TRIMETHOPRIM-INDUCED ACCUMULATION OF GUANOSINE TETRAPHOSPHATE (ppGpp) IN *Escherichia coli*

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Summary Trimethoprim, which is known to restrict formylation of methionyl-$tRNA_f$, caused (i) equivalent inhibition of protein accumulation in a *rel*$^+$ and *rel*$^-$ strain of *E. coli* K-12, (ii) severe and rapid inhibition of RNA accumulation in the *rel*$^+$ strain but a much less severe and more gradual inhibition in the *rel*$^-$ strain, and (iii) most significantly appreciable accumulation of ppGpp and MS II in the *rel*$^+$ strain but not in the *rel*$^-$ strain. This observation is consistent with the general notion that whenever formation of aminoacylated tRNA or N-formylmethionyl-$tRNA_f$ is restricted in *rel*$^+$ strains, it evokes ppGpp accumulation as well as inhibition of RNA accumulation.

When stringent (*rel*$^+$) *Escherichia coli* cells are deprived of an essential amino acid or are unable to carry out the amino-acylation of tRNA, two seemingly interrelated phenomena become apparent: (i) there is a severe and preferential inhibition of ribosomal and transfer RNA synthesis, and (ii) coincidental with this inhibition is the immediate and rapid accumulation of two unusual nucleotides of guanine, originally referred to as MS I and MS II (1,2). The structure of MS I has been established as guanosine 5'-diphosphate 2'- or 3'-diphosphate (ppGpp) whereas MS II, which is produced in lesser amounts, has not yet been fully characterized (3). These phenomena constitute the typical stringent response (2). Mutants carrying the recessive *rel*$^-$ allele (*rel*$^-$ strains) fail to exhibit the stringent response.

The dependence of RNA accumulation on the availability of N-formylmethionyl-$tRNA_f$ has been examined through the use of trimethoprim, a specific inhibitor of dihydrofolate reductase

(4,5). 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. Recently it has been claimed that the severe inhibition of RNA synthesis caused by trimethoprim in rel$^+$ strains is not accompanied by the accumulation of ppGpp (6). Such a result is, however, inconsistent with the general notion that the stringent response is always characterized by a significant increase over the basal cellular level of ppGpp (2).

In order to reconcile this apparently anomalous behaviour of trimethoprim we have reinvestigated the effect of trimethoprim on accumulation of RNA and ppGpp in rel$^+$ and rel$^-$ strains of Escherichia coli K-12. This paper presents conclusive evidence to show that in all rel$^+$ strains examined inhibition of RNA accumulation by trimethoprim is always accompanied by an immediate and significant accumulation in the level of ppGpp.

Materials and Methods:

The following K-12 strains were used: CP 78 (rel$^+$)and CP 79 (rel$^-$) (7); NF 161 (rel$^+$) (8); prototrophic strain (rel$^+$) (National Research Council of Canada Culture Collection No. 2012).

Two growth media were used: (i) Tris-maleate minimal medium (TMM) which contains (grams/liter), tris (hydroxymethyl) aminomethane, 6.05; maleic acid, 5.8; NaCl, 2.5; KCl, 2.0; NH_4Cl, 1.0; $MgCl_2.6H_2O$, 1.0; Na_2HPO_4, 0.142; Na_2SO_4, 0.142 (final pH adjusted to 7.3) and (ii) Tris-buffered minimal medium A (6). Both media were supplemented with glucose (0.4 %), 100 µg/ml of each of the 19 common acids and 10 µg/ml of tyrosine, 50 µg/ml each of thymine, uracil, adenine and guanine, and when CP 78 and CP 79 were used, thiamine-HCl was also present at 10 µg/ml. TMM contains 10^{-3}M phosphate and medium A, 5 X 10^{-3}M.

Bacteria were grown at 37°C and growth was followed by measuring absorbance at 500 nm (A_{500}).

Trimethoprim (2,4-diamino-5(3',4',5'-trimethoxybenzyl) pyrimidine) (5) obtained from Calbiochem and Dr. G.H. Hitchings, Burroughs Wellcome Co. was used at a final concentration of 50 µg/ml.

RNA accumulation was measured by following ^{32}P-orthophosphate incorporation according to the procedure of Roodyn and Mandel (9) as modified by Watson and Yamazaki (unpublished) and by the orcinol colorimetric assay as described by Lazzarini, Cashel and Gallant (8).

ppGpp and MS II were assayed one-dimensionally as previously described (2,10). ppGpp and MS II were also identified by two-dimensional thin-layer chromatography (3).

Protein accumulation was determined colorimetrically (11) and by following ^{14}C-arginine incorporation.

Results and Discussion:

In order to preferentially inhibit the formylation of methionyl-$tRNA_f$, the effect of trimethoprim was studied in the presence of all common amino acids, purines and pyrimidines (6). Since the efficiency of antibacterial agents is often subject to growth conditions, we examined the effect of trimethoprim on growth and protein accumulation under our growth conditions (Fig. 1). Growth and protein accumulation were severely inhibited in both CP 78 (*rel*$^+$) and CP 79 (*rel*$^-$). Identical degree of inhibition of growth was observable at any given concentration between 25 µg/ml and 100 µg/ml of trimethoprim. Similarly, trimethoprim severely inhibited the incorporation of ^{14}C-arginine in both strains (data not shown). However, trimethoprim exhibited a differential effect on the accumulation of RNA and ppGpp in *rel*$^+$ and *rel*$^-$ strains (Fig. 2). As previously observed (4), RNA

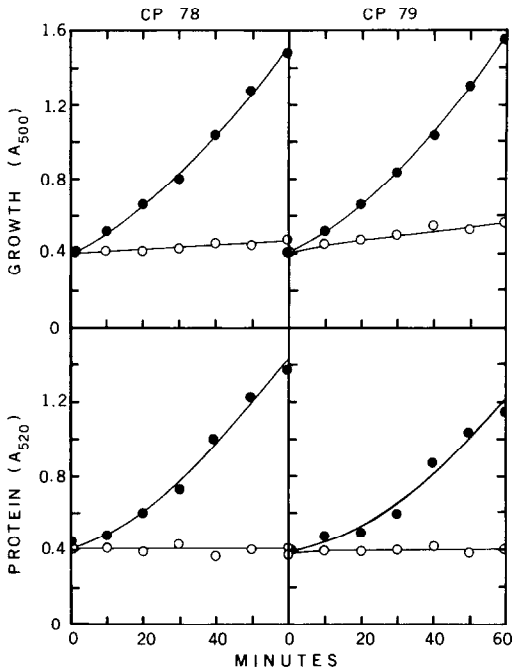

Fig. 1.

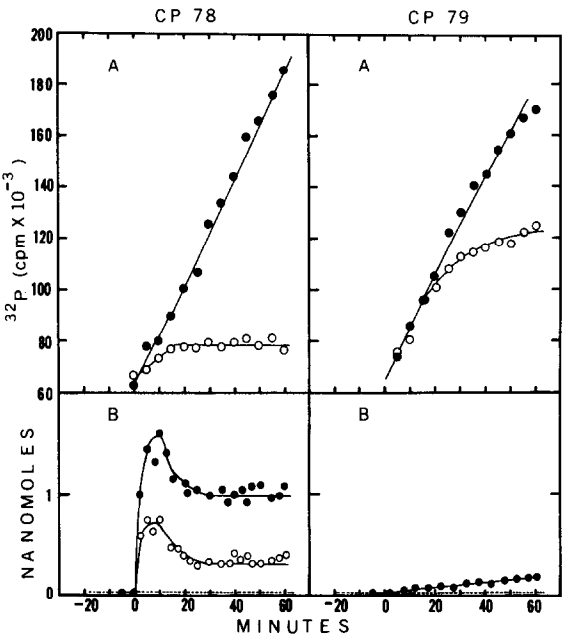

Fig. 2.

Figure 1 Effect of trimethoprim on growth and protein accumulation in CP 78 (rel$^+$) and CP 79 (rel$^-$).

Trimethoprim (50 µg/ml) was added to log-phase cultures of CP 78 and CP 79 in TMM at 0 time. Samples were withdrawn periodically from the treated culture (o) and from the untreated control (●). Growth was determined by measuring absorbance at 500 nm and protein accumulation was assayed colorimetrically at 520 nm (11).

Figure 2 Effect of trimethoprim on accumulation of RNA, ppGpp, and MS II in CP 78 (rel$^+$) and CP 79 (rel$^-$).

CP 78 and CP 79 were grown in TMM to an A_{500}=0.25. The culture was divided into two portions. ^{32}P-orthophosphate (carrier-free) was added at 5 µCi/ml for RNA accumulation (a final specific activity of 5 µCi/µmole) and at 250 µCi/ml (a final specific activity of 250 µCi/µmole) for the assay of ppGpp and MS II. After a 30 minute equilibration, each portion of the culture was further divided into two aliquots. One aliquot was mixed with 1/10 volume of trimethoprim (500 µg/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 ppGpp and MS II (Part B). In Part A, closed circles indicate the control and open circles, the trimethoprim-treated culture. In Part B, closed circles represent ppGpp and open circles, MS II. 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.

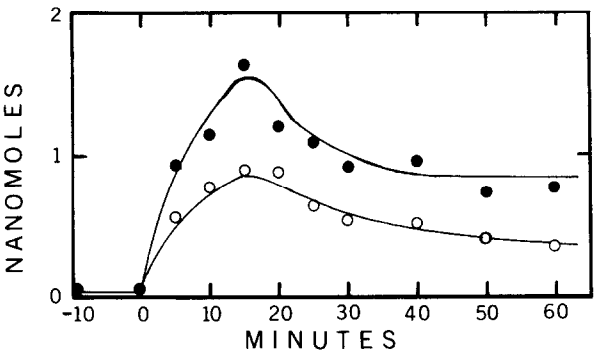

Figure 3 Trimethoprim-induced accumulation of ppGpp and MS II in CP 78 (rel$^+$) grown in medium A.

CP 78 was grown in medium A as described by Chen et al. (6). ^{32}P-orthophosphate (370 μCi/ml) was added to the culture (a final specific activity of 74 μCi/μmole) at A_{500}=0.3. After a 30 minute equilibration, trimethoprim (50 μg/ml) was added at 0 time. The levels of ppGpp and MS II were determined as described in Fig. 2. Closed circles represent ppGpp and open circles, MS II.

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accumulation in the rel$^+$ strain was immediately reduced and ceased completely within 10 minutes of addition of trimethoprim, whereas in the rel$^-$ strain it continued at the same rate as the control for at least 15 minutes and then gradually declined. Similar results were obtained with the orcinol colorimetric assay. Both ppGpp and MS II rapidly accumulated in the rel$^+$strain whereas only a small and gradual increase in the level of ppGpp was observed in the rel$^-$ strain. The level of MS II did not increase to measurable amounts.

Since trimethoprim-induced accumulation of ppGpp was not observed by Chen et al. (6), we examined the possibility that this difference might arise from differences in growth conditions. Therefore, we have used their growth conditions and Fig. 3 clearly shows that significant amounts of ppGpp and MS II did accumulate in CP 78 (rel$^+$) following the addition of trimethoprim. These results were reproducible using trimethoprim obtained from two different sources

(see Materials and Methods). Therefore, it is unlikely that the difference between our result and those of Chen et al. (6) are caused by either the source of trimethoprim or the growth conditions employed. Furthermore, trimethoprim-induced accumulation of ppGpp is not specific to CP 78 (rel$^+$) because two other K-12 rel$^+$ strains (2012 and NF 161) also accumulate ppGpp following addition of trimethoprim.

In summary, the present results are consistent with the general notion that whenever formation of aminoacylated tRNA or N-formylmethionyl-tRNA$_f$ is restricted in rel$^+$ strains, it evokes accumulation of ppGpp as well as inhibition of RNA accumulation in these strains.

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