INHIBITION OF THE TETRAHYDROFOLATE-DEPENDENT BIOSYNTHESIS OF RIBOTHYMIDINE IN tRNAs OF *B. SUBTILIS* AND *M. LYSODEIKTICUS* BY TRIMETHOPRIM

H. H. ARNOLD and H. KERSTEN

Physiologisch-Chemisches Institut, 852 Erlangen, Wasserturmstrasse 5, West Germany

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1. Introduction

In a previous paper we have shown that ribothymidine (m^5U) in tRNA of *B. subtilis* is not labeled when the cells are grown in the presence of methyl-[^{14}C]methionine [1]. However m^5U is present in most tRNAs of *B. subtilis* [2–4]. Recently we have presented evidence that the methylgroups of m^5U are derived from formate or serine and suggested that tetrahydrofolate is involved in the biosynthesis of ribothymidine in tRNA of *B. subtilis* [4]. To establish that tetrahydrofolate in fact serves as coenzyme in this transmethylation reaction was investigated by using trimethoprim which inhibits the dihydrofolate reductase in bacteria thus preventing the synthesis of tetrahydrofolate [5]. As a consequence the syntheses of purines, thymidine, methionine, glycine are blocked. Moreover the formylation of methionyl-$tRNA^{met}_f$ which initiates protein synthesis in procaryotic organisms is inhibited [6,7]. In *E. coli* the requirements induced by trimethoprim can be satisfied by supplying the growth-medium with purines, thymidine and amino acids [8]. Only the deficiency of formylmethionyl-$tRNA^{met}_f$ cannot be released, resulting in an inhibition of protein synthesis [9].

In contrast *B. subtilis* and *M. lysodeikticus* during treatment with trimethoprim continue to accumulate protein and RNA at normal rates for at least two hours when purines, thymidine, methionine and glycine are present in the growth medium. From the continuation of protein synthesis under these conditions it is suggested that the initiation of protein synthesis can be mediated by an unformylated methionyl-$tRNA^{met}_f$

The synthesis of m^5U in tRNAs of *B. subtilis* and *M. lysodeikticus* is almost totally inhibited in the presence of trimethoprim indicating that tetrahydrofolate is the coenzyme in this transmethylation reaction.

2. Materials and methods

2.1. *Chemicals*

[^{14}C]Uridine (50 mCi/mmol), [^{3}H]uridine (6.4 Ci/mmol), [^{14}C]phenylalanine (522 mCi/mmol): Radiochemical Centre Amersham, England. RNase T_2: Sigma. Purine nucleosides, thymidine and L-amino acids: Boehringer Mannheim, West Germany. Trimethoprim was a kind gift from Hoffmann-La Roche AG, Grenzach, West Germany. All other reagents, solvents and cellulose thin-layer plates have been described previously [1,4].

B. subtilis W 23 and *M. lysodeikticus* were cultured in a medium described earlier [1].

2.2. *Methods*

RNA and protein syntheses were measured by the incorporation of [^{3}H]uridine (2 mCi/mmol) and [^{14}C]phenylalanine (2 mCi/mmol) into acid precipitable material. Usually 0.1 ml of dual-labeled cultures were poured into 5 ml ice-cold 10% trichloracetic acid (TCA). The precipitates were collected on Satorius membrane filters, washed twice with 5 ml 5% TCA, dried and counted in a Packard liquid scintillation spectrometer Modell 3003, at a channel-setting appropriate for double labeling experiments. More details are described in the legend to fig.2.

For labeling experiments with [^{14}C]uridine 100 ml cultures were grown in the minimal medium [1]

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containing 2 μmol [^{14}C] uridine (50 mCi/mmol), 10 mg each of guanosine, adenosine, thymidine and 2 mg each of methionine and glycine. When the cells were inhibited by trimethoprim 5 mg of the inhibitor was added simultaneous with the [^{14}C] uridine at the mid-log-phase. The cells were harvested two hr after addition of [^{14}C] uridine respectively of trimethoprim. The isolated tRNA [1] was hydrolyzed to nucleotides by RNase T_2 at 37°C for 4 hr according to the method of Nishimura et al. [10] or to bases by formic acid at 180°C for 2 hr according to the method of Munns et al. [11]. Autoradiography and quantitative analysis of labeled compounds: see legend to fig.1 and [1].

3. Results and discussion

The effect of trimethoprim on the synthesis of ribothymidine in tRNAs of *B. subtilis* and *M. lysodeikticus* was investigated. Trimethoprim was used at concentrations which totally inhibit the growth of the microorganisms in unsupplemented media. Cultures of *B. subtilis* or *M. lysodeikticus* which were first grown in supplemented media were split into two parts. To one portion trimethoprim and [^{14}C] uridine were added, the other portion received [^{14}C] uridine alone. After two hr of further incubation the cells were harvested, the tRNA isolated and hydrolyzed to nucleotides or bases by T_2 RNase or by formic acid. The components of the hydrolysates were separated on thin layer plates and identified after autoradiography. Mainly uracil and cytosine are labeled (fig.1). Hydrolysates of tRNA from trimethoprim treated cells contain only traces of radioactivity in the position of m^{5}U (fig.1A) whereas in tRNA hydrolysates from untreated cells a radioactive spot was always found in the position of m^{5}U (fig.1B). The ^{14}C-incorporation into uridylic acid or uracil, respectively into ribothymidylic acid or 5-methyluracil was determined and the ratios of labeled thymine to uracil were calculated (table 1). The ratios were found to be decreased by about 90% upon analyses of tRNAs from trimethoprim treated organisms. Thus the methylation of uridine to ribothymidine is drastically inhibited by trimethoprim. These results confirm our previous proposal [4] that the biosynthesis of ribothymidine for *B. subtilis* and

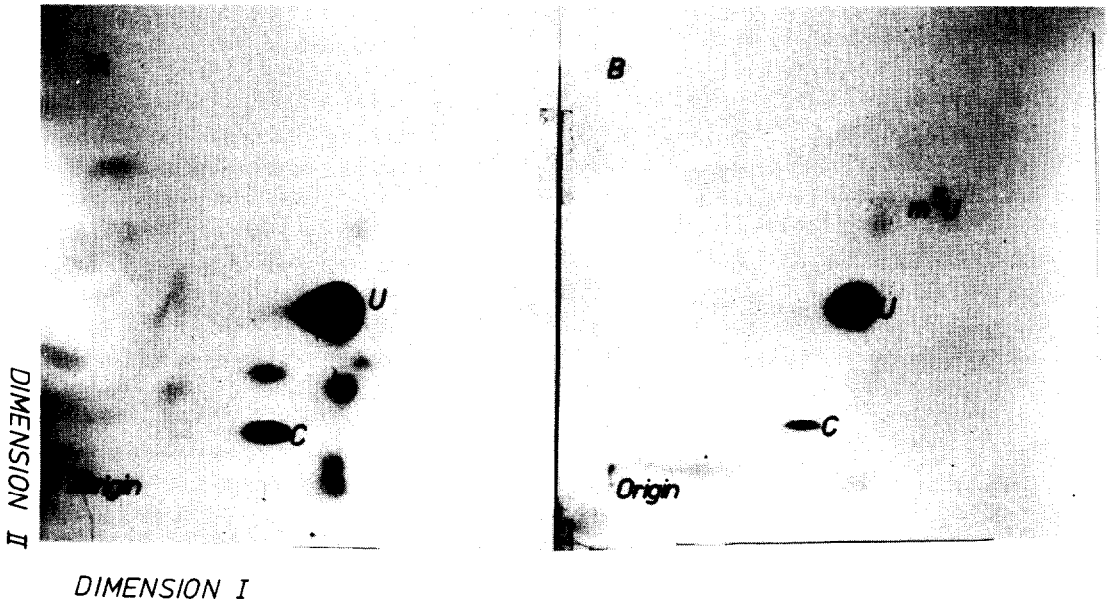

Fig.1. Autoradiographic maps of two dimensional thin-layer chromatographies of tRNA digests of *B. subtilis*, labeled with [^{14}C]-uridine in the presence, (A) and the absence of trimethoprim, (B). tRNAs were hydrolyzed by 88% formic acid at 180°C for 120 min in sealed glass tubes or by RNase T_2 at 37°C for 4 hr. For chromatography the following solvents were used: I. methanol/HCl/water 65:17:18, II. *n*-butanol/acetic acid/water 4:1:1. For quantitation see table 1. Further details see Materials and methods.

Table 1
[^{14}C] Uridine incorporation into thymine residues of tRNAs in *B. subtilis* and *M. lysodeikticus* in the presence of trimethoprim

	B. subtilis			*M. lysodeikticus*		
	Thymine cpm	Uracil cpm	Ratio T/U	Thymine cpm	Uracil cpm	Ratio T/U
Control	320	6780	1: 21	150	5820	1: 39
Trimethoprim added* 50 μg/ml	90	14 920	1:165	160	63 200	1:395

* To ensure the detection of trace amounts of thymine, formed in tRNAs of trimethoprim inhibited cells, for *B. subtilis* a two-fold and for *M. lysodeikticus* a ten-fold amount of cpm was applied to the TLC-plates. Experimental details see Materials and methods.

M. lysodeikticus involves a tetrahydrofolate derivative as coenzyme. The very low radioactivity found in ribothymidine of tRNAs from trimethoprim inhibited cells (10% of the controls) may be caused by a residual synthesis of ribothymidine, shortly after the simultaneous additions of [^{14}C] uridine and trimethoprim. The results presented here cannot exclude the possibility that in *B. subtilis* and *M. lysodeikticus*

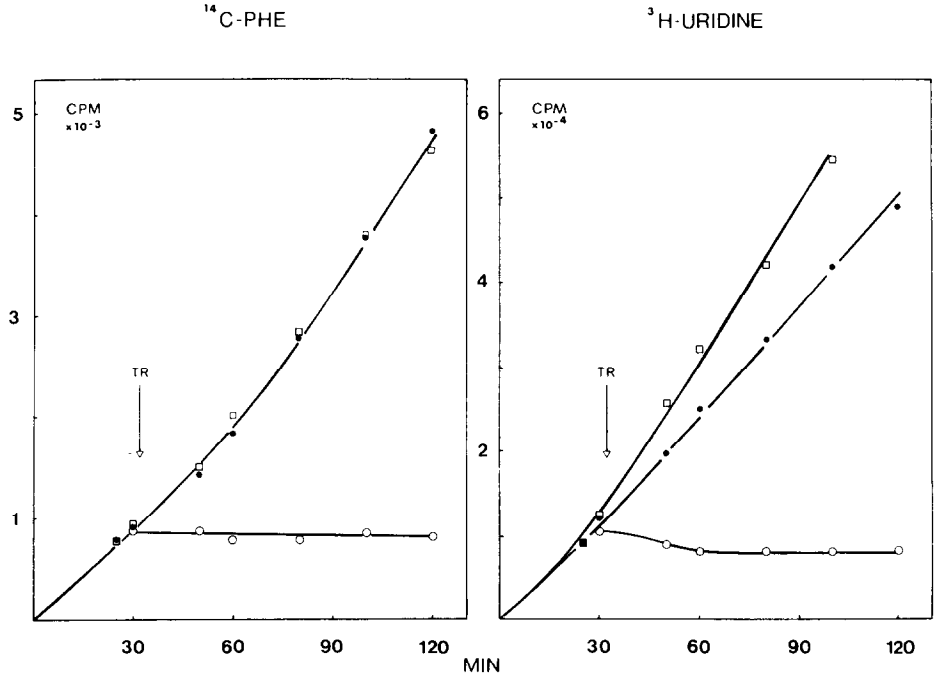

Fig.2. Effect of trimethoprim (50 μg/ml) on protein and RNA syntheses of *B. subtilis* W 23 in minimal medium without supplements (○——○) and in medium supplied with guanosine, adenosine, thymidine (100 μg/ml each), methionine and glycine (2 μg/ml each) (□——□). As control cells growing in the supplemented minimal medium were used (●——●). The incorporation on [^{14}C] phenylalanine 0.25 μCi/ml (2 mCi/mmol) and [^{3}H] uridine 2.5 μCi/ml (2 mCi/mmol) was measured as described in Materials and methods. The required compounds were added 10 min before addition of trimethoprim indicated by the arrow.

ribothymidine is synthesized at the level of nucleotides as thymine for DNA. Nevertheless this seems to be unlikely, since all known modifications of tRNA are posttranscriptional events.

The growth of *B. subtilis* or *M. lysodeikticus* is completely inhibited by trimethoprim at concentrations of 5 μg/ml or more. Both organisms, however, are able to grow at essentially the normal rates when the medium contains purines, thymidine, methionine and glycine. *B. subtilis* synthesize RNA and protein at the same rates as normally growing cells when the medium is supplemented by the low molecular weight products of folate one-carbon metabolism (see fig.2). The same result was obtained for *M. lysodeikticus*. The formation of *N*-formylmethionyl-$tRNA_f^{met}$, considered to be essential for initiation of protein synthesis in procaryotes [12,13] is efficiently blocked by trimethoprim [8,9,14,15]. Therefore it is surprising that under the conditions described the synthesis of protein in *B. subtilis* continues at the normal rate for at least two hours. It might be possible that a very low level of residual synthesis of tetrahydrofolate is sufficient to formylate the initiator tRNA as shown for trimethoprim inhibited *E. coli* growing in minimal medium [8]. Furthermore it cannot be excluded that in *B. subtilis* and *M. lysodeikticus* an alternative acylation of methionyl-$tRNA_f^{met}$ occurs.

An interesting exception exists to the general accepted opinion that the formylation of methionyl-$tRNA_f^{met}$ is an essential step in the initiation of protein synthesis in procaryotes. In the folate dependent *Streptococcus faecalis* grown in folate deficient medium but supplemented with the required compounds the synthesis of protein is initiated by an unformylated methionyl-$tRNA_f^{met}$. This specific tRNA does not contain ribothymidine in the TΨCG-loop [16]. The results presented in this paper are strikingly similar to the findings in *Streptococcus faecalis* during folate deficiency. It is therefore possible that in the presence of trimethoprime the two grampositive organisms, *B. subtilis* and *M. lysodeikticus* are able to initiate the synthesis of protein by a ribothymidine deficient unformylated methionyl $tRNA_f^{met}$.

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References

[1] Arnold, H. H. and Kersten, H. (1973) FEBS Lett. 36, 34–38.
[2] Randerath, E., Chia, L.-L. S. Y., Morris, H. P. and Randerath, K. (1974) Biochim. Biophys. Acta 366, 159–167.
[3] Romeo, J. M., Delk, A. S. and Rabinowitz, J. C. (1974) Biochem. Biophys. Res. Comm. 61, 1256–1261.
[4] Arnold, H. H., Schmidt, W. and Kersten, H. (1975) FEBS Lett., in press.
[5] Burchall, I. I. and Hitchings, G. M. (1965) Mol. Pharmacol. 1, 126–136.
[6] Clark, B. F. C. and Marcker, K. A. (1966) J. Mol. Biol. 17, 394–406.
[7] Eisenstadt, J. and Lengyel, P. (1966) Science 154, 524–527.
[8] Harvey, R. J. (1973 J. of Bacteriol. 114, 309–322.
[9] Smith, R. J. and Midgley, J. E. M. (1973) Biochem. J. 136, 225–234.
[10] Nishimura, S., Harada, F., Narushima, U. and Seno, T. (1967) Biochim. Biophys. Acta 142, 133–148.
[11] Munns, T. W., Podratz, K. C. and Katzman, P. A. (1974) Biochemistry 13, 4409–4416.
[12] Marcker, K. A. and Smith, A. E. (1969) Bull. Soc. Chim. Biol. 51, 1453–1458.
[13] Lucas-Lenard, J. and Lipman, F. (1971) Ann. Rev. Biochem. 40, 409–448.
[14] Pine, M. J., Gordon, B. and Sarimo, S. S. (1969) Biochim. Biophys. Acta 179, 439–447.
[15] Samuel, Ch. E., D'Ari, L. and Rabinowitz, J. C. (1970) J. Biol. Chem. 245, 5115–5121.
[16] Delk, A. S. and Rabinowitz, J. C. (1971) Nature 252, 106–109.