and agitated gently on a vortex mixer. The tubes were capped with marbles and placed in a 90-95°C water bath for 20 min to hydrolyze the nucleic acids. After cooling to room temperature, the supernates were transferred to 15 ml

conical centrifuge tubes and the glass fibers were removed by centrifugation at 2000 rpm for 5-10 min at 5°C. A 0.2 ml aliquot of the clear supernatant fluid was taken for the orcinol colorimetric assay for RNA (Brown, 1946; Schneider, 1945) and a 1.0 ml portion was taken for the diphenylamine assay for DNA (Burton, 1956).

(i) Orcinol assay. 0.2 ml of the supernate was diluted with 1.8 ml of H_2O and mixed with 2.0 ml of freshly prepared orcinol reagent which contained 6.8 mg of $CuCl_2 \cdot 2H_2O$ and 0.2 g of reagent grade orcinol (Fisher Scientific Co.) in 100 ml concentrated HCl. The tubes were placed in a water bath at 100°C for 20 min and the resulting blue color was measured at 620 nm.

(ii) Diphenylamine assay. 2.0 ml diphenylamine reagent was added to 1.0 ml of the supernatant fluid and the samples were incubated for 15 hr at 30°C. The intensity of the blue color produced was measured at 600 nm. Diphenylamine reagent contains 1.5 g diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of concentrated H_2SO_4 . Just before use, 0.1 ml of 1.6% aqueous acetaldehyde is added to every 20 ml of the diphenylamine reagent.

Colorimetric Estimation of Protein Accumulation

Protein accumulation was determined, in some instances, by the Lowry colorimetric method (Lowry *et al.*, 1951). At intervals, 5.0 ml portions of a culture were mixed with 1.0 ml ice-cold 30% (w/v) TCA in conical 15 ml

centrifuge tubes, and the collected samples were left on ice for at least 30 min. The samples were then heated at 80°C for 30 min to solubilize precipitated nucleic acids and centrifuged at 2000 rpm for 5 min to pellet the precipitated proteins. The pellets were resuspended in 5 ml of 5% TCA, centrifuged again, and the supernates were poured off. All traces of TCA were removed from the inside of the centrifuge tubes by leaving the tubes in an inverted position on a sheet of absorbent paper and by wiping it dry with a tissue. The pellets were suspended in 0.5 ml of 1.0N NaOH and heated at 90°C for 10 min. After cooling to room temperature, the protein content of the samples was determined by the Lowry colorimetric assay (Lowry et al., 1951) as modified by us. 5.0 ml H₂O followed by 0.5 ml "concentrated" Lowry reagent (C) was added and the samples were left at room temperature for 10 min. 0.5 ml of the 1N Folin-phenol reagent was then added and the samples were left at room temperature for 30 min to allow color development. The absorbance of the resulting blue color was measured at 520 nm. "Concentrated" Lowry reagent (C) (500 ml) is prepared by mixing 300 ml reagent (A) (containing per 300 ml, 20g NaOH and 100g Na₂CO₃) with 200 ml reagent (B) (containing per 200 ml, 1.0g sodium tartrate or 1.2g sodium tartrate.2H₂O and 0.5g CuSO₄.5H₂O). 1N Folin phenol reagent is prepared by diluting 2N phenol reagent 1:1 (v/v) with H₂O.

Viable Counts

Viable cell counts were determined by plating 0.1 ml aliquots of appropriately diluted bacterial cultures onto plates of LB medium which contains (grams/liter), Bacto-tryptone, 10; Bacto yeast extract, 5; NaCl, 10; and Bacto-agar, 15.

Determination of "Total" Nucleotides during Various Treatments.

The nucleotides found per unit volume of culture were assayed by ^{32}P -orthophosphate labelling of cells followed by extraction of the acid-soluble pools with formic acid and separation of the nucleotides by thin layer chromatography (Cashel, 1969). Carrier-free ^{32}P -orthophosphoric acid was added to the cultures to final specific activities of 150-300 Ci/mole, and the cultures were equilibrated with the label for at least one doubling or more prior to the addition of trimethoprim, L-valine or β -2-thienylalanine. 50 μl samples were withdrawn at various times before and after treatment of the culture, and pipetted directly into ice-cold microfuge tubes containing 50 μl of 2M formic acid. Following extraction of the acid-soluble pools for 30 min at 0°C and centrifugation for 5 min in a Beckman Model 152 Microfuge to pellet the cells, 5 μl aliquots of each sample were applied to polyethyleneimine-cellulose thin-layer plates (MN-Polygram CEL 300 from Brinkmann Instruments or Baker-flex

cellulose PEI from Canlab) which had been washed in H_2O for 15 min and air-dried prior to use. The chromatograms were developed in 1.5M KH_2PO_4 (pH adjusted to 3.4 with conc. H_3PO_4) and the ppGpp spots were located following autoradiography using 8 x 10 inch Kodak No-Screen Medical X-ray films (Eastman Kodak Co.). The radioactive spots were cut out from the plates and their radioactivity was determined using 5 ml of toluene-based scintillation fluid as described before. The procedure used to assay nucleotide triphosphates was identical to the one described except that the chromatograms were developed in 0.75M KH_2PO_4 (pH 3.4).

Determination of Nucleotide Levels during Balanced Growth

(i) "Total" nucleotides. ^{32}P -Orthophosphoric acid was added to cultures in exponential growth at final specific activities of 350-500 Ci/mole. After one doubling, duplicate samples (50 μ l) were withdrawn and mixed with an equal volume of 2M formic acid at $0^\circ C$ in microfuge tubes. After at least 30 min at $0^\circ C$, samples were centrifuged for 5 min in a Beckman Model 152 Microfuge. 20 μ l amounts of the supernates were spotted onto Polyethyleneimine cellulose sheets (Baker-flex; J.T. Baker Chemical Co.) and the nucleotides were separated by means of two-dimensional chromatography (Cashel and Kalbacher, 1970). The chromatograms were first developed in 3.3M ammonium formate containing 4.2% boric acid (pH

adjusted to 7.0 with conc. NH_4OH). The developed chromatograms were then soaked in 80% methanol for 30 min at room temperature and air-dried before being developed with 1.5M KH_2PO_4 (pH 3.4) in the second dimension. The nucleotides were located by radioautography and the radioactivity in the ppGpp and pppGpp spots was determined by liquid scintillation counting as described previously.

(ii) "Extracellular" nucleotides. Cultures were equilibrated with ^{32}P -orthophosphate as described above. After one doubling, duplicate 0.2 ml portions of the culture were withdrawn and suction-filtered through a Millipore filter (10 mm diameter; 0.45 μ pore size) on a Swinnex-13 filter unit (Millipore Corporation, Bedford, Mass.) which was mounted on a test tube (20 x 150 mm) with a side arm, which was held in ice. 50 μ l of the filtrate was mixed with 50 μ l of 2M formic acid. The samples were kept for 30 min at 0°C , centrifuged, and 20 μ l of the diluted filtrates were chromatographed two-dimensionally as described in the preceding assay. The extracellular amounts of the nucleotides were subtracted from the total (extra- and intracellular) amounts of nucleotides determined in the preceding assay to obtain the intracellular basal nucleotide levels during balanced growth.

RESULTS

I. The validity of the filter paper disk method
for the fractionation of cellular RNA and DNA

Throughout the present investigation, whenever the examination of the fine kinetics of macromolecular accumulation was required, radioactive assays were preferred over colorimetric methods because of their greater sensitivity. However, serious problems are sometimes encountered in the use of radioactive assays, for example, amino acid starvation drastically reduces the rate of entry of radioactive uracil into the UTP pool in stringent strains (Lazzarini and Dahlberg, 1971). Thus, the use of uracil as a label to measure RNA accumulation during amino acid starvation is not reliable unless it is corrected for the specific activity of the cellular UTP pool (Lazzarini and Dahlberg, 1971). This problem is alleviated if ^{32}P -orthophosphate is used as a label instead. However, the use of ^{32}P -orthophosphate necessitates a fractionation of the cellular RNA and DNA. Recently, Watson and Yamazaki (1973) reported a simple filter paper disk technique for the fractionation of RNA and DNA in Escherichia coli. RNA is hydrolyzable by alkali whereas DNA is not. Since whole cells labelled with ^{32}P -orthophosphate are to be assayed, it was necessary to confirm that the alkali-soluble radioactivity released from the cells indeed represents RNA. Rifampicin is an effective inhibitor of bacterial RNA synthesis (Mauro et al., 1969),

whereas DNA synthesis continues for at least one doubling after the addition of rifampicin (Lark, 1972). Therefore, rifampicin should exhibit a differential effect on the accumulation of the alkali-soluble versus the alkali-insoluble radioactivity of a ^{32}P -orthophosphate labelled culture. Fig. 1 demonstrates that in a culture labelled with ^{32}P -orthophosphate, only the alkali-soluble portion of the total acid-insoluble radioactivity ceased to accumulate within 2 min after the addition of rifampicin, whereas the alkali-insoluble radioactivity due to DNA increased at the same rate as the untreated control for at least 15 min before it gradually declined and finally ceased. Therefore, it is concluded that the alkali-soluble radioactivity measures RNA. In addition, the observed effect of rifampicin on DNA accumulation (Fig. 1B) is consistent with the conclusion of others (Lark, 1972; Brutlag *et al.*, 1971; Sugino *et al.*, 1972) that a transcriptional event is required for the initiation of a new round of DNA replication.

II. An analysis of the trimethoprim-imposed stringent response

Trimethoprim is a potent inhibitor of dihydrofolate reductase (Burchall and Hitchings, 1966), the enzyme which catalyzes the regeneration of tetrahydrofolate. Tetrahydrofolate is required for the formylation of a specific methionyl-tRNA which participates in polypeptide chain initiation. The dependence of RNA accumulation on the

Figure 1. Effect of rifampicin on RNA and DNA accumulation in HY 1.

HY 1 (rel⁺) was grown in completely supplemented TMM at 37°C. Carrier-free ³²P-orthophosphate was added to the log phase culture to a final specific activity of 5 Ci per mole (5 µCi per ml culture). After at least one doubling, the labelled culture was divided into two portions. At 0 time, the first portion was mixed with one-tenth the volume of a rifampicin solution in 20% dimethylsulfoxide (2 mg/ml) and the second portion was mixed with one-tenth the volume of 20% dimethylsulfoxide which served as a control. Two 50 µl samples were removed at intervals from both the control (●) and the rifampicin-treated (○) cultures and assayed for the acid-insoluble (total nucleic acid) and alkali-insoluble (DNA) radioactivity (Part B) by the filter paper disk method as described under Materials and Methods. The radioactivity due to RNA (Part A) was determined by subtracting the alkali-insoluble radioactivity from the total acid-insoluble radioactivity. Preliminary experiments showed that the addition of 2% (v/v) dimethylsulfoxide had no effect on RNA and DNA accumulation in the E. coli K-12 strains used.

availability of N-formylmethionyl-tRNA has been demonstrated by Shih, Eisenstadt and Lengyel (1966). They found that trimethoprim causes a rapid and severe inhibition of RNA accumulation in rel⁺ strains but the effect in rel⁻ strains is more gradual and much less severe despite the fact that protein accumulation is equally inhibited in both strains. Recently, Chen, Weissman and Lengyel (1972) claimed that the severe inhibition of RNA accumulation caused by trimethoprim in rel⁺ strains is not accompanied by the accumulation of ppGpp. Such a result is, however, inconsistent with the general notion that the stringent response is characterized not only by a preferential inhibition of RNA accumulation but also by a rapid and significant increase over the basal cellular level of ppGpp (Cashel, 1969).

In order to reconcile this apparently anomalous behaviour of trimethoprim, the effect of trimethoprim on RNA and ppGpp accumulation was reinvestigated in several rel⁺ and rel⁻ strains of Escherichia coli K-12. In order to preferentially inhibit the formylation of methionyl-tRNA, the effect of trimethoprim was studied in the presence of all common amino acids, purines and pyrimidines (Chen et al., 1972).

(A) Effect of trimethoprim on bacterial mass. The absorbance of a culture is known to be proportional to bacterial mass (Maaløe and Kjeldgaard, 1966). The effect of trimethoprim concentrations, ranging from 0 to 100

$\mu\text{g/ml}$, on the increase in total cell mass was examined. A concentration of 50 $\mu\text{g/ml}$ was found to give maximal inhibition of bacterial mass increase in both CP 78 (rel⁺) and CP 79 (rel⁻). Trimethoprim was slightly less effective in suppressing the increase of bacterial mass in CP 79 (rel⁻) as shown in Fig. 2, presumably because of continued RNA accumulation as will be demonstrated below.

(B) Effect of trimethoprim on protein and RNA accumulation. The effect of trimethoprim (50 $\mu\text{g/ml}$) on protein accumulation in CP 78 (rel⁺) and CP 79 (rel⁻) was determined colorimetrically. Fig. 2 shows that protein accumulation was severely inhibited in both strains. However, trimethoprim exhibited a differential effect on the accumulation of RNA in CP 78 (rel⁺) and CP 79 (rel⁻) as shown in Fig. 3. Consistent with the earlier observation of Shih et al. (1966), RNA accumulation in the rel⁺ strain was immediately reduced and ceased completely within 10 min of the addition of trimethoprim, whereas in the rel⁻ strain it continued at the same rate as the control for at least 15 min and then gradually declined, confirming the dependence of RNA accumulation in rel⁺ strains on the presence of N-formylmethionyl-tRNA.

(C) Effect of trimethoprim on ppGpp accumulation. The addition of trimethoprim also evoked a rapid and substantial accumulation of both ppGpp and pppGpp in CP 78 (rel⁺) whereas only a small and gradual increase in the level of ppGpp was observed in CP 79 (rel⁻) as shown in Fig. 3B.

Figure 2. Effect of trimethoprim on growth and protein accumulation in CP 78 and CP 79.

CP 78 (rel⁺) and CP 79 (rel⁻) were grown in completely supplemented TMM at 37°C. At 0 time, trimethoprim was added to log-phase cultures at a final concentration of 50 µg/ml. Samples were withdrawn periodically from the treated culture (○) and from the untreated control (●). Growth was determined by measuring absorbance at 500 nm and protein accumulation was assayed colorimetrically at 520 nm as described under Materials and Methods.

Figure 3. Effect of trimethoprim on accumulation of RNA, ppGpp and pppGpp in CP 78 and CP 79.

CP 78 (rel^+) and CP 79 (rel^-) were grown into the log-phase at 37°C in completely supplemented TMM and the cultures were divided into two portions. ^{32}P -Orthophosphate (carrier-free) was added to the first portion at $5\ \mu\text{Ci/ml}$ for RNA accumulation (a final specific activity of $5\ \text{Ci/mole}$) and to the second portion at $250\ \mu\text{Ci/ml}$ (a final specific activity of $250\ \text{Ci/mole}$) for the assay of ppGpp and pppGpp. After equilibrating for one doubling, each portion of the culture was further divided into two aliquots. One aliquot was mixed with one-tenth the volume of trimethoprim ($500\ \mu\text{g/ml}$) in TMM at 0 time, and the second aliquot, with the same volume of basal TMM only. Samples were withdrawn periodically and assayed for accumulation of RNA (Part A) and for "total" ppGpp and pppGpp (Part B). In Part A, closed circles indicate the control and open circles, the trimethoprim-treated culture. The data represents the amount of radioactivity of ^{32}P -labelled RNA in $50\ \mu\text{l}$ of the culture. In Part B, closed circles represent ppGpp and open circles, pppGpp. The broken line indicates the basal level of ppGpp. The amounts of phosphate incorporated into these nucleotides were calculated as nanomoles of phosphate per milliliter of culture at $A_{500} = 1.0$.

The level of pppGpp in CP 79 (rel⁻) did not increase to measurable amounts.

Since trimethoprim-induced accumulation of ppGpp in CP 78 (rel⁺) was not observed by Chen *et al.* (1972), the possibility that this difference might arise from differences in growth conditions was examined. Therefore, the effect of trimethoprim on ppGpp accumulation in CP 78 (rel⁺) grown in their medium A (as described under Materials and Methods) was examined. Fig. 4 clearly shows that even under their growth conditions significant amounts of ppGpp and pppGpp did accumulate in CP 78 (rel⁺) following the addition of trimethoprim. These results were reproducible using trimethoprim obtained from two different sources, as indicated under Materials and Methods. Therefore, it is unlikely that the difference between the results presented here and those of Chen *et al.* (1972) were caused by either the growth conditions employed or the source of the trimethoprim used. Furthermore, trimethoprim-induced accumulation of ppGpp was not specific to CP 78 (rel⁺) because two other K-12 rel⁺ strains, namely, NF 161 and the standard wild-type K-12 strain, 2012 also accumulated ppGpp immediately following the addition of trimethoprim (Fig. 5). Finally, the only plausible reason remaining for the discrepancy between the results presented here and those of Chen *et al.* (1972) could be that the CP 78 (rel⁺) strain used in the experiments reported here was not identical to the CP 78 (rel⁺) used

Figure 4. Trimethoprim-induced accumulation of ppGpp in CP 78 grown in medium A.

CP 78 (rel⁺) was grown in completely supplemented medium A at 37°C as described by Chen et al. (1973). ³²P-Orthophosphate was added to the log-phase culture at a final specific activity of 74 Ci/mole and a final concentration of 370 μ Ci/ml. After equilibration for one-doubling, trimethoprim was added at 0 time to a final concentration of 50 μ g/ml. The "total" levels of ppGpp and pppGpp were determined as described under Materials and Methods. Closed circles represent ppGpp and open circles, pppGpp. The amounts of phosphate incorporated into these nucleotides were calculated as nanomoles of phosphate per milliliter of culture at $A_{500} = 1.0$.

Figure 5. Effect of trimethoprim on ppGpp accumulation in strains 2012 and NF 161.

2012 (rel⁺) and NF 161 (rel⁺) were grown in completely supplemented TMM at 37°C. Carrier-free ³²P-orthophosphate was added to log-phase cultures to a final specific activity of 150-300/Ci per mole. After at least one doubling, trimethoprim was added at 0 time to a final concentration of 50 µg/ml. The "total" levels of ppGpp (●) and pppGpp (○) were determined as described under Materials and Methods. The amounts of phosphate incorporated into these nucleotides were calculated as nanomoles of phosphate per milliliter of culture at A₅₀₀ = 1.0.

by Chen et al. (1972). Therefore, the CP 78 (rel⁺) and CP 79 (rel⁻) pair which was purportedly used in their study was obtained from Dr. John Chen, and another CP 78 (rel⁺) and CP 79 (rel⁻) pair was kindly provided by Dr. Peter Lengyel. CP 78 (rel⁺) strains obtained from both sources were found to be indistinguishable from our own CP 78 (rel⁺) strain in all characteristics tested such as growth rates in glucose-minimal medium and enriched glucose-minimal medium, auxotrophic markers and inhibition of protein accumulation by trimethoprim. Most significantly, both strains were also indistinguishable from our strain in their capacity to accumulate ppGpp and pppGpp following the addition of trimethoprim. Trimethoprim-evoked ppGpp accumulation under relatively similar growth conditions has now ~~been~~ confirmed independently in CP 78 (rel⁺) (Midgley, 1972) and also in Escherichia coli B (Lund and Kjeldgaard, 1972A): Thus, inhibition of peptide chain initiation by restricting the formylation of methionyl -tRNA in rel⁺ strains causes the accumulation of ppGpp as well as inhibition of RNA accumulation.

(D) Effect of antibiotics. The following antibiotics were used to characterize the trimethoprim-induced stringent response: (i) rifampicin, which inhibits RNA chain initiation by interacting specifically with the β subunit of the RNA polymerase (Mauro et al., 1969); (ii) chloramphenicol, which binds specifically to the 50S ribosomal subunit and inhibits transpeptidation by inhibiting the functional attachment of the aminoacyl end of

aminoacyl-tRNA to the 50S subunit (Pestka, 1971); and (iii) oxytetracycline which prevents the binding of the Tu-GTP-aminoacyl-tRNA complex to the attachment site on the 30S ribosomal subunit (Pestka, 1971). Fig. 6 shows that protein accumulation was completely inhibited within 5 min of the addition of either oxytetracycline (Part A) or chloramphenicol (Part B). The effect of rifampicin on macromolecular synthesis is illustrated in Fig. 7. RNA accumulation (Part A) was rapidly and completely inhibited on the addition of rifampicin, whereas DNA accumulation (Part B) continued for approximately one doubling as previously demonstrated (Fig. 1). Protein accumulation (Fig. 7C) continued for approximately 10 min before being completely arrested. This continuation of protein synthesis can be ascribed to residual mRNA activity.

The effect of chloramphenicol and oxytetracycline on the level of ppGpp accumulated during an 11 min treatment with trimethoprim in CP 78 (rel⁺) is illustrated in Fig. 8. The addition of either oxytetracycline (Fig. 8A) or chloramphenicol (Fig. 8B) caused the accumulated ppGpp to decay rapidly to the initial basal level with a half-life of 20 to 30 sec. Trimethoprim-induced ppGpp accumulation might result either from an increased synthesis or decreased degradation of ppGpp or a combination of both processes. Since tetracyclines directly inhibit the synthesis of ppGpp (Pedersen et al., 1973), the rapid disappearance of ppGpp following the addition of

Figure 6. Effect of oxytetracycline and chloramphenicol on protein accumulation in CP 78.

CP 78 (rel⁺) was grown into the logarithmic phase at 37°C in completely supplemented TMM. In Part A, the culture was divided into two portions. At 0 time, the first portion was mixed with one-tenth the volume of ¹⁴C-arginine (10 µCi/ml) and one-tenth the volume of oxytetracycline hydrochloride (2.5 mg/ml). The second portion was similarly labelled in the absence of oxytetracycline and served as the untreated control. In part B, the culture was divided into three portions. ¹⁴C-arginine was added at 0 time to all three portions at a final concentration of 1 µCi/ml. At 0 time, chloramphenicol was added at a final concentration of 100 µg/ml to one portion of the culture and 200 µg/ml to the second portion of the culture. The third portion served as the untreated control. Fifty microliter aliquots of the control cultures (●) and the cultures treated with 250 µg/ml oxytetracycline (○), 100 µg/ml chloramphenicol (△) or 200 µg/ml chloramphenicol (▲) were periodically withdrawn and assayed for the hot TCA-insoluble radioactivity by the filter paper disk method as described under Materials and Methods.

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Figure 7. Effect of rifampicin on RNA, DNA and protein accumulation in CP 78.

CP 78 (rel⁺) was grown into the log-phase in completely supplemented TMM at 37°C. Carrier-free ³²P-orthophosphate was added to one portion of the culture at a final specific activity of 5 Ci per mole (5 µCi per ml culture). After one doubling, 50 µl samples were removed at intervals from the culture starting at time 0. Rifampicin was added to the culture at a final concentration of 200 µg/ml at 11 min as denoted by the arrows. The samples were assayed for the acid-insoluble (total nucleic acid) and alkali-insoluble (DNA) radioactivity (Part B) by the filter paper disk method as described under Materials and Methods. The radioactivity due to RNA (Part A) was determined by subtracting the alkali-insoluble radioactivity from the total acid-insoluble radioactivity. In Part C, to another portion of the culture, ¹⁴C-arginine and rifampicin were added at 0 time to final concentrations of 1 µCi/ml and 200 µg/ml, respectively. Fifty microliter samples were removed at intervals from the culture and assayed for the hot TCA-insoluble radioactivity also as described under Materials and Methods.

Figure 8. Effect of oxytetracycline, chloramphenicol and rifampicin on trimethoprim-induced ppGpp accumulation.

CP 78 (rel⁺) was grown in completely supplemented TMM at 37°C. When the cells had reached logarithmic growth, carrier-free ³²P-orthophosphate was added to the culture at a final specific activity of 150-300 Ci per mole.

After at least one doubling, trimethoprim was added to the culture at a final concentration of 50 µg/ml at time 0. Oxytetracycline (Part A), chloramphenicol (Part B), or rifampicin (Part C) were added to portions of the trimethoprim-treated culture at 11 min as indicated by the arrows. The final concentrations of oxytetracycline, chloramphenicol and rifampicin added were 250, 200 and 200 µg/ml, respectively. Samples were removed at appropriate intervals before and after the addition of the antibiotics and were assayed for the "total" ppGpp (●) and pppGpp (○) levels as described under Materials and Methods. The amounts of phosphate incorporated into these nucleotides were calculated as nanomoles of phosphate per milliliter of culture at $A_{500} = 1.0$.

oxytetracycline (Fig. 8A) indicates that trimethoprim causes ppGpp accumulation by stimulating its synthesis rather than by retarding its degradation as it appears to be the case during amino acid starvation (Gallant et al., 1972). It is well established that chloramphenicol causes a resumption of RNA synthesis (Aronson and Spiegelman, 1961; Kurland and Maaløe, 1962) and a rapid disappearance of the accumulated ppGpp (Cashel, 1969) in amino acid starved E. coli cells. Fig. 9 shows that chloramphenicol also relieved the inhibition of RNA accumulation by trimethoprim. Therefore, the trimethoprim-evoked stringent response is identical to that of classical amino acid starvation.

Since chloramphenicol does not inhibit the in vitro synthesis of ppGpp (Pedersen et al., 1973), the reason for the rapid decay of ppGpp on the addition of chloramphenicol remains to be explained. Fig. 10 shows that some residual protein synthesis was occurring in the presence of trimethoprim indicating some residual formylation of methionyl-tRNA. The addition of chloramphenicol completely inhibited this residual protein synthesis (Fig. 10). This complete inhibition of protein synthesis could lead to an accumulation of N-formylmethionyl-tRNA which would abolish the trimethoprim-evoked ppGpp accumulation. An additional interesting observation is that the addition of chloramphenicol to the trimethoprim-inhibited culture at 31 min also resulted in a resumption of RNA accumulation but in a very peculiar manner: RNA accumulation appeared to

Figure 9. Resumption of trimethoprim-inhibited RNA accumulation in CP 78 following the addition of chloramphenicol.

CP 78 (rel⁺) was cultured in completely supplemented TMM at 37°C. When the cells had reached logarithmic growth, carrier-free ³²P-orthophosphate was added to the culture at a final specific activity of 5 Ci/mole. After at least one doubling, the culture was divided into two portions. Trimethoprim was added at time 0 to one portion of the culture at a final concentration of 50 µg/ml while the second portion of the culture served as the untreated control. Chloramphenicol was added at a final concentration of 200 µg/ml to portions of the trimethoprim-treated culture either at 11 min or at 31 min as indicated by the arrows. Fifty microliter samples of the control (●), trimethoprim-treated culture (○) and the culture treated with both trimethoprim and chloramphenicol (▲) were withdrawn and assayed for RNA accumulation as described under Materials and Methods.

Figure 10. Inhibition of residual protein accumulation by chloramphenicol in a trimethoprim-inhibited CP 78 culture.

CP 79 (rel⁺) was grown in completely supplemented TMM at 37°C. When the cells had reached logarithmic growth, the culture was divided into two portions. ¹⁴C-Arginine at a final concentration of 1 µCi/ml and trimethoprim at a final concentration of 50 µg/ml were added to one portion of the culture at 0 time whereas the second portion of the culture was similarly labelled in the absence of trimethoprim. At the time indicated by the arrow (11 min after the addition of trimethoprim), chloramphenicol was added to a portion of the trimethoprim-treated culture at a final concentration of 200 µg/ml. Fifty microliter aliquots were removed periodically from the control (●), trimethoprim-inhibited culture (Δ), and the culture inhibited with both trimethoprim and chloramphenicol (○) and assayed for the hot TCA-insoluble radioactivity as described under Materials and Methods.

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proceed "cyclically" (Fig. 9). The reason for this phenomenon is not known.

Watson and Yamazaki (1972) suggested that ppGpp can accumulate only when valine-imposed isoleucine starvation is maintained by the presence of mRNA. The same conclusion was also derived from a similar in vivo study (Lund and Kjeldgaard, 1972B). During the trimethoprim-imposed stringent response, mRNA synthesis is expected to continue at a significant rate (Lazzarini and Winslow, 1970; Lazzarini and Dahlberg, 1971). The addition of rifampicin to a trimethoprim-treated culture would lead to the gradual depletion of the unstable mRNA (due to residual protein synthesis) since rifampicin would inhibit further RNA synthesis. If the presence of mRNA is required for the maintenance of the trimethoprim-evoked ppGpp accumulation, then depletion of the intracellular mRNA level would result in a gradual decay of the accumulated ppGpp. This prediction was substantiated by the result shown in Fig. 8C. The addition of rifampicin to the trimethoprim-inhibited culture caused a gradual decline in the level of ppGpp which was preceded by an initial rise. The reason for the initial rise is not clear. Recent in vitro studies have demonstrated the absolute requirement for mRNA in the synthesis of ppGpp during amino acid starvation (Pedersen et al., 1973; Haseltine and Block, 1973).

III. Intracellular ppGpp levels and the regulation of RNA synthesis during balanced growth

The excellent kinetic correlation between ppGpp accumulation and the cessation of RNA accumulation during the stringent response caused by amino acid starvation or by trimethoprim addition, as well as during various other physiological stress conditions, suggests a possible inhibitory role of this nucleotide in the regulation of the synthesis of stable bacterial RNA (rRNA and tRNA). This proposed regulatory role of ppGpp received additional support when Lazzarini et al. (1971) reported that the basal levels of ppGpp observed during balanced growth vary inversely with the growth rate and RNA content per unit DNA suggesting a more general role of ppGpp in the regulation of stable RNA synthesis.

However, Lazzarini et al. (1971) demonstrated this relationship using NF 161 (rel⁺) which is auxotrophic for arginine and methionine. The inherent danger in the use of such an auxotroph for this purpose is that the auxotroph when grown on a poor carbon source, might utilize the amino acids added as a growth requirement for a preferred carbon source. Moreover, NF 161 (rel⁺), unlike a wild-type rel⁺ strain, possesses an additional mutation which causes a drastically reduced cellular capacity to accumulate ppGpp during the stringent response (J. Gallant, personal communication). Therefore, in view of the significance of the work of Lazzarini

et al. (1971), it was necessary to reinvestigate this relationship in a prototrophic wild-type rel⁺ strain over a wider range of growth rates created by utilizing different carbon sources.

Prototrophic strain HY 1 (rel⁺) was grown in TMM supplemented with eight selected carbon sources to obtain eight different growth rates. The growth curves on these carbon sources are shown in Fig. 11. This strain maintained exponential growth for at least two cell doublings on each carbon source and the doubling times ranged from 35 to 330 min (Table I). Furthermore, Table I also shows that cell mass was larger at the faster growth rates and smaller at the slower growth rates, in agreement with an earlier report that cell mass depends in a simple manner on the growth rate afforded by the chemical composition of the medium (Schaechter, Maaløe and Kjeldgaard, 1958).

It has been previously assumed that ppGpp accumulates only intracellularly. Thus, all the ppGpp found in the acidified culture (referred to as "total" ppGpp here) has been taken as the intracellular level of ppGpp. However, it was found in the present study that significant quantities of ppGpp (as well as trace quantities of triphosphates) accumulated also extracellularly in all cultures. Table II shows the "total" and intracellular amounts of ppGpp, GTP and ATP. The differences between the "total" and intracellular amounts must be due to the

Figure 11. Growth of HY 1 on eight different carbon sources.

HY 1 (rel⁺) was cultured at 37°C on eight different carbon sources: (1) glucose plus 20 common amino acids, (2) glucose, (3) succinate, (4) lactate, (5) pyruvate, (6) acetate, (7) alanine, and (8) aspartate as described under Materials and Methods. The numbers in parentheses correspond to the numbers in the figure indicating the logarithmic growth phase of HY 1 on each carbon source. The doubling times of HY 1 on the eight selected carbon sources were determined from these semi-logarithmic plots and are listed in Table I. Each division on the abscissa corresponds to 60 min.

Table I. The relationship of RNA/DNA ratios and cell mass to the growth rate.

HY 1 (rel⁺) was grown into the logarithmic phase on various carbon sources at 37°C as indicated. For the determination of the RNA/DNA ratios, samples were removed in triplicate and assayed for RNA (orcinol assay) and DNA (diphenylamine assay) as described under Materials and Methods. The RNA/DNA ratios (average of three determinations) represent ~~the relative~~ color intensities: orcinol color (A_{620})/diphenylamine color (A_{600}). For the determination of relative cell mass, samples were removed from the cultures at a known optical density (A_{500}) and the number of viable cells/ml was determined as described under Materials and Methods. The data represents the A_{500} of 10^9 cells/ml of each culture under the experimental conditions used.

TABLE I

Carbon Source	Doubling Time (min)	RNA/DNA Ratio	Relative Cell Mass
Glucose enriched with amino acids	35-40	0.860	2.49
Glucose	55	0.616	1.13
Succinate	70-80	0.425	1.03
Lactate	90	0.368	1.04
Pyruvate	95	0.348	0.984
Acetate	120	0.306	0.813
Alanine	130	0.257	0.658
Aspartate	300-330	--	0.704

Table II. Total and intracellular distribution of ppGpp, GTP and ATP

HY 1 (rel⁺) was grown at 37°C on various carbon sources as indicated and NF 161 (rel⁺) was cultured on alanine. When the cultures had attained logarithmic growth, carrier-free ³²P-orthophosphate was added to final specific activities of 350 to 500 Ci per mole. After one doubling, samples were withdrawn in duplicate and assayed for total and extracellular ppGpp, GTP and ATP levels as described under Materials and Methods. The intracellular amounts were calculated by subtracting the extracellular amounts from the "total" amounts. The total and intracellular levels of ppGpp, GTP and ATP are presented as the amounts of ³²P-orthophosphate incorporated into these nucleotides as nanomoles of phosphate per milliliter of culture at A₅₀₀ = 1.0. The doubling times of HY 1 on these carbon sources are shown in Table I. The doubling time of NF 161 was 165 min on alanine as a carbon source.

TABLE II

Carbon Source	ppGpp		GTP		ATP	
	Total	Intra-cellular	Total	Intra-cellular	Total	Intra-cellular
Strain: HYL ₁ (rel ⁺)						
Glucose + amino acids	0.100 0.109	0.086 0.086	2.62 2.86	2.53 2.73	7.52 7.92	7.18 7.52
Glucose	0.151 0.138	0.137 0.126	3.87 3.51	3.80 3.46	9.28 8.45	9.11 8.35
Succinate	0.091 0.091	0.068 0.068	2.48 2.49	2.46 2.47	7.39 7.59	7.29 7.50
Lactate	0.101 0.100	0.062 0.050	1.98 1.75	1.89 1.69	5.03 4.98	4.83 4.87
Pyruvate	0.122 0.133	0.068 0.071	1.67 1.75	1.63 1.68	4.50 4.63	4.32 4.41
Acetate	0.176 --	0.153 --	1.43 --	1.41 --	5.85 --	5.77 --
Alanine	0.256 0.196	0.195 0.153	2.60 2.71	2.51 2.67	7.48 8.06	7.35 7.94
Aspartate	0.164 0.169	0.115 0.119	0.856 0.676	0.821 0.634	3.57 2.99	3.46 2.89
Strain: NF 161 (rel ⁺)						
Alanine	0.520 0.682	0.506 0.662	3.07 3.42	3.03 3.40	8.80 9.72	8.61 9.53

excretion of these nucleotides. The relative extra- and intracellular distribution of these nucleotides was calculated from the data in Table II and is shown as a percentage of the "total" amounts in Table III. The extracellular amounts of ppGpp varied considerably with the carbon source used. Its extracellular accumulation is likely due to the excretion of this nucleotide rather than to the lysis of a fraction of the cells, since its extracellular distribution (relative to the intracellular amount) was much greater than that of ATP or GTP (Table III). If it had been a result of lysis, the extracellular distribution of all three nucleotides would have been similar. Therefore, it seems obvious that the intracellular ppGpp level must be determined in order to study its intracellular metabolic role.

Fig. 12 illustrates that there is no correlation between the intracellular level of ppGpp (Part B) and the growth rate (correlation coefficient = -0.248 ; not significant at 5% level). Since the RNA/DNA ratios are directly correlatable with the growth rate (Part C), it is concluded that the intracellular ppGpp levels do not vary inversely with the RNA content per unit DNA. Thus, the basal levels of ppGpp appear to have no direct relation to the cellular capacity to accumulate RNA during balanced growth. The basal ppGpp level during balanced growth may then merely reflect the sum of the metabolism of ppGpp in different carbon sources.

Table III. Extra- and intracellular distribution of ppGpp, GTP and ATP.

HY 1 (rel⁺) was grown at 37°C on various carbon sources as indicated, and NF 161 (rel⁺) was grown on alanine. When the cultures had attained logarithmic growth, ³²P-orthophosphate was added to final specific activities of 350 to 500 Ci per mole. After one doubling, samples were withdrawn in duplicate and assayed for extra- and intracellular ppGpp, GTP and ATP as described under Materials and Methods. The extra- and intracellular levels (average of two determinations) are presented as a percentage of the total amounts.

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TABLE III

Carbon Source	ppGpp		GTP		ATP	
	Extra-	Intra-cellular	Extra-	Intra-cellular	Extra-	Intra-cellular
Strain: HY 1 (rel ⁺)						
Glucose + amino acids	18	82	4	96	5	95
Glucose	9	91	2	98	2	98
Succinate	25	75	1	99	1	99
Lactate	45	55	4	96	3	97
Pyruvate	45	55	3	97	4	96
Acetate	13	87	2	98	2	98
Alanine	23	77	2	98	2	98
Aspartate	30	70	5	95	3	97
Strain: NF 161 (rel ⁺)						
Alanine	3	97	1	99	2	98

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Figure 12. Levels of ppGpp and RNA/DNA ratios at different growth rates in HY 1

Strain HY 1 was cultured in each of the following carbon sources: glucose plus 20 common amino acids (●); glucose (○); succinate (▲); lactate (△); pyruvate (□); acetate (◻); alanine (×); aspartate (◐). When the cultures had attained logarithmic growth, carrier-free ^{32}P -orthophosphate was added to final specific activities of 350 to 500 Ci per mole. After one doubling, samples were removed in duplicate for the assay of total (Part A) and intracellular ppGpp (Part B) as described under Materials and Methods. The data (average of two determinations) represents the molar quantities of phosphate in ppGpp per ml of culture at $A_{500} = 1.0$. For the determination of the RNA/DNA ratios (Part C), samples were removed in triplicate and assayed for RNA (orcinol assay) and DNA (diphenylamine assay) also as described under Materials and Methods. The data (average of three determinations) represents the relative color intensities (orcinol color/diphenylamine color). The RNA/DNA ratio for aspartate is not included since growth on this carbon source consisted of an initial fast logarithmic phase followed by a final slow logarithmic phase, resulting in an overestimated value.

The present conclusion is based upon the examination of this relationship in a wild-type rel⁺ bacterium over a wider range of growth rates. It was found that NF 161 (rel⁺) grown in a medium containing alanine as a carbon source (doubling time of 165 min) contained approximately a three times greater intracellular level of ppGpp than in HY 1 (rel⁺), similarly grown in the alanine medium. Moreover, the excretion of ppGpp from NF 161 cells was negligibly small in this medium unlike HY 1 (Table III). Whether the difference between HY 1 and NF 161 is due to the mutation (with respect to the metabolism of guanosine polyphosphates) in NF 161 remains to be clarified. Furthermore, in strain HY 1 (rel⁺), the levels of GTP and ATP, the normal precursors of ppGpp and pppGpp (Haseltine et al., 1972) do not exhibit any apparent correlation with the growth rate either (Table II). However, the ppGpp levels are generally correlatable with the GTP and ATP levels (Table II).

IV. Effect of growth rate on the phenotypic expression of the rel gene

There is very little data in the literature concerning the effect of growth rates on the phenotype of the rel gene. The investigation of this problem has demonstrated further the correlation between the accumulation of ppGpp and amino acid control of RNA accumulation as will be shown below. The phenotypic expression of the rel⁺ or rel⁻ genotype at different growth rates was compared.

The growth rates of rel⁺ and rel⁻ strains were adjusted by either of three different ways: (i) using different carbon sources (ii) lowering the temperature of cultivation from 37°C to 20°C or (iii) limiting a nutritional requirement.

The degree of stringency or relaxedness has been conventionally determined by comparing the incorporation of a radioactive precursor into RNA during amino acid deprivation with that in the unstarved control (Stent and Brenner, 1961; Fiil and Friesen, 1968; Swanton and Edlin, 1972). Amino acid deprivation is normally accomplished by removing a required amino acid from a culture of an amino acid requiring mutant by means of filtration and resuspension in a medium lacking the required amino acid. However, the addition of β -2-thienylalanine or valine to an E. coli K-12 culture has been found to be a convenient and rapid means of creating phenylalanine (Sköld, 1970) or isoleucine starvation (Cashel and Gallant, 1969; Nierlich, 1968; Fiil, 1969), respectively, since they both interfere with the biosyntheses of these amino acids in susceptible cells (Ezekiel, 1965; Leavitt and Umbarger, 1962). However, in these cases, it is necessary to assess the degree of relaxedness relative to the degree of amino acid starvation caused by the analogue. Thus, the ratio of the amount of precursor incorporation into RNA and protein (RNA/protein) during the analogue treatment has been used as a measure of relaxedness (Fiil, 1969).

The effect of valine-imposed isoleucine starvation on the accumulation of RNA and ppGpp was measured in rel⁺ cells at different growth rates which were created by using different carbon sources or by limiting the intracellular biosynthesis of thiamine. Using the conventional methods discussed above, it was found that a rel⁺ cell exhibits significantly less stringency during nutritionally slowed growth as will be demonstrated below.

(A) Effect of carbon sources on stringency. Prototrophic strain HY 1 (rel⁺) was grown on three different carbon sources to obtain three distinct growth rates. The doubling times of HY 1 were approximately 60 min in glucose, 120 min in acetate, and 300 min in the aspartate medium at 37°C. Fig. 13 shows the effects of valine-imposed isoleucine starvation on the accumulation of ¹⁴C-arginine labelled protein in HY 1 at the three growth rates. In order to permit comparison of the three cultures, the amounts of the labelled proteins are expressed as percentages of the amounts in the unstarved control of each culture at one quarter of the doubling time after the addition of the label. The addition of valine caused similar inhibition of accumulation of labelled protein in the three cultures indicating that the degree of isoleucine starvation was approximately the same. In contrast, the effect of isoleucine starvation on RNA accumulation was profoundly different as shown in Fig. 14. As expected, RNA accumulation in HY 1 (rel⁺) grown in the glucose medium (Part A) was severely inhibited during isoleucine

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Figure 13. Effect of valine-imposed isoleucine starvation on protein accumulation in HY 1 grown on three different carbon sources.

Strain HY 1 (rel⁺) was cultured at 37° in glucose (Part A), acetate (Part B), or aspartate (Part C). When the cells had reached logarithmic growth, each culture was divided into two equal portions. At 0 time, the first portion was mixed with one-tenth the volume of a protein-labelling mixture (5 μ Ci/ml ¹⁴C-arginine; 100 μ g/ml L-arginine) and one-tenth the volume of L-valine (5 mg per ml). The second portion was similarly labelled in the absence of valine, thus serving as an unstarved control. Fifty microliter portions of the labelled cultures were periodically withdrawn from the starved (○) and unstarved (●) cultures and assayed for the hot trichloroacetic acid-insoluble radioactivity by the filter paper disk method as described under Materials and Methods. The data is expressed as a percentage of the radioactivity in the unstarved control culture at one-quarter doubling time after the addition of valine.

Figure 14. Effect of valine-imposed isoleucine starvation on RNA accumulation in HY 1 grown on three different carbon sources.

Strain HY 1 (rel⁺) was cultured at 37° in glucose (Part A), acetate (Part B), or aspartate (Part C).

Carrier-free ³²P-orthophosphate was added to the log-phase cultures to a final specific activity of 5 Ci per mole (5 μ Ci per ml culture). After at least one doubling, the labelled culture was divided into two equal portions. At 0 time, L-valine was added to the first portion to a final concentration of 500 μ g per ml. The second portion served as an unstarved control. Two 50 μ l portions were removed at intervals from both the starved (○) and unstarved (●) cultures and assayed for the acid-insoluble (total nucleic acid) and alkali-insoluble (DNA) radioactivity by the filter paper disk method as described under Materials and Methods. The radioactivity due to RNA was determined by subtracting the alkali-insoluble radioactivity from the acid-insoluble radioactivity. The data is presented as a percentage of the RNA radioactivity in the control culture at one-quarter doubling time after the addition of valine.

starvation whereas the same strain growing at the slower rates exhibited considerable degrees of "relaxedness". Namely, RNA accumulation in the acetate and aspartate cultures continued at significant rates up to one-quarter doubling times after the onset of amino acid starvation (Parts B and C), beyond which time RNA accumulation rapidly declined and ceased completely after one-half doubling times (data not shown).

The determination of relaxedness is valid only for an early period of amino acid starvation since even "high-relaxed" rel⁻ strains (Fiil and Friesen, 1968) accumulate RNA at an uninhibited rate only up to one-third doubling time after the onset of amino acid starvation. In order to closely examine the kinetics of the degree of "relaxedness" shown by the stringent strain, the RNA to protein ratios are given in Fig. 15, which were calculated from the data in Fig. 13 and Fig. 14. The higher the value of such a ratio during amino acid starvation indicates greater relaxedness; and the lower the value, greater stringency. On isoleucine starvation, the RNA/protein ratio rapidly fell to nearly zero in the glucose culture (Part A), whereas the ratio in the acetate or aspartate culture rapidly rose to a maximum and subsequently declined to values higher than the level in the unstarved control (Parts B and C). The maximal ratio obtained in the aspartate culture was significantly higher than that in the acetate culture. This phenotypic "relaxedness"

Figure 15. Effect of valine-imposed isoleucine starvation on the RNA/protein ratio in HY 1 grown on three different carbon sources.

The RNA/protein ratios in the starved (○) and unstarved (●) cultures of HY 1 (rel⁺) were calculated directly from the data shown in Fig. 13 and Fig. 14. Part A shows the ratio in glucose, Part B in acetate, and Part C in aspartate. The time zero indicates the time of addition of valine.

was observable also in the medium containing alanine as a carbon source which yielded a doubling time of approximately 130 min. (data not shown).

The second hallmark of the stringent/relaxed phenotype is the property of ppGpp accumulation during amino acid starvation. Fig. 16 shows the effect of carbon sources on the capacity to accumulate ppGpp upon isoleucine starvation. In all three cultures, isoleucine starvation immediately elicited the accumulation of ppGpp. However, the amounts of ppGpp that accumulated above the basal levels were considerably different: the largest in the glucose culture, intermediate in acetate, and the least in aspartate. The amounts of pppGpp accumulation (Cashel and Gallant, 1969; Cashel, 1969) were also different: greater in the glucose culture than that in the acetate culture. No detectable amounts of pppGpp accumulated in the aspartate culture. Therefore, by this criterion (ppGpp accumulation) as well, the cells grown in aspartate again exhibited the highest "relaxedness"; the acetate culture, the intermediate; and the glucose culture, the highest stringency.

(B) Effect of temperature on stringency. In order to determine if the primary cause for the observed "relaxedness" was the slow growth rate per se or the nutritional composition of the medium, strain HY 1 (rel⁺) was grown on glucose as a carbon source at a lower incubation temperature which resulted in a considerable

Figure 16. Effect of valine-imposed isoleucine starvation on accumulation of ppGpp by HY 1 grown on three different carbon sources.

Strain HY 1 (rel⁺) was grown at 37° on glucose (Part A), acetate (Part B), or aspartate (Part C) as a carbon source. ³²P-Orthophosphate was added to log phase cultures at a final specific activity of 150 - 300 Ci per mole. After at least one doubling, L-valine was added to a final concentration of 500 µg per ml at 0 time. Fifty microliter portions of the culture were removed periodically and were assayed for the "total" ppGpp (●) and pppGpp (○) levels as described under Materials and Methods. The data is presented as the amount of phosphate incorporated into these nucleotides in one ml of the culture of A₅₀₀ = 1.0.

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reduction in the growth rate of HY 1. For example, the doubling time of HY 1 (rel⁺) was approximately 160 min at 20°C as compared to 60 min at 37°C. Fig. 17 shows that the growth slowed by lowering the incubation temperature to 20°C did not appreciably affect the degree of stringency. This is in contrast to the "relaxed" RNA accumulation caused by a change in the growth rate when the carbon source is changed (Fig. 14). Thus, it appears that the chemical environment of the cells is primarily responsible for the degree of stringency/relaxedness.

(C) Effect of vitamin deprivation on stringency. In order to examine whether the observed phenomenon of phenotypic "relaxedness" exhibited by rel⁺ strains was specific to growth regulated by a change in the carbon source, the degree of stringency was studied in CP 78 Thi^L, a rel⁺ strain leaky for the thiamine requirement, grown in the absence of thiamine.

Since CP 78 Thi^L was maintained on thiamine-containing media, transfer of the cells to thiamine-free media did not immediately yield thiamine-limited growth because of an intracellular reserve of thiamine. Therefore, it was necessary to determine an experimental condition which would ensure the depletion of this reserve. For this purpose, thiamine-requiring CP 78 was grown in glucose-minimal medium with all supplements including thiamine (10 µg/ml) for a few doublings and the cells were collected by filtration, washed copiously to remove the extra-

Figure 17: Effect of low incubation temperature on accumulation of RNA and protein in HY 1 during isoleucine starvation.

HY 1 (rel⁺) was cultured at 20° on glucose as a carbon source. The effect of valine-imposed isoleucine starvation on accumulation of RNA (Part A) and protein (Part B) was studied in the same manner as described in Fig. 13 (protein) and Fig. 14 (RNA). Valine was added at 0 time. The data indicates the amounts of radioactivity in 50 μ l portions of starved (○) and unstarved (●) cultures.

cellular thiamine and resuspended in a medium containing all supplements except thiamine. Fig. 18 shows that once the strain had grown on thiamine, removal of the thiamine showed little effect on the growth for at least three doublings whereas removal of any one of the required amino acids by this process significantly affected the growth of CP 78. This suggests that CP 78 Thi^L must be grown in the absence of thiamine for many doublings before the thiamine-limited growth can be observed. In subsequent experiments, the final thiamine-limited growth of CP 78 Thi^L was obtained after prolonged growth in the absence of thiamine. In the thiamine-free glucose medium, the doubling times were approximately 330 min for CP 78 Thi^L and 70 min for CP 78 Thi^+ . In the presence of thiamine, both grew at the same rate as their parent CP 78 with a doubling time of 60 min.

Since the determination of the stringent/relaxed phenotype depends upon the equilibration of the exogenous ^{32}P -orthophosphate with the intracellular triphosphate pools, it was necessary to establish that this equilibration was not affected by thiamine-limitation. Fig. 19 shows that complete equilibration was reached within one-tenth of the doubling time in CP 78 Thi^L growing exponentially in the absence of thiamine. Moreover, the levels of ATP and GTP (the normal precursors of ppGpp) were equivalent to those found in the presence of thiamine (data not included).

Figure 18. Effect of removal of thiamine and individual amino acid requirements by filtration and resuspension on the growth of CP 78

CP 78 (rel⁺), a thiamine-requiring strain, was grown on glucose as a carbon source in fully supplemented TMM at 37°C. A log-phase culture was collected by filtration on a membrane filter, washed thoroughly with basal TMM and resuspended in the original volume of the basal medium. At 0 time, aliquots of the cell suspension were diluted with the following glucose-containing media to obtain an initial cell density of $A_{500} = 0.02$: fully supplemented TMM (●); TMM supplemented with all requirements except thiamine (○); arginine (▲); leucine (■); or threonine (Δ). The cultures were incubated at 37°C and growth was measured by following the absorbance of the cultures at 500 nm.

Fig. 20 illustrates the effects of thiamine deprivation on the degree of stringency. β -2-thienylalanine (500 μ g/ml) was used to create phenylalanine starvation since the medium contained isoleucine and therefore valine could not be used. In the presence of thiamine, the leaky thi mutant, CP 78 Thi^L , exhibited typical stringency in terms of accumulation of RNA and ppGpp (Fig. 20C and E) whereas in the absence of thiamine it exhibited a high degree of "relaxedness" in terms of the accumulation of RNA and ppGpp (Fig. 20D and F). In the absence of thiamine, an unusually high basal level of ppGpp was observed (Part F) which did not appear to change when the cells were subjected to phenylalanine starvation. The amount of pppGpp was either undetectable or immeasurably low. In order to appreciate the degree of "relaxedness" exhibited by this rel⁺ strain, the typical relaxed response of CP 79 (rel⁻) is shown in Fig. 21. The "relaxed" accumulation of RNA caused by thiamine-limitation was not greatly different from that shown by this high-relaxed mutant grown in a fully supplemented medium.

The thiamine revertant, CP 78 Thi^+ , which has regained the capacity to synthesize thiamine, exhibited typical stringency when the cells were grown in the absence of thiamine (Fig. 22). CP 78 Thi^+ had recovered the capacity to accumulate ppGpp and inhibit RNA accumulation upon amino acid starvation (Parts B and C). Therefore, it was the limitation of the intracellular supply of thiamine that was a primary cause of the "relaxed" response observed in

Figure 19. Equilibration of ^{32}P -orthophosphate with the intracellular ATP and GTP pools in CP 78 Thi^L during thiamine-limited growth.

CP 78 Thi^L was cultured at 37°C in TMM containing glucose and all required supplements except thiamine. When the cells had reached the thiamine-limited logarithmic growth phase (doubling time of 330 min), carrier-free ^{32}P -orthophosphate was added at time 0 to a final specific activity of 150-300 Ci per mole. Fifty microliter samples were withdrawn at 30 min intervals and the ATP and GTP levels were assayed as described under Materials and Methods. The data is presented as the amount of phosphate incorporated into ATP (●) and GTP (○) in one ml of a culture of $A_{500} = 1.0$.

Figure 20. Effect of β -2-thienylalanine-imposed phenylalanine starvation on accumulation of protein, RNA and ppGpp in CP 78 Thi^L

CP 78 Thi^L (thiamine leaky mutant) was grown into the log-phase on glucose in the presence of thiamine, and then divided into two portions. The effect of β -thienylalanine-imposed phenylalanine starvation was studied on the first portion in the continued presence of thiamine (the left-hand panel): Part A indicates protein accumulation; Part C, RNA accumulation; Part E, ppGpp accumulation. The second portion was transferred to a thiamine-free medium. The cells were cultured for several generations until the final thiamine-limited logarithmic growth (doubling time of 330 min) was obtained. The effect of β -thienylalanine-imposed phenylalanine starvation was then studied on this thiamine-limited culture (the right-hand panel): Part B indicates protein accumulation; Part D, RNA accumulation; Part F, ppGpp accumulation. β -2-thienylalanine was added at 0 time to cause phenylalanine starvation (Ezekiel, 1965). Protein and RNA accumulation were determined as described under Materials and Methods. The data in Parts A, B, C and D represents the amounts of radioactivity in 50 μ l of the labelled cultures. The open circles represent the starved samples; the closed circles; the unstarved samples. The accumulation of ppGpp (Parts E and F) was determined as described under Materials and Methods. The data is presented as the amount of phosphate incorporated into ppGpp (●) and pppGpp (○) in one ml of the culture of $A_{500} = 1.0$. No detectable amount of pppGpp was accumulated in the culture grown in the absence of thiamine (Part F).

Figure 21. Effect of β -2-thienylalanine-imposed phenylalanine starvation on protein, RNA and ppGpp accumulation in CP 79

CP 79 (rel) was grown on glucose as a carbon source in fully supplemented TMM at 37°C. The log-phase culture was divided into three portions. The first portion was used to study the effect of phenylalanine starvation on RNA accumulation (Part A). The effect on protein accumulation was studied on the second portion (Part B). The effect on "total" ppGpp accumulation was examined in the third portion of the culture (Part C). RNA, protein and ppGpp accumulation were determined as described under Materials and Methods. β -2-Thienylalanine was added at time 0 to a final concentration of 500 μ g/ml. The data in Part A (RNA) and Part B (Protein) represents the amount of radioactivity in 50 μ l of the labelled starved (○) and unstarved (●) cultures. The closed circles in Part C represent ppGpp. The data is presented as the amount of phosphate incorporated into ppGpp in one ml of the culture of $A_{500} = 1.0$. No detectable amount of pppGpp accumulated in the culture.

Figure 22. Effect of β -2-thienylalanine-imposed phenylalanine starvation on protein, RNA and ppGpp accumulation in CP 78 Thi⁺

CP 78 Thi⁺ was cultured at 37°C on glucose as a carbon source in TMM containing all supplements and no thiamine. When the cells had reached the logarithmic phase (doubling time of 70 min), the culture was divided into three portions. The first portion was used to study the effect of phenylalanine starvation on protein accumulation (Part A). The effect on RNA accumulation was studied on the second portion (Part B) and the effect on "total" ppGpp accumulation was examined in the third portion of the culture (Part C). RNA, protein and ppGpp accumulation were assayed as described under Materials and Methods. β -2-Thienylalanine was added at time 0 to a final concentration of 500 μ g/ml. The data in Part A (Protein) and Part B (RNA) represents the amount of radioactivity in 50 μ l of the labelled starved (○) and unstarved (●) cultures. The closed circles in Part C represent ppGpp. The data is presented as the amount of phosphate incorporated into ppGpp in one ml of the culture of $A_{500} = 1.0$. The level of pppGpp in the culture did not increase to measurable amounts.

CP 78 Thi^L. Thus, it is concluded that this phenomenon is not unique to the regulation of growth rates by carbon sources. Classical amino acid starvation (withdrawal of a required amino acid) was attempted to cause a greater degree of starvation but CP 78 Thi^L grown in the absence of thiamine was found to be extremely sensitive to membrane filtration and resuspension (growth did not resume for a long time even in a fully supplemented medium).

(D) Effect of carbon sources on relaxedness in a relaxed mutant. Since rel⁺ strains were found to exhibit "relaxedness" during nutritionally slowed growth, it was interesting to determine the effect of carbon sources on the degree of relaxedness in a genetically relaxed mutant. The effect of valine-imposed isoleucine starvation on a relaxed mutant, CSH 62 (which is auxotrophic only for thiamine) was studied. This strain has a doubling time of approximately 60 min in glucose and 240 min in acetate. Fig. 23 illustrates the effect of isoleucine starvation on protein and RNA accumulation in CSH 62 at the two different growth rates. Again the amount of labelled protein or RNA accumulating in the starved culture is expressed as a percentage of the amount in the unstarved culture at one-quarter doubling time. The effect of isoleucine starvation on protein accumulation was similar in both cultures (Parts A and B). The relative rate of RNA accumulation during isoleucine starvation was significantly greater in

Figure 23. Effect of valine-imposed isoleucine starvation on protein and RNA accumulation in CSH 62 grown on two different carbon sources.

CSH 62 (rel) was cultured on either glucose or acetate as a carbon source. When the cells had reached logarithmic growth, the culture was divided into two portions. The first portion was used to study the effect of isoleucine starvation on protein accumulation. The effect on RNA accumulation was studied on the second portion. RNA and protein accumulation were determined as described under Materials and Methods. Valine was added at 0 time. The open circles represent the starved samples; the closed circles, the unstarved samples. The data is expressed as a percentage of radioactivity in the unstarved culture at one-quarter doubling time after valine addition. Parts A (glucose) and B (acetate) represent protein accumulation; Parts C (glucose) and D (acetate), RNA accumulation.

acetate than in glucose, both exceeding the rate in the unstarved culture (Parts C and D).

The RNA/Protein plot shown in Fig. 24 was calculated from the data in Fig. 23 and permits a quantitative comparison of relaxedness at the two growth rates. A faster rise to a higher value of relaxedness was observed in acetate than in glucose. However, the effect of carbon source in the rel⁻ strain was far less dramatic as compared to that in the rel⁺ strains (cf Fig. 14).

Figure 24. Effect of valine-imposed isoleucine starvation on the RNA/protein ratio in CSH 62 grown on two different carbon sources.

The RNA/protein ratios in the starved (○) and unstarved (●) cultures of CSH (rel⁻) were calculated from the data shown in Fig. 23. Part A shows the ratio in the glucose culture, Part B in the acetate culture. The time zero indicates the time of valine addition.

DISCUSSION

(A) Inhibition of peptide chain initiation triggers ppGpp accumulation

Whenever protein accumulation is inhibited by amino acid starvation or restricted aminoacylation of tRNA, both the cessation of RNA accumulation and the rapid accumulation of ppGpp and pppGpp are observed in stringent (rel⁺) but not relaxed (rel⁻) cells (Cashel, 1969). These two phenomena together constitute the typical stringent response. Inconsistent with this generalization is the report that inhibition of protein accumulation by trimethoprim (which inhibits formylation of methionyl-tRNA) was not accompanied by ppGpp accumulation in rel⁺ cells though RNA accumulation was more severely inhibited in rel⁺ than in rel⁻ cells (Chen et al., 1972). It is conclusively shown here that inhibition of protein and RNA accumulation by trimethoprim in a rel⁺ strain is always accompanied by an immediate and significant accumulation in the level of ppGpp (Fig. 3). Thus, this result is consistent with the general notion that whenever formation of aminoacylated tRNA or N-formylmethionyl-tRNA is restricted in rel⁺ strains, it evokes accumulation of ppGpp as well as inhibition of RNA accumulation in these strains. The discrepancy between the earlier report (Chen et al., 1972) and the present result is not due to differences either in the growth conditions employed (Fig. 4) or the strain used. Closer examination of the

report by Chen et al. (1972) reveals an error in their experimental design, i.e. trimethoprim was added to an amino acid starved culture.

The ppGpp which accumulates on the addition of trimethoprim decays rapidly with a half-life of 20-30 sec when either chloramphenicol or oxytetracycline is added to the culture (Fig. 8). This is accompanied by a resumption of RNA accumulation with a lag of one min (Fig. 9). Some residual protein synthesis occurs in trimethoprim-inhibited cultures, indicating some residual formylation of methionyl-tRNA. Since chloramphenicol further inhibits this residual protein synthesis (Fig. 10), it is conceivable that N-formylmethionyl-tRNA accumulates when chloramphenicol is added, thus relieving the trimethoprim evoked ppGpp accumulation. This idea is supported by the recent observation that chloramphenicol has no inhibitory effect on the in vitro synthesis of ppGpp (Pedersen et al., 1973). However, in vivo studies (De Boer et al., 1971; Gallant et al., 1972) suggest that chloramphenicol may directly inhibit ppGpp synthesis and such a possibility is not ruled out by the data presented.

(B) Presence of messenger RNA is required for ppGpp accumulation

Wong and Nazare (1970) found that in the presence of rifampicin amino acid starvation does not cause ppGpp accumulation. Since rifampicin inhibits RNA chain

initiation (Wehrli et al., 1968), they suggested that ppGpp synthesis depends upon nascent RNA synthesis. This interpretation was disputed by Erlich et al., (1971) who provided a different explanation for the inhibitory effect of rifampicin on ppGpp biosynthesis. They showed that ppGpp can accumulate normally in the presence of rifampicin if an aminoacyl-tRNA synthetase is simultaneously inactivated, even though RNA synthesis is completely inhibited by rifampicin. They concluded, therefore, that the effect of rifampicin on ppGpp synthesis is indirect. Inhibition by rifampicin of RNA chain initiation will lead to a decline in the level of the unstable mRNA due to continued protein synthesis which utilizes mRNA. When mRNA is depleted, protein synthesis ceases, which in turn would lead to the accumulation of charged tRNA due to protein turnover, thus, abolishing the effect of amino acid starvation. This would account for the observation of Wong and Nazar (1970).

The dependence of ppGpp accumulation directly on the presence of mRNA was implied by the observation of Watson and Yamazaki (1972) that, although rifampicin inhibited ppGpp accumulation during isoleucine starvation in an uninfected host, the same host infected with RNA phage R17 did accumulate a significant amount of ppGpp in the presence of rifampicin which inhibits host RNA and protein synthesis. On the basis of this observation, it was suggested that ppGpp accumulation in the rifampicin-

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treated infected cell was a result of the continuing presence of the rifampicin-insensitive phage mRNA which maintains the valine-imposed isoleucine starvation. Confirmation of this idea is provided by the data shown in Fig. 7 (Part A) and Fig. 8 (Part C). Rifampicin induces a slow decay of ppGpp (Fig. 8, Part C) with a half-life of 5 min whereas RNA accumulation is completely inhibited within 1.5 min of the addition of rifampicin (Fig. 7, Part A), confirming the dependence of ppGpp biosynthesis on the presence but not the continued synthesis of mRNA. A similar conclusion was reached by Lund and Kjeldgaard (1972 B) who studied the effect of rifampicin on ppGpp accumulation in *E. coli* infected with phage T7. Very recently, an absolute requirement for mRNA, uncharged tRNA, 70S ribosomes, rel⁺ protein factor, ATP and GTP has been demonstrated for the in vitro synthesis of ppGpp (Haseltine et al., 1972; Pedersen et al., 1973; Haseltine and Block, 1973).

(C) Role of ppGpp?

Although the requirements and mechanism of ppGpp synthesis in vitro (Haseltine et al., 1972; Pedersen et al., 1973) and the conditions governing its synthesis and decay in vivo (Lund and Kjeldgaard, 1972 A) are known, very little is understood about the function of ppGpp in the regulation of rRNA synthesis. All the experimental evidence suggesting that ppGpp is involved in the

regulation of rRNA synthesis has so far been indirect.

A mechanism directly relating ppGpp to the regulation of rRNA synthesis was proposed by Travers et al. (1970).

Its essential feature is that the transcription of rRNA cistrons by the RNA polymerase is specifically stimulated by a positive control element, ψ , which in turn is antagonized by ppGpp. In this model, changes in the intracellular ppGpp concentration would directly modulate RNA synthesis through a direct effect on the positive control element. Several attempts have been made to test this model. Purified E. coli RNA polymerase is capable of a limited transcription of rRNA cistrons of E. coli DNA.

Only 2-10% of the RNA synthesized in vitro can be identified as rRNA by hybridization competition (Haseltine, 1972; Pettijohn, 1972; Murooka and Lazzarini, 1972)

whereas 30-50% of the RNA synthesized during balanced growth corresponds to rRNA species (Lazzarini and

Dahlberg, 1971; Salser et al., 1968; Pettijohn et al., 1970). Several factors that stimulate bulk RNA synthesis have been isolated. However, none of these factors has a specific stimulative effect on rRNA synthesis

(Haseltine, 1972; Murooka and Lazzarini, 1972). RNA

polymerizing systems which contain such factors show only limited inhibition by ppGpp and the relative proportion of rRNA synthesized in these systems is not significantly affected by the addition of ppGpp (Haseltine, 1972;

Murooka and Lazzarini, 1972). The low relative rates of

rRNA synthesis in these in vitro systems are remarkably similar to those observed in amino acid starved rel⁺ E. coli cells (Lazzarini and Dahlberg, 1971) and may correspond to a basal or unstimulated rRNA synthesis that would occur in the absence of a positive regulatory element. Thus, it is conceivable that these in vitro systems are lacking in regulatory elements. Permeabilized E. coli cells exhibit a high relative rate of rRNA synthesis similar to that found in growing cells. Again, ppGpp does not specifically inhibit rRNA synthesis in this system although a limited inhibition of total RNA is observed (Lazzarini and Johnson, 1973). These authors, therefore, have concluded that ppGpp does not directly inhibit the enzymatic elements necessary for rRNA synthesis. Thus, both in vitro and semi in vivo systems have not provided any clear-cut information regarding the specific role of ppGpp in the regulation of rRNA synthesis.

(i) RNA regulation during balanced growth. The most compelling evidence for the involvement of ppGpp in the regulation of rRNA synthesis was provided by Lazzarini et al. (1971) who demonstrated that basal levels of ppGpp vary inversely with both growth rate and the RNA content of the cells. Because of its relative importance, this relationship was reinvestigated over a wider range of growth rates created by utilizing different carbon sources. No apparent correlation was found between the intracellular level of ppGpp and the RNA/DNA ratios

(Fig. 12). Thus, it is concluded that the basal levels of ppGpp have no direct relation to the cellular capacity to accumulate RNA during balanced growth.

Recently, the synthesis of ppGpp in vitro has been shown to require the interaction between an uncharged tRNA and the ribosomes carrying the codon specific to the tRNA (Pedersen et al., 1973; Haseltine and Block, 1973). These nucleotides do not accumulate when the ribosomes are actively engaged in polypeptide synthesis both in vitro and in vivo. These results suggest a possibility that these nucleotides are normal intermediates in protein synthesis. Since there is no correlation between the basal ppGpp levels and the growth rates (Fig. 12) and the rate of protein synthesis is proportional to the growth rate (Maaløe and Kjeldgaard, 1966), it appears there is no direct relationship between the basal ppGpp levels and the rate of protein synthesis. Thus, the basal ppGpp level during balanced growth appears to be incidental; either simply reflecting the turnover of ppGpp in different carbon sources and different strains or differences in the pool sizes of ATP and GTP, the normal precursors of ppGpp (Haseltine et al., 1972). In fact, there is some general correlation between the ATP and GTP levels and the ppGpp levels (Table II). Since the basal ppGpp levels per se do not provide any information regarding their metabolic role, one may then have to determine the turnover rates of these nucleotides in order to reveal their precise intracellular role.

It has been previously assumed that ppGpp accumulates only intracellularly (Cashel and Gallant, 1969; Cashel, 1969; Cashel and Kalbacher, 1970). However, the present investigation demonstrates that significant quantities of ppGpp (as well as trace quantities of triphosphates) accumulate also extracellularly as shown in Table III. The extracellular amounts of ppGpp varied considerably with the carbon source used. Thus, all the ppGpp found in the acidified culture (defined as "total" ppGpp here) does not necessarily represent the intracellular level of ppGpp as it has been previously assumed. Therefore, it now seems obvious that one must determine the intracellular ppGpp level if one wishes to investigate its intracellular role.

An additional interesting observation is that the RNA/DNA ratio versus growth rate (doublings/hour) plot is linear and can be extrapolated to the RNA/DNA ratio of zero when the growth rate reaches zero (Fig. 12, Part C). The linearity of such a plot has been well documented for several kinds of bacteria at various growth rates, whereas the extrapolation to zero growth rate has always yielded a finite and significant value of the RNA/DNA ratio (Rosset et al., 1966; Schaechter et al., 1958; Kjeldgaard and Kurland, 1963). On the other hand, Koch (1970) theoretically predicted that the RNA/DNA ratio would be directly proportional to the growth rate. The RNA/DNA ratio would then become zero when the growth rate is zero. The present results are consistent with this prediction.

The data shown in Fig. 12 provides strong evidence for the "constant efficiency theory" (Maaløe and Kjeldgaard, 1966) extending its applicability to all growth rates. This theory states that protein synthesis per unit stable RNA is constant during balanced growth at any rate.

(ii) Degree of stringency and ppGpp accumulation.

The present investigation also demonstrates that rel⁺ strains of E. coli, when growing slowly on poor carbon sources, exhibit a less stringent control of RNA accumulation (Fig. 14) and a lesser extent of ppGpp accumulation (Fig. 16) during amino acid starvation. An even greater degree of "relaxedness" was observed, in terms of RNA accumulation, when the growth rate was greatly reduced due to the limited intracellular supply of thiamine (Fig. 20). The rate of RNA accumulation in a rel⁻ cell during amino acid starvation is also enhanced when growth rate is slowed by cultivation on a poor carbon source (Fig. 23), though the effect was less evident than in rel⁺ cells presumably due to its already-expressed relaxed phenotype. Since slow growth created by a lower incubation temperature did not cause a substantial increase in "relaxedness" in the rel⁺ cells (Fig. 17), this new phenomenon is attributed to the pattern of biochemical activities imposed by the nutritional environment of the cell rather than the growth rate per se.

It has been previously shown that incubation temperature does not greatly influence cellular mass and composition (Schaechter et al., 1958). The present data indicates that temperature exerts little effect on the amino acid dependent control mechanism involved in RNA accumulation. Inhibition of RNA accumulation in stringent cells during amino acid starvation has been ascribed to the greatly reduced capacity to synthesize ribosomal and transfer RNA species (Lazzarini and Dahlberg, 1971) whereas relaxed mutants continue to synthesize these RNA species accounting for continued RNA accumulation. On the other hand, the "degradative control" model (Koch, 1971; Donini, 1973) states that during amino acid starvation ribosomal and transfer RNA are normally made and then broken down in a rel⁺, but not in a rel⁻ cell. However, this model lacks direct evidence. Various possibilities can be speculated to account for the nutritionally imposed "relaxedness" reported here. For example, a poor nutritional condition might reduce the efficiency of RNA control by the rel gene. However, a significant increase in relaxedness in a relaxed cell under a similar condition may have to be accounted for. On the other hand, the "degradative control" model may ascribe the present phenomenon to a reduced rate of RNA degradation. Amino acid starvation is known to cause a similar increase in instability of proteins in stringent and relaxed cells (Brunschede and Bremer, 1971). It is conceivable that a

nutritionally poor condition additionally enhances protein turnover. The increased degradation would then reduce the effect of amino acid starvation by recharging of transfer RNA in a stringent cell.

The degree of "relaxedness" observed (Fig. 14) can be correlated to the amount of ppGpp accumulated above the basal level (Fig. 16). Although, this again suggests the possible involvement of ppGpp in the regulation of RNA synthesis during amino acid starvation, alternate explanations are also possible. For example, if ppGpp is a normal intermediate in protein synthesis, the intracellular requirement for this nucleotide is expected to be smaller in a slowly growing culture. The extent of ppGpp accumulation at different growth rates may merely reflect the synthetic rate of this compound prior to the onset of amino acid starvation, and therefore may not have direct bearing on the control of RNA accumulation.

(D) Current Status and Future Investigation

In Escherichia coli growing on glucose minimal medium 30-50% of the RNA synthesized at any instant corresponds to rRNA species (Lazzarini and Dahlberg, 1971; Salser et al., 1968; Pettijohn et al., 1970). When protein accumulation is inhibited by the removal of a required amino acid, rRNA synthesis is preferentially restricted and its rate of synthesis falls to 5-10% (Lazzarini and Dahlberg, 1971; Stamato and Pettijohn, 1971). At the

same time the unusual nucleotide ppGpp accumulates intracellularly (Cashel and Gallant, 1969; Cashel, 1969). Much experimental evidence suggests that ppGpp is involved in the regulation of rRNA synthesis. Most important are the demonstrations that (1) ppGpp accumulation precedes the shut-off of rRNA accumulation during amino acid starvation of stringent cells and, on readdition of the deprived amino acid, a substantial reduction in the ppGpp concentration precedes the resumption of rRNA accumulation (Cashel, 1969); (2) relaxed cells fail to accumulate ppGpp and to halt RNA accumulation on amino acid deprivation (Cashel, 1969); (3) chloramphenicol simultaneously interferes with ppGpp synthesis and allows RNA accumulation (Cashel, 1969 and Discussion, Section A); (4) when relaxed cells do accumulate ppGpp, as during step-down transitions, they preferentially restrict their rRNA synthesis (Lazzarini and Winslow, 1970; Lazzarini et al., 1971; Winslow, 1971; Harshman and Yamazaki, 1971).

Although the mode of synthesis of ppGpp is now understood, its proposed function in the regulation of rRNA synthesis is still unknown. Repeated attempts by several investigators to show a direct inhibition of rRNA synthesis by ppGpp in various in vitro and semi in vivo systems have failed. Therefore, the mechanism of the rel gene dependent regulation of RNA synthesis remains unresolved at the present time. It is hoped that the

following lines of future investigation will help to elucidate the mechanism of regulation of rRNA and tRNA synthesis by amino acids in Escherichia coli:

(1) Isolation and characterization of an element regulating stable RNA: The permeabilized cell system of Lazzarini and Johnson (1973) appears to possess the hypothetical positive control element required for rRNA synthesis, since the relative rate of rRNA synthesis is much greater than that observed in the in vitro systems. However, such an element is not antagonized by ppGpp. Therefore, it may be necessary to look for a second regulatory element which interacts with ppGpp to repress rRNA synthesis. However, any future system developed to test the effect of ppGpp must possess all regulatory elements in their functional state.

(2) The mechanisms governing the synthesis and decay of ppGpp in vivo: It has been suggested that amino acid starvation primarily affects the synthesis of ppGpp rather than its degradation (Gallant et al., 1972). However, this proposal must be substantiated by direct evidence.

Although, relaxed strains do not accumulate ppGpp as a response to amino acid starvation, they do accumulate ppGpp in response to other changes in cell conditions such as carbon source downshift or a high salt shock. Whether this synthesis is a consequence of the ribosomal mechanism responding to a different signal or of a

completely different synthetic pathway remains to be established. Another important problem which has not been seriously investigated is the mechanism of breakdown of ppGpp in the cell. This problem is relevant since the intracellular accumulation of ppGpp depends not only on the synthesis but also the breakdown of this nucleotide.

(3) Mode of action of the rel gene product: Although, the mode of action of the rel gene product in the synthesis of ppGpp is being investigated, one must consider the possibility that the rel gene product exerts its regulatory role in the synthesis of rRNA in an entirely different manner, for example, a coupling between some step in translation and the transcription of rRNA may be governed by the rel gene product.

(4) Involvement of other genetic loci: Recent communications from other laboratories indicate that genetic loci other than the rel gene might also be involved in the regulation of rRNA synthesis. Genetic studies will doubtless provide the information required for the identification of these loci, their gene products and the possible role of these elements in the overall process of regulation of ribosomal RNA synthesis.

(5) Turnover of ppGpp: The determination of the turnover rate of ppGpp during balanced growth might prove useful to an understanding of its normal metabolic role.

(6) Rate of rRNA synthesis and intracellular ppGpp levels: RNA accumulation is the net result of its synthesis and degradation. It is conceivable that the rate of RNA degradation increases with decreasing growth rate. Although no apparent correlation was observed between the amount of RNA accumulated per unit DNA and the basal intracellular level of ppGpp, the possibility still exists that the rate of rRNA synthesis is modulated by the intracellular ppGpp levels. This possibility should be examined.

LITERATURE CITED

- Alfoldi, L., Stent, G.S., and Clowes, R.C. (1962) The chromosomal site of the RNA control (RC) locus in E. coli. J. Mol. Biol. 5, 348-355.
- Alfoldi, L., Stent, G.S., Hoogs, M., and Hill, R. (1963) Physiological effects of the PNA control (RC) gene in E. coli. Z. Vererbungslehre 94, 285-302.
- Alfoldi, L., and Kerekes, E. (1964) Neutralization of the amino acid sensitivity of RC^{rel} Escherichia coli. Biochim. Biophys. Acta 91, 155-157.
- Aronson, A.I., and Spiegelman, S. (1961) Protein and ribonucleic acid synthesis in a chloramphenicol-inhibited system. Biochim. Biophys. Acta 53, 70-84.
- Bollum, F.J. (1968) Filter paper disk techniques for assaying radioactive macromolecules in Methods in Enzymology (Grossman, L., and Moldave, K., eds.), Vol. XII, Part B, pp. 169-173, Academic Press, New York.
- Borek, E., Rockenbach, J., and Ryan, A. (1956) Studies on a mutant of Escherichia coli with unbalanced ribonucleic acid synthesis. J. Bacteriol. 71, 318-323.
- Brown, A.H. (1946) Determination of pentose in the presence of large amounts of glucose. Arch. Biochem. Biophys. 11, 269-278.
- Brunschede, H., and Bremer, H. (1971) Synthesis and breakdown of proteins in Escherichia coli during amino-acid starvation. J. Mol. Biol. 57, 35-57.
- Brutlag, D., Schekman, R. and Kornberg, A. (1971) A possible role for RNA polymerase in the initiation of M 13 DNA synthesis. Proc. Nat. Acad. Sci. U.S.A. 68, 2826-2829.
- Burchall, J.J., and Hitchings, G.H. (1966) Inhibitor binding analysis of dihydrofolate reductases from various species. J. Mol. Pharmacol. 1, 126-136.
- Burton, K. (1956) A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62, 315-

- Cashel, M., and Gallant, J. (1968) Control of RNA synthesis in Escherichia coli. I. Amino acid dependence of the synthesis of the substrates of RNA polymerase. J. Mol. Biol. 34, 317-330.
- Cashel, M. and Gallant, J. (1969) Two compounds implicated in the function of the RC gene of Escherichia coli. Nature 221, 838-841.
- Cashel, M. (1969) The control of ribonucleic acid synthesis in Escherichia coli IV. Relevance of unusual phosphorylated compounds from amino acid-starved stringent strains. J. Biol. Chem. 244, 3133-3141.
- Cashel, M., and Kalbacher, B. (1970) The control of ribonucleic acid synthesis in Escherichia coli. V. Characterization of a nucleotide associated with the stringent response. J. Biol. Chem. 245, 2309-2318.
- Chen, J.H., Weissman, S.M. and Lengyel, P. (1972) RC⁺ gene-dependent inhibition of RNA synthesis without ppGpp accumulation. Biochem. Biophys. Res. Commun. 46, 785-789.
- DeBoer, H.A., Raué, H.A., Ab, G., and Gruber, M. (1971) Role of the ribosome in stringent control of bacterial RNA synthesis. Biochim. Biophys. Acta 246, 157-160.
- Donini, P. (1972) Turnover of ribosomal RNA during the stringent response in Escherichia coli. J. Mol. Biol. 72, 553-569.
- Edlin, G., and Neuhard, J. (1967) Regulation of nucleoside triphosphate pools in Escherichia coli. J. Mol. Biol. 24, 225-230.
- Edlin, G., and Broda, P. (1968) Physiology and genetics of the "Ribonucleic acid control" locus in Escherichia coli. Bacteriol. Revs. 32, 206-226.
- Edlin, G., Stent, G.S., Baker, R.F., and Yanofsky, C. (1968) Synthesis of a specific messenger RNA during amino acid starvation of Escherichia coli. J. Mol. Biol. 37, 257-268.
- Edlin, G. and Donini, P. (1971) Synthesis of guanosine 5'-diphosphate, 2'-(or 3'-) diphosphate and related nucleotides in a variety of physiological conditions J. Biol. Chem. 246, 4371-4373.

- Eidlic, L., and Neidhardt, F.C. (1965) Protein and nucleic acid synthesis in two mutants of Escherichia coli with temperature-sensitive aminoacyl ribonucleic acid synthetases. J. Bacteriol. 89, 706-711.
- Erlich, H., Laffler, T., and Gallant, J. (1971) ppGpp formation in Escherichia coli treated with rifampicin. J. Biol. Chem. 246, 6121-6125.
- Ezekiel, D.H. (1965) False-feedback inhibition of aromatic amino acid biosynthesis by B-2-thienylalanine. Biochim. Biophys. Acta 95, 54-62.
- Ezekiel, D.H., and Elkins, B.N. (1968) The stimulation of ribonucleic acid synthesis by ribosome inhibitors in amino acid-starved Escherichia coli. Biochim. Biophys. Acta 166, 466-474.
- Fangman, W.L., and Neidhardt, F.C. (1964) Protein and ribonucleic acid synthesis in a mutant of Escherichia coli with an altered aminoacyl ribonucleic acid synthetase. J. Biol. Chem. 239, 1844-1847.
- Fiil, N. (1969) A functional analysis of the rel gene in Escherichia coli. J. Mol. Biol. 45, 195-203.
- Fiil, N., and Friesen, J.D. (1968) Isolation of "relaxed" mutants of Escherichia coli. J. Bacteriol., 95 729-731.
- Forchhammer, J., and Kjeldgaard, N.O. (1968) Regulation of messenger RNA synthesis in Escherichia coli. J. Mol. Biol. 37, 245-255.
- Friesen, J.D. (1966) Control of messenger RNA synthesis and decay in Escherichia coli. J. Mol. Biol. 20, 559-573.
- Gale, E.F., and Folkes, J.P. (1953) The assimilation of amino acids by bacteria. Biochem. J. 53, 493-498.
- Gallant, J., and Cashel, M. (1967) On the mechanism of amino acid control of ribonucleic acid biosynthesis. J. Mol. Biol. 25, 545-553.
- Gallant, J., Irr, J. and Cashel, M. (1971) The mechanism of amino acid control of guanylate and adenylate biosynthesis. J. Biol. Chem. 246, 5812-5816.
- Gallant, J., Margason, G., and Finch, B. (1972) On the turnover of ppGpp in Escherichia coli. J. Biol. Chem. 247, 6055-6058.

- Gallant, J., and Margason, G. (1972) Amino acid control of messenger ribonucleic acid synthesis in Bacillus subtilis. J. Biol. Chem. 247, 2289-2294.
- Golden, N.G., and Powell, G.L. (1972) Stringent and relaxed control of phospholipid metabolism in Escherichia coli. J. Biol. Chem. 247, 6651-6658.
- Gros, F., and Gros, F. (1956) Role des aminoacides dans la synthèse des acides nucléiques chez Escherichia coli. Biochim. Biophys. Acta 22, 200-201.
- Harshman, R.B., and Yamazaki, H. (1971) Formation of ppGpp in a relaxed and stringent strain of Escherichia coli during diauxie lag. Biochemistry 10, 3980-3982.
- Harshman, R.B., and Yamazaki, H. (1972) MS I accumulation induced by sodium chloride. Biochemistry 11, 615-618.
- Haseltine, W.A. (1972) In vitro transcription of Escherichia coli ribosomal RNA genes. Nature 235, 329-333.
- Haseltine, W.A., Block, R., Gilbert, W., and Weber, K. (1972) MS I and MS II are made on the ribosomes in an idling reaction in protein synthesis. Nature 238, 381-385.
- Haseltine, W.A., and Block, R. (1973) Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific uncharged transfer ribonucleic acid in the acceptor site of ribosomes. Proc. Nat. Acad. Sci. U.S.A. 70, 1564-1568.
- Hochstadt-Ozer, J., and Cashel, M. (1972) The regulation of purine utilization in bacteria. V. Inhibition of purine phosphoribosyltransferase activities and purine uptake in isolated membrane vesicles by guanosine tetraphosphate. J. Biol. Chem. 247, 7067-7072.
- Julien, J., Rosset, R., and Monier, R. (1967) Métabolisme des acides ribonucléiques chez Escherichia coli carencé en phosphate. Bull. Soc. Chim. biol. 49, 131-145.
- Koch, A.L. (1970) Overall controls on the biosynthesis of ribosomes in growing bacteria. J. Theor. Biol. 28, 203-231.

- Koch, A.L. (1971) Evaluation of the rates of biological processes from tracer kinetic data. III. The net synthesis lemma and exchangeable pools. *J. Theor. Biol.* 32, 429-450.
- Kjeldgaard, N.O., and Kurland, C.G. (1963) The distribution of soluble and ribosomal RNA as a function of growth rate. *J. Mol. Biol.* 6, 341-348.
- Kurland, C.G., and Maaløe, O. (1962) Regulation of ribosomal and transfer RNA synthesis. *J. Mol. Biol.* 4, 193-210.
- Lark, K.G. (1972) Evidence for the direct involvement of RNA in the initiation of DNA replication in Escherichia coli 15 T⁻. *J. Mol. Biol.* 64, 47-60.
- Lavallé, R., and DeHauwer, G. (1968) Messenger RNA synthesis during amino acid starvation in Escherichia coli. *J. Mol. Biol.* 37, 269-288.
- Lazzarini, R.A., Nakata, K., and Winslow, R.M. (1969) Coordinate control of ribonucleic acid synthesis during uracil deprivation. *J. Biol. Chem.* 244, 3092-3100.
- Lazzarini, R.A. and Winslow, R.M. (1970) The regulation of RNA synthesis during growth rate transitions and amino acid deprivation in E. coli. Cold Spring Harbor Symp. Quant. Biol. 35, 383-390.
- Lazzarini, R.A., Cashel, M., and Gallant, J. (1971) On the regulation of guanosine tetraphosphate levels in stringent and relaxed strains of Escherichia coli. *J. Biol. Chem.* 246, 4381-4385.
- Lazzarini, R.A. and Dahlberg, A.E. (1971) The control of ribonucleic acid synthesis during amino acid deprivation in Escherichia coli. *J. Biol. Chem.* 246, 420-429.
- Lazzarini, R.A. and Johnson, L.D. (1973) Regulation of ribosomal RNA synthesis in cold-shocked E. coli. *Nature New Biology* 243, 17-20.
- Leavitt, R.I., and Umbarger, H.E. (1962) Isoleucine and valine metabolism in Escherichia coli. XI. Valine inhibition of the growth of Escherichia coli strain K-12. *J. Bacteriol.* 83, 624-630.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

- Lund, E. and Kjeldgaard, N.O. (1972A) Metabolism of guanosine tetraphosphate in Escherichia coli Europ. J. Biochem. 28, 316-326.
- Lund, E., and Kjeldgaard, N.O. (1972B) Protein synthesis and formation of guanosine tetraphosphate. FEBS Letters 26, 306-310.
- Maaløe, O., and Kjeldgaard, N.O. (1966) Control of macromolecular synthesis: A study of DNA, RNA, and protein synthesis in bacteria. W.A. Benjamin, Inc. New York.
- Mandelstam, J., and Halvorson, H. (1960) Turnover of protein and nucleic acid in soluble and ribosome fractions of non-growing Escherichia coli. Biochim. Biophys. Acta 40, 43-49.
- Mans, R.J., and Novelli, G.D. (1961) Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch. Biochem. Biophys. 94, 48-53.
- Martin, R.G. (1968) Polarity in relaxed strains of Salmonella typhimurium. J. Mol. Biol. 31, 127-134.
- Mauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo, A. and Tocchini-Valentini, G.P. (1969) Rifampicin sensitivity of the components of DNA-dependent RNA polymerase. Nature 222, 533-537.
- Merlie, J.P., and Pizer, L.I. (1973) Regulation of phospholipid synthesis in Escherichia coli by guanosine tetraphosphate. J. Bacteriol. 116, 355-366.
- Midgley, J.E.M. (1972) The formation of MS nucleotides in Escherichia coli inhibited by trimethoprim. Biochem J. 129, 39P.
- Morris, D.W., and DeMoss, J.A. (1966) Polysome transitions and the regulation of ribonucleic acid synthesis in Escherichia coli. Proc. Nat. Acad. Sci. U.S.A. 56, 262-268.
- Morris, D.W., and Kjeldgaard, N.O. (1968) Evidence for the non-coordinate regulation of ribonucleic acid synthesis in stringent strains of Escherichia coli. J. Mol. Biol. 31, 145-148.
- Murooka, Y., and Lazzarini, R.A. (1972) Stimulation of RNA synthesis by two protein factors in extracts of Escherichia coli. Proc. Nat. Acad. Sci. U.S.A. 69, 2336-2340.

- Neidhardt, F.C. (1966) Roles of amino acid activating enzymes in cellular physiology. *Bacteriol. Revs.* 32, 206-222.
- Nierlich, D.P. (1968) Amino acid control over RNA synthesis: a re-evaluation. *Proc. Nat. Acad. Sci. U.S.A.* 60, 1345-1352.
- Pardee, A.B., and Prestidge, L.S. (1956) The dependence of nucleic acid synthesis on the presence of amino acids in *Escherichia coli*. *J. Bacteriol.* 71, 677-683.
- Pedersen, F.S., Lund, E. and Kjeldgaard, N.O. (1973) Codon specific, tRNA dependent *in vitro* synthesis of ppGpp and pppGpp. *Nature New Biology* 243, 13-15.
- Pestka, S. (1971) Inhibitors of ribosome function *Ann. Rev. Microbiol.*, 25, 487-562.
- Pettijohn, D.E., Clarkson, K., Kossman, C.R., and Stonington, O.G. (1970) Synthesis of ribosomal RNA on a protein-DNA complex isolated from bacteria: A comparison of ribosomal RNA synthesis *in vitro* and *in vivo*. *J. Mol. Biol.* 52, 281-300.
- Pettijohn, D.E. (1972) Ordered and preferential initiation of ribosomal RNA synthesis *in vitro*. *Nature New Biology* 235, 204-206.
- Primakoff, P., and Berg, P. (1970) Stringent control of transcription of phage $\phi 80$ psu₃. *Cold Spring Harbor Symp. Quant. Biol.* 35, 391-396.
- Que, L., Jr., Willie, G.R., Cashel, M., Bodley, J.W., and Gray, G.R. (1973) Guanosine 5'-diphosphate, 3'-diphosphate: assignment of structure by ¹³C nuclear magnetic resonance spectroscopy. *Proc. Nat. Acad. Sci. U.S.A.* 70, 2563-2566.
- Raina, A., and Cohen, S.S. (1966) Polyamines and RNA synthesis in a polyauxotrophic strain of *Escherichia coli*. *Proc. Nat. Acad. Sci. U.S.A.* 55, 1587-1593.
- Raina, A., Jansen, M., and Cohen, S.S. (1967) Polyamines and the accumulation of ribonucleic acid in some polyauxotrophic strains of *Escherichia coli*. *J. Bacteriol.* 94, 1684-1696.
- Roodyn, D., and Mandel, G. (1960) A simple membrane fractionation method for determining the distribution of radioactivity in chemical fractions of *Bacillus cereus*. *Biochim. Biophys. Acta* 41, 80-88.
- Rosset, R., Julien, J., and Monier, R. (1966) Ribonucleic acid composition of bacteria as a function of growth rate. *J. Mol. Biol.* 18, 308-320.

- Ryan, A.M., and Borek, E. (1971) The relaxed control phenomenon. Prog. Nucleic Acid Res. Molec. Biol. (Davidson, J.N., and Cohn, W.E., eds.), Vol. XI, pp. 193-228, Academic Press, New York.
- Sands, M.K., and Roberts, R.B. (1952) The effects of a tryptophan-histidine deficiency in a mutant of Escherichia coli. J. Bacteriol. 63, 505-511.
- Salser, W., Janin, J., and Levinthal, C. (1968) Measurement of the unstable RNA in exponentially growing cultures of Bacillus subtilis and Escherichia coli. J. Mol. Biol. 31, 237-266.
- Sarkar, S., and Moldave, K. (1968) Characterization of the ribonucleic acid synthesized during amino acid-deprivation of a stringent auxotroph of Escherichia coli. J. Mol. Biol. 33, 213-224.
- Schaechter, M., Maaløe, O., and Kjeldgaard, N.O. (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of Salmonella typhimurium. J. Gen. Microbiol. 19, 592-606.
- Schneider, W.C. (1945) Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161, 293-303.
- Shih, An-Ya, Eisenstadt, J., and Lengyel, P. (1966) On the relation between ribonucleic acid synthesis and peptide chain initiation in E. coli. Proc. Nat. Acad. Sci. U.S.A. 56, 1599-1605.
- Sköld, O. (1970) Effect of the "ribonucleic acid control" locus in Escherichia coli on T4 bacteriophage-specific ribonucleic acid synthesis. J. Virol. 5, 718-725
- Sokawa, Y., Nakao, E., and Kaziro, Y. (1968) On the nature of the control by RC gene in Escherichia coli: amino acid-dependent control of lipid synthesis. Biochem. Biophys. Res. Commun. 33, 108-112.
- Sokawa, Y., and Kaziro, Y. (1969) Amino acid dependent control of the transport of alpha-methyl glucoside in Escherichia coli. Biochem. Biophys. Res. Commun. 34, 99-103.
- Stamato, T.D., and Pettijohn, D.E. (1971) Regulation of ribosomal RNA synthesis in stringent bacteria. Nature New Biology 234, 99-102.

- Stent, G.S., and Brenner, S. (1961) A genetic locus for the regulation of ribonucleic acid synthesis. Proc. Nat. Acad. Sci. U.S.A. 47, 2005-2014.
- Stubbs, J.D., and Hall, B.D. (1968) Effects of amino acid starvation upon constitutive tryptophan messenger RNA synthesis. J. Mol. Biol. 37, 303-312.
- Sugino, A., Hirose, S. and Okazaki, R. (1972) RNA-linked nascent DNA fragments in Escherichia coli. Proc. Nat. Acad. Sci. U.S.A. 69, 1863-1867.
- Swanton, M., and Edlin, G. (1972) Isolation and characterization of an RNA relaxed mutant of B. subtilis. Biochem. Biophys. Res. Commun. 46, 583-588.
- Sy, J., and Lipmann, F. (1973) Identification of the synthesis of guanosine tetraphosphate (MS I) as insertion of a pyrophosphoryl group into the 3'-position in guanosine 5'-diphosphate. Proc. Nat. Acad. Sci. U.S.A. 70, 306-309.
- Tissieres, A., Bourgeois, S., and Gros, F. (1963) Inhibition of RNA polymerase by RNA. J. Mol. Biol. 7, 100-103.
- Travers, A., Kamen, R., and Cashel, M. (1970) The in vitro synthesis of ribosomal RNA. Cold Spring Harbor Symp. Quant. Biol. 35, 415-418.
- Watson, R. and Yamazaki, H. (1972) Expression of the rel gene during R 17 phage infection. Biochemistry 11, 611-614.
- Watson, R. and Yamazaki, H. (1973) Alkali hydrolysis of RNA of Escherichia coli deposited on filter paper disks. Anal. Biochem. 51, 312-314.
- Wehrli, W., Knüsel, F., Schmid, K., and Staehelin, M. (1968) Interaction of rifamycin with bacterial RNA polymerase. Proc. Nat. Acad. Sci. U.S.A. 61, 667-673.
- Winslow, R.M. (1971) A consequence of the rel gene during a glucose to lactate downshift in Escherichia coli. The rates of ribonucleic acid synthesis. J. Biol. Chem. 246, 4872-4877.
- Wong, J.T.-F., and Nazar, R.N. (1970) Relationship of the MS nucleotides to the regulation of ribonucleic acid synthesis in Escherichia coli. J. Biol. Chem., 245, 4591-4595.

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