Biosynthesis of Ribothymidine in the Transfer RNA of 
*Streptococcus faecalis* and *Bacillus subtilis*

A METHYLATION OF RNA INVOLVING 5,10-METHYLENETETRAHYDROFOLATE*

(Received for publication, July 28, 1976)

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The methyl moiety of ribothymidine in the tRNA of *Streptococcus faecalis*, *Bacillus subtilis*, and probably *Bacillus cereus* is derived from a 1-carbon folate derivative and not S-adenosylmethionine. *Micrococcus luteus* (M. lysodeikticus) tRNA appears to be almost devoid of ribothymidine.

*S. faecalis* tRNA lacking ribothymidine can be methylated in vitro with formation of ribothymidine. 5,10-Methylenetetrahydrofolate has been identified as the 1-carbon donor involved in ribothymidine formation in *S. faecalis* and implicated in the analogous reaction in *B. subtilis*. The reaction requires FADH₂ and/or another reducing agent present in cell extracts for the reduction of the methylene moiety.

As we reported previously (1, 2), ribothymidine of the tRNA of *Streptococcus faecalis* and *Bacillus subtilis* does not derive its methyl moiety from methionine—and consequently not from S-adenosylmethionine. Since ribothymidine of *S. faecalis* tRNA is synthesized only in the presence of folate (1, 3) and formate is used for the biosynthesis of ribothymidine in this tRNA, but not of other methylated nucleic acid residues (1), it was suggested that a 1-carbon folate derivative is involved in this tRNA methylation (1, 2).

These were the first reports of methylation of RNA by a compound other than Ado-Met, which has been assumed to be the only methyl donor involved in this type of RNA modification (4, 5). Two known folate derivatives are recognized as biological methyl donors in other reactions. 5,10-Methylenetetrahydrofolate is the source of the methyl moiety of deoxythymidic acid, the deoxyribonucleotide; 5-methyltetrahydrofolate is the methyl donor used in methionine biosynthesis (6).

Through experiments carried out in vivo, we have examined more extensively the occurrence of folate-mediated methylations of RNA in *S. faecalis*, *B. subtilis*, and other bacteria and, through analyses performed in vitro, identified the folate derivative involved in ribothymidine formation in *S. faecalis* and determined several other characteristics of the reaction.

MATERIALS AND METHODS

Unless stated otherwise, experimental procedures were the same as those described previously (1).

Tetrahydrofolate derivatives were prepared as follows: (d)-l-tetrahydropteroyltriglutamate from the 5,10-methylene derivative isolated from *Clostridium acidi-urici* as previously described (7); (d)-l-tetrahydrofolate by hydrogenation of folic acid (Sigma) over platinum oxide (Matheson, Coleman and Bell) in neutral solution (8) followed by chromatography on DEAE-cellulose (9); (d)-l-5,10-methylenetetrahydrofolate by incubation of tetrahydrofolate (in 0.2 M Tris/Cl, pH 7.5, and 0.5 mM mercaptoethanol) with a 1.5-fold excess of formaldehyde and its identity confirmed by ultraviolet spectroscopy (10); (d)-l-5,10-[¹⁴C]methylenetetrahydrofolate in a similar manner using [¹⁴C]formaldehyde (15 mCi/mmol, Amersham/Searle). (d)-l-5-Methyltetrahydrofolate (Sigma) and (d)-l-5-[¹⁴C]methyltetrahydrofolate (50 mCi/mmol, Amersham/Searle) were purchased as the barium salts and barium removed by precipitation with potassium phosphate (pH 7). (d)-l-[²H₂]Tetrahydrofolate (1.2 Ci/mmol, 1.34 x 10⁶ m in 0.2 mM mercaptoethanol and 0.007 mM formaldehyde) was a gift of Dr. D. V. Santi of the University of California, San Francisco.

For analysis of ribothymidine synthesized in vivo, *Streptococcus faecalis* grown in folate-sufficient medium containing [methyl-¹⁴C]methionine (Amersham/Searle, 50 mCi/mmol) was incubated with lysozyme for 5 to 10 min and disrupted in a French pressure cell at 20,000 psi. After removing cell debris by centrifugation at 13,000 x g, the suspension was centrifuged at 200,000 x g for 3 h. The supernatant fraction was extracted with phenol and tRNA isolated by chromatography on DEAE-cellulose. The pelleted material, after suspension in 1 M NH₄Cl and recentrifugation, was extracted with phenol. The RNA recovered by precipitation with ethanol was assumed to be primarily tRNA. Following digestion with pancreatic,
U$_1$ and T$_2$ RNases, the material was chromatographed on thin layer cellulse plates (1). Brutanolysis of various strains (2) were grown with [methyl$-^{14}$C]methionine as previously described (2); *Bacillus cereus*, ATCC 10876, was grown in a similar medium supplemented with amino acids (except methionine) and vitamins; *Micrococcus luteus*, ATCC 4698 (Frøm, formerly *M. lysodeikticus* (11)) and ATCC 12095, was grown in TrypCibase Soy Broth (BBL). *B. subtilis* W23 was also grown in medium (2) containing 10 μCi of [3$^{14}$C]serine (Amersham/Searle, 50 μCi/ml) and supplemented with vitamins and amino acids (except serine). After phenol extraction, tRNA and rRNA were separated by chromatography on Sephadex G-100 or DEAE-cellulose as described above.

These organisms were also grown in medium containing no labeled compound; tRNA was extracted and analyzed by the $^3$H postlabeling method of Randerath and Randerath (2, 12).

In one experiment, *S. faecalis* was grown in medium (5 ml) containing 0.25% sodium ascorbate, 0.5 mg of thymidine, and 25 μg of (dl)-t$^{16}$H]-threonyltrasferylolate (65 μCi) in the presence or absence of [H]$^3$H]formate (50 μCi, 250 μCi/ml). DNA, as well as RNA, was extracted by the phenol method at pH 8.5 (13). RNA was digested by RNases and the sample was chromatographed (1) and analyzed by fluorography (12). Material remaining at the origin was analyzed by scraping the cellulose from that area, incubating the material extensively with pancreatic DNase and snake venom phosphodiesterase, and rechromatographing the soluble material with authentic thymidine $^5$-monophosphate.

For analysis of ribothymidine formation in vitro, crude extracts of *S. faecalis* grown by suspending cells harvested during exponential growth (2 g wet weight/ml) in a buffer containing 0.01 M sodium bicarbonate, pH 7.5 (Calbiochem), 0.01 M MgCl$_2$, 0.01 M NH$_4$Cl, 0.001 M sodium EDTA, and 0.02 M mercaptoethanol, disrupting the cells in the presence of 2 μg/ml of pancreatic DNase (Worthington, RNase free) in a French pressure cell; and centrifuging the broken cell suspension at 13,000 $\times$ g for 60 min. Supernatant fractions (designated S$^{200}$ supernatant) obtained by centrifuging crude extracts at 200,000 $\times$ g for 3 h, were either passed through a column of DEAE-cellulose (Cl$^-$, 1 ml of packed material/ml of extract) equilibrated with Tris/EDTA buffer (15) at pH 7.5, or dialyzed against 0.5 M NaCl, or fractionated with addition of (NH$_4$)$_2$SO$_4$, and dialyzed versus the cell breakage buffer described above.

$[^3$H]tRNA (UWC$^3$) specifically lacking ribothymidine (1, 3) was prepared from *S. faecalis* grown under folate-free conditions in 400 ml of medium containing 6 μCi of [H]$^3$H]uracil (Amersham/Searle or New England Nuclear, 25 to 28 Ci/mmol) and unlabeled uracil, 10$^{-6}$ M final concentration, by phenol extraction and chromatography on DEAE-cellulose. $[^3$H]tRNA (rTWC$^3$) was prepared in the same way from cells grown in folate-containing medium. In some experiments $[^3$H]tRNA was diluted with unlabeled tRNA of similar ribothymidine content.

Early assays for ribothymidine formation based on release of $^3$H from $[^3$H]tRNA (15) were carried out at 37$^\circ$ for 60 min in a 0.1-ml reaction mixture containing 10 μm sodium bicarbonate (pH 9), 5 μm NH$_4$Cl, 5 μm KC1, 5 μm MgCl$_2$, 0.5 μm EDTA, 25 μm mercaptoethanol, 0.4 μm (dl)-t$^{16}$H]-threonyltrasferylolate, 0.022 AS units of [H]$^3$H]methyltetrahydrofolate, TPNH, and FAD ("initial" conditions). $[^3$H]tRNA (UWC$^3$) specifically lacking ribothymidine (1, 3) was incubated with enzyme fraction or from $[^3$H]tRNA (UWC$^3$) in the absence of enzyme and rRNA, which contains substantial quantities of ribothymidine, did not become labeled when cells are grown in folate-sufficient medium containing [methyl-1$^4$C]methionine because it derives its methyl moiety from a 1-carbon folate derivative and not from methionine (1). However, formation of ribothymidine of rRNA does involve methionine as the methyl donor (Fig. 1b) presumably in the form of Ado-Met (4, 5), as do the biosyntheses of other methylated residues in tRNA (Fig. 1e) and rRNA (Fig. 1b). Similar results were obtained with four strains of *Bacillus subtilis* and one of *Bacillus cereus*. In each of these organisms, ribothymidine appears to be the most abundant methylated residue in rRNA. In tRNA there is a substantial amount of an unidentified methylated residue with chromatographic properties similar, but not identical, to those of Up, which is also present in *Bacillus steatorrhombophilus* tRNA (21). We have not attempted to identify either this or all of the other minor nucleic acid residues in these organisms.

When *B. subtilis* was grown in medium containing [3$^{14}$C]serine, label was incorporated into the methyl moiety of ribothymidine of tRNA under conditions where no label was incorporated into the pyrimidine ring (Fig. 2). Analysis of the radioactivity in the two major purines and methylated purines, which were detected after extensive autoradiography, indicated that label was not incorporated into the methyl moiety of other methylated residues.

We examined two strains of *Micrococcus luteus* and found each to be essentially devoid of ribothymidine in tRNA as illustrated in Fig. 2e. In this chromatography system, ribothymidine derivative is found to the right of the glyceral derivative (2, 12) as indicated in the figure. Quantitation of the radioactivity in this area (12) indicated that the tRNA of strain 4098 and 12098 contains 0.153 and 0.036 μmol of ribothymidine, respectively. However, analyses of an unknown strain of *M. lysodeikticus*, stored at $-20^\circ$ in powder form for more than 20 years, indicate that certain strains of this organism could contain more ribothymidine (in this case, 0.040 to 0.138 mol %) or that contamination of tRNA samples with degraded rRNA, which contains substantial quantities of ribothymidine (29), can increase the value for ribothymidine by a significant amount. After extended autoradiography, analysis of chromatograms of nucleotides obtained from tRNA of *M. luteus* labeled in vivo with [methyl-1$^4$C]methionine revealed radioactive ribothymidine in ribonucleic acid (Fig. 3b). The amount of ribothymidine relative to several other methylated residue determined

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3 Since ribothymidine of tRNA is known to occur only in the sequence GrTYCPu in loop IV (14) as a result of methylation of uridine at that position (5) we will assume that ribothymidine occurs only in this sequence in *Streptococcus faecalis* tRNA.
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Fig. 1 (left and center). Autoradiograph of fractionated 3'-nucleotides obtained by enzymatic hydrolysis of (a) tRNA and (b) rRNA of Streptococcus faecalis grown in the presence of methyl-[14C]methionine. Solvents: (1) isobutyric acid/NH₄OH; (2) isopropyl alcohol/HCl/H₂O (1, 19). Outlines indicated positions of nucleotides located by ultraviolet absorption. Standard letter designations are used for the nucleotides. T, ribothymidine.

Fig. 2 (right) Autoradiograph of 3'-nucleotides obtained from tRNA of Bacteriopia stulils W23 grown in medium containing [3-¹⁴C]serine. Solvents as in Fig. 1. T, ribothymidine.

Fig. 3 (left and center). a, fluorograph of fractionated ³H post-labeled nucleoside derivatives of Micrococcus luteus (ATCC 4698) tRNA. Values, expressed as mol %, determined for some of the minor bases were as follows: ribothymidine (T), 0.015; ψ, 1.30; D, 1.42; mA, 0.61; mG, 0.50; mG, 0.25. Solvents: (1) acetonitrile/ethyl acetate/1-butanol/isopropanol/6 N ammonia; (2) t-amyl alcohol/methyl ethyl ketone/acetonitrile/ethyl acetate/water/formic acid (2, 12). Standard nomenclature is used to identify the bases, B stands for blank and glyc for glycerol (2, 12). b, autoradiograph of fractionated 3'-nucleotides of M. luteus (ATCC 4698) tRNA labeled in vivo with [methyl-¹⁴C]methionine. Values, expressed as percentage total radioactivity recovered, were as follows: ribothymidine (T), 1.5; m'Ac, 93.8; mG, 30.5; mG, 14.9; m'6A, 5.7; m'C, 0.6. Solvents as in Fig. 1.

Fig. 4 (right) Release of ³H from [³H]tRNA(UlyC) as a function of time in the presence and absence of enzyme. Reaction conditions were similar to those described under "Materials and Methods" for "early" assays, except TPNH (6 mM) and FAD (0.05 mM) were also present. The counting efficiency for ³H in the filtrate was 16.8%, with a 93 ± 1% recovery of ³H.

As illustrated in Fig. 4, release of ³H was shown to be dependent upon incubation of the substrate with an enzyme fraction. The small amount of ³H release at zero time and after a 90-min incubation in the absence of enzyme represents about 0.2% of the total ³H in the tRNA, which was released by nonenzymatic base hydrolysis (23). In this experiment, after a 90-min incubation with enzyme, ³H release equivalent to a 90% conversion of tRNA(UlyC) to tRNA(r'TAC) was obtained.

³H release from [³H]tRNA(r'TAC) was also examined. As illustrated in Table I, after a 60-min incubation there was no enzymatically catalyzed release of ³H from [³H]tRNA(r'TAC), in contrast to that observed from [³H]tRNA(UlyC). In the presence of added tetrahydropteroylglutamate, extracts prepared from folate-free, as well as folate-containing, cells by this procedure (legend, Fig. 3b) agreed reasonably well with that determined by the postlabeling method (legend, Fig. 3c).

In attempting to identify the folate derivative involved in ribothymidine formation in the tRNA of the organisms mentioned above, we developed an assay based on enzymatic release of ³H from [³H]tRNA(UlyC) by extracts of S. faecalis. Under standard conditions, adsorption of the [³H]tRNA to charcoal was greater than 99.8% complete and reproducible within 0.01%. This level of efficiency and reproducibility of adsorption is important since the assay involves detection of a small amount of released ³H relative to [³H]tRNA added to the reaction. Assuming that all uracil and cytosine residues are labeled equally and that only 1 in 40 labeled residues is substrate for ribothymidine modification (14), complete ribothymidine formation would account for release of only 2.5% of the ³H from the substrate. The counting efficiency for ³H in the filtrate was 16.8%, with a 93 ± 1% recovery of ³H.
can catalyze release of $^3$H from [PHl]tRNA(UWC), indicating
that the enzyme is present in folate-free cells and that added
tetrahydrofolate can apparently mediate the transfer of a 1-
carbon unit from some component in the extract to
tRNA(UWC). The apparent reduced activity of extracts from
folate-free cells can probably be attributed to unlabeled
tRNA(UWC) in the extract which competes with [PHl-
tRNA(UWC)] for enzyme.

It was necessary to add tetrahydrofolate to all extracts to
obtain maximum $^3$H release. In the complete absence of folate
derivatives, little release of $^3$H was observed from [PHl-
tRNA(UWC)]. Under no conditions was $^3$H release above the
background level observed with [PHl]tRNA(UWC).

Direct base analysis of unlabeled tRNA(UWC) in extracts
from folate-free cells before and after incubation with tetra-
hydropteroyltriglutamate demonstrated that ribothymidine
was being made under the conditions of the $^3$H release assay
(Fig. 5). No other significant difference in base composition
was detected. The amount of ribothymidine formed in this
reaction was sufficient to equate $^3$H release with addition of a
methyl moiety to uridine.

The $^3$H release assay was used to obtain the data shown in
Table II. It is difficult to compare the quantitative aspects of
one experiment with another because the reaction mixture
was modified on the basis of the results of each experiment.
Among major alterations made were the increase in amount
and decrease in specific activity of the [PHl]tRNA substrate and
the use of several different enzyme samples obtained under
slightly varied conditions from a number of cultures of folate-
containing S. faecalis.

It should be noted that in this assay ribothymidine forma-
tion is detectable regardless of the nature of the methyl donor.
Although this compound is not present in extracts in sufficient
quantity for maximum ribothymidine formation, added tetra-
hydrofolate can mediate the transfer of a 1-carbon unit from
some component in the extract to tRNA(UWC) (Table I, Fig. 5,
and below).

The reaction was found to be optimum at about pH 9.
Addition of TPNH and FAD greatly stimulated ribothymidine
formation (Table II, Experiment 1). Removal of ribosomes and
cell debris led to an increase in the specific activity of the
enzyme sample (Experiment 2). After passing this fraction
over a DEAE-cellulose column to remove endogenous tRNA, a
substantial portion of the activity was lost; however, addition
of boiled extract restored the activity (Experiment 3). Since
boiled extract alone was inactive in releasing $^3$H from
[PHl]tRNA(UWC), these data indicate that the chromatog-
graphic procedure had removed a cofactor or some other com-
ponent necessary for the reaction and had not removed or
destroyed the enzyme.

Attempts to identify this component are summarized in
Experiment 4. It could not be replaced by Ado-Met, but addi-
tion of pyridoxal-P led to a marked increase in ribothymidine
formation. However, if boiled extract was added as well, fur-
ther activity was observed. Likewise, addition of serine in-
creased ribothymidine formation, but addition of boiled ex-
tract was necessary for maximum activity. Addition of satu-
rating amounts of TPNH, FAD, pyridoxal-P, and serine were
ineffective in overcoming the stimulatory effect of boiled ex-
tract.

Attempts to replace boiled extract with any one of the
following compounds were not successful: ATP, GTP, Ado-
Met, ppGpp, cAMP, B$_5$-coenzyme, biotin, lipoate, reduced
glutathione, spermidine, or a water extract of ashed boiled
extract. The component(s) is present in extracts from folate-
free, as well as folate-containing, cells. It was lost upon dia-
lysis, but recoverable in the dialysate. It was partially re-
moved or destroyed by passage of boiled extract over BioRad
AG-1 (Cl$^-$) or AG-50 (Na$^+$) at neutral pH, but apparently not
adsorbed to charcoal. It has not yet been possible to identify
the component or to determine if it is essential for activity or
merely has a stimulatory effect.

The function of pyridoxal-P and serine, which had been

![Fig. 5. Fluorograph of $^3$H postla-
beled nucleoside derivatives of tRNA
from extract of folate-free cells (a) be-
fore and (b) after incubation with tet-
rahydropteroyltriglutamate. (See "Ma-
terials and Methods" for reaction con-
ditions and Fig. 3o for analysis meth-
ods.) T, ribothymidine.]
Table II

Requirements for methylation of \([\text{tRNA(UUC)}]\) by Streptococcus faecalis extracts

<table>
<thead>
<tr>
<th>Enzyme fraction and cofactors</th>
<th>Tritium release$^a$</th>
<th>Enzyme fraction and cofactors</th>
<th>Tritium release$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1.</strong> Crude extract with tetrahydropteroyltetrahydrofolate</td>
<td>360</td>
<td><strong>Experiment 5.</strong> DEAE-cellulose pass-through with boiled extract, TPNH, and FAD</td>
<td>614</td>
</tr>
<tr>
<td>+ TPNH</td>
<td>1060</td>
<td>+ Tetrahydrofolate</td>
<td>2928</td>
</tr>
<tr>
<td>+ TPNH and FAD</td>
<td>2009</td>
<td>+ 5,10-Methylenetetrahydrofolate</td>
<td>1419</td>
</tr>
<tr>
<td>Experiment 2.** S200 supernatant with tetrahydropteroyltetrahydrofolate, TPNH, and FAD</td>
<td>3746</td>
<td>+ 5,10-Methylenetetrahydrofolate, pyridoxal-P, serine</td>
<td>4414</td>
</tr>
<tr>
<td>+ Boiled extract</td>
<td>955</td>
<td>Experiment 6.** 50-60% ammonium sulfate precipitate with boiled extract and 5,10-methylenetetrahydrofolate</td>
<td>1220</td>
</tr>
<tr>
<td>As in b except 0.63 mg of protein.</td>
<td>5775</td>
<td>+ FAD</td>
<td>2747</td>
</tr>
<tr>
<td><strong>Experiment 3.</strong> DEAE-cellulose pass-through with tetrahydropteroyltetrahydrofolate, TPNH, and FAD</td>
<td>532</td>
<td>+ FAD and TPNH</td>
<td>2963</td>
</tr>
<tr>
<td>+ Boiled extract</td>
<td>1650</td>
<td>+ FADH$_2$</td>
<td>1220</td>
</tr>
<tr>
<td>As in b except 0.15 mg of protein and 40 µl of boiled S200 supernatant.</td>
<td>2257</td>
<td>Experiment 7.** 50-60% ammonium sulfate precipitate with boiled extract, TPNH, and FAD</td>
<td>1276</td>
</tr>
<tr>
<td>+ Pyridoxal-P</td>
<td>4125</td>
<td>+ 5,10-Methylenetetrahydrofolate</td>
<td>263</td>
</tr>
<tr>
<td>+ Pyridoxal-P and boiled extract</td>
<td>6555</td>
<td>+ 5-Methyltetrahydrofolate</td>
<td>60</td>
</tr>
<tr>
<td>+ Pyridoxal-P and serine</td>
<td>8765</td>
<td>+ 5,10-Methylenetetrahydrofolate and 5-methyltetrahydrofolate</td>
<td>1328</td>
</tr>
<tr>
<td><strong>Experiment 4.</strong> DEAE-cellulose pass-through with tetrahydropteroyltetrahydrofolate, TPNH, and FAD</td>
<td>1650</td>
<td>As under &quot;Materials and Methods,&quot; &quot;final&quot; assay, except 1.8 mM tetrahydrofolate or methylenetetrahydrofolate; 0.4 mg of protein.</td>
<td>6</td>
</tr>
<tr>
<td>+ Boiled extract</td>
<td>1850</td>
<td>+ FAD and 5,10-Methylenetetrahydrofolate</td>
<td>9</td>
</tr>
<tr>
<td>As in f except 0.34 mg of protein.</td>
<td>532</td>
<td>+ FADH$_2$</td>
<td>19</td>
</tr>
<tr>
<td>+ Pyridoxal-P</td>
<td>2257</td>
<td>Experiment 8.** 50-60% ammonium sulfate precipitate with boiled extract, TPNH, and FAD</td>
<td>263</td>
</tr>
<tr>
<td>+ Pyridoxal-P and boiled extract</td>
<td>4125</td>
<td>+ 5-Methyltetrahydrofolate</td>
<td>60</td>
</tr>
<tr>
<td>As in h except 0.68 mg of protein, 1.8 mM methyl or methylene-tetrahydrofolate.</td>
<td>6555</td>
<td>+ 5,10-Methylenetetrahydrofolate and 5-methyltetrahydrofolate</td>
<td>1328</td>
</tr>
<tr>
<td>+ Pyridoxal-P and serine, and boiled extract</td>
<td>8765</td>
<td>As in i except 0.34 mg of protein.</td>
<td>6</td>
</tr>
</tbody>
</table>

* After subtracting background.

* Conditions as under "Materials and Methods," "early" assay, except 6 mM TPNH and 0.05 mM FAD, as indicated; with 1.4 mg of protein.

* As in b except 0.63 mg of protein.

* As in b except 0.15 mg of protein and 40 µl of boiled S200 supernatant.

* As in b except 0.40 mg of protein, 0.5 mM FAD; 0.02 mM Ado-Met added to the reaction mixture because of their role in the synthesis of 5,10-methylenetetrahydrofolate by the enzyme serine hydroxymethyltransferase (6) is illustrated by Experiment 5. Although these two compounds stimulated ribothymidine formation when tetrahydrofolate was used, when 5,10-methylenetetrahydrofolate was prepared chemically and added to the reaction mixture, the requirement for pyridoxal-P and serine was overcome.

As shown in Experiment 6, TPNH appears to be involved in reduction of FAD. TPNH alone was less effective than TPNH and FAD (Experiment 1); FADH$_2$ was as effective as the latter combination (Experiment 6). Boiled extract was still required for maximum activity.

As shown in Experiment 7, 5-methyltetrahydrofolate was not capable of replacing 5,10-methylenetetrahydrofolate in stimulating ribothymidine formation. The small amount of ribothymidine formation observed with 5-methyltetrahydrofolate probably results from free tetrahydrofolate, which was shown to be present in the preparation by analysis with 10-formyltetrahydrofolate synthetase (16). Neither 5-methyltetrahydrofolate nor any other component in the sample appears to inhibit ribothymidine formation by 5,10-methylenetetrahydrofolate.

Under optimal conditions established by the $^3$H release assay, a radioactive 1-carbon unit from $^{[14]}$Cformaldehyde, presumably via 5,10-methylenetetrahydrofolate (10, 24) was transferred to tRNA(UUC) with formation of tRNA(rTV'C) labeled in the methyl moiety of thymine, as illustrated in Fig. 6. Label from $^{[14]}$Cmethyltetrahydrofolate could not be incorporated into ribothymidine either in the presence or the absence of TPNH and FAD (data not shown).

The small amount of radioactivity derived from $^{[14]}$Cformaldehyde (between uracil and thymine, Fig. 6) not corresponding to the free base comigrated with ribothymidine and appears to be the result of incomplete hydrolysis rather than formation of another methylated base. However, after enzymatic (rather than acid) hydrolysis and chromatography, in addition to radioactive material corresponding to rTv, a minor radioactive component accounting for about 3% of the
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of PHlformate. (Solvent system as in Fig. 1.) T, ribothymidine. 
late remained at or near the origin (Fig. 7~). It was shown to 
reside in thymidyl residues of DNA as illustrated in Fig. 8.

As shown in Fig. 7u, no label from [6-3H]tetrahydrofolate 
analyzing tRNA for incorporation of label into ribothymidine.
growing S. fuecalis with excess [6-3H]tetrahydrofolate and 
methyl moiety of ribothymidine but not the pyrimidine ring or 
finding that carbon 3 of serine is used for the synthesis of the 
uracil moiety; the reducing hydride is transferred from 
position 6 of tetrahydrofolate (25). The formation of ribothymi-
dine in tRNA by a similar mechanism was examined by 
Kersten and co-workers (22, 27) have reported that the 
biosynthesis of ribothymidine in tRNA of Micrococcus lyso-
deikticus (reclassified M. luteus (11)) also involves a folate 
derivative. However, data presented here (Fig. 3) are in sub-
stantial variance with their data (22, 27). We found a very 
small amount (< 0.04 mol%) of ribothymidine in the tRNA of 
two strains of M. luteus, including strain 4698, formerly re-
designated M. lysoedikticus. These data indicate that less than 1 in 
30 tRNA molecules contains ribothymidine. We also found 
small amounts of ribothymidine that had derived its methyl 
moiety from methionine in our tRNA samples (Fig. 3b) and 
feel that we cannot exclude the possibility that all of the 
ribothymidine in our tRNA preparations was derived from methionine. These results are in considerable disagreement 
with those of Kersten’s group (22) who reported that tRNA of 
M. lysoedikticus (strain not specified) contains one ribothymi-
dine for every 2 molecules. This value, equivalent to 0.65 mol 
%, compared to 0.57 for m7G and 0.77 for D (221, is difficult to 
reconcile with data in Fig. 3, unless one assumes major strain 
variability. Their conclusion that the methyl moiety of this 
residue is derived from a folate derivative is based on (a) 
failure to observe incorporation of label from [methyl-
14C]methionine into ribothymidine of tRNA and (b) finding a 
decrease in the amount of ribothymidine relative to uridine 
following treatment of the organism with the folate antago-
nist, trimethoprim (22, 27).

In view of the significant discrepancy in data on the ribo-
thymidine content of M. lysoedikticus (luteus) tRNA, we feel that the possibility of occasional contamination of tRNA with 
degraded rRNA (29) which does contain ribothymidine (22), 
the limitations in quantitatively analyzing trace components

DISCUSSION

As we reported earlier (1, 2) ribothymidine of the tRNA of 
Streptococcus faecalis and Bacillus subtilis does not derive its 
methyl moiety from Ado-Met (Fig. 1a) but from a 1-carbon 
folate derivative. Until this observation was made, it was 
assumed that Ado-Met is the only methyl donor involved in 
methylations of nucleic acids (4, 5). The involvement of folate 
in ribothymidine biosynthesis in B. subtilis has been con-
firmed by others (22, 26-29) and further strengthened by our 
finding that carbon 3 of serine is used for the synthesis of the 
methyl moiety of ribothymidine but not the pyrimidine ring or 
the methyl moieties of other residues (Fig. 2).

We have investigated folate-mediated methylations in other 
bacteria and found that whereas another Gram-positive bacte-
rion, Bacillus cereus, probably synthesizes ribothymidine of 
DNA, polymicrobial ribothymidine derivative, this phenomenon is 
neither an invariant property of Gram-positive bacteria nor of 
Bacilli, since Clostridium acidi-urici (ATCC 7906) and Bacil-
lus stearothermophilus (21) utilize methionine for the biosyn-
thesis of this residue.

As illustrated in Fig. 6, the product of the reaction is a 5-
methyluracil and not a 5-hydroxymethyluracil moiety. No 
evidence for the formation of a 5-hydroxymethyluracil residue 
as an intermediate in this reaction has been obtained either in 
the presence or the absence of FADH2.

5,10-Methylene tetrahydrofolate is the methyl donor in-
volved in the analogous conversion of deoxuryridylate to thymi-
dylate, the deoxyribonucleotide (6). In this reaction thymidylate 
synthetase transfers the methylene moiety to position 5 of 
the uracil moiety; the reducing hydride is transferred from 
position 6 of tetrahydrofolate (25). The formation of ribothymi-
dine in tRNA by a similar mechanism was examined by 
growing S. faecalis with excess [6-3H]tetrahydrofolate and 
analyzing tRNA for incorporation of label into ribothymidine.

As shown in Fig. 7a, no label from [6-3H]tetrahydrofolate could be detected in ribothymidine of tRNA, although label 
(5,800 cpm) from [14H]formate was incorporated into ribothymi-
dine under these growth conditions (Fig. 7b). A large amount 
(41,700 cpm) of radioactivity derived from [6-3H]-tetrahydrofo-
late remained at or near the origin (Fig. 7a). It was shown to 
reside in thymidyl residues of DNA as illustrated in Fig. 8.

4 J. M. Romeo and J. C. Rabinowitz, unpublished data.
of RNA, and the complexity and current uncertainty of the overall effect of trimethoprim on cell metabolism (30, 31) should be considered and examined in detail before concluding that ribothymidine of *M. lysodeikticus* (*luteus*) tRNA derives its methyl moiety from a folate derivative.

In all of the organisms described above, ribothymidine of rRNA and probably all other methylated residues of rRNA and tRNA derive their methyl moieties from methionine as shown in Fig. 1 and reported previously (1, 2, 22). With *S. faecalis* tRNA we have observed small amounts of label derived from [¹⁴C]formate in vivo that do not co-chromatograph with rTp (Fig. 2 of Ref. 1). However, the detection of these labeled compounds has not been consistent, which along with their chromatographic properties, suggests that they are ribothymidine containing dinucleotides resulting from incomplete hydrolysis. Labeled material which was occasionally observed at the origin has been identified by digestion with DNase and snake venom phosphodiesterase as DNA containing labeled dT. In enzymatically hydrolyzed samples of tRNA isolated from cell extracts after an *in vitro* incubation with 5,10-[¹⁴C]methylene-tetrahydrofolate containing free [¹⁴C]-formaldehyde, we consistently observed a labeled compound other than ribothymidine with chromatographic properties different from those of the putative dinucleotides (data not presented). However, after acid hydrolysis of this tRNA, thymine and a small amount of ribothymidine were the only labeled compounds detected (Fig. 6). We feel that the unknown labeled compound observed in enzymatic hydrolysates is probably the product of nonenzymatic addition of formaldehyde, although the occurrence of a tolute-mediated methylation of another residue cannot be excluded by data currently available.

The development of a cell-free assay based on release of [³H]from position 5 of the uridine moiety in the sequence GUP in found in loop IV of tRNA (14) obtained from *S. faecalis* grown in the absence of folate (3) has allowed us to identify the 1-carbon folate derivative involved in ribothymidine formation. We have examined primarily the reaction catalyzed by the *S. faecalis* enzyme.

We have demonstrated specific, enzymatic release of [³H]from [³H]tRNA(UWC), but not from [³H]tRNA(rUC), which is dependent upon the presence of tetrahydrofolate (Fig. 4 and Table I) as is the *in vivo* biosynthesis of ribothymidine (1, 3). The enzyme that causes this [³H]release is present in extracts from folate-free, as well as folate-containing, cells (Table I and Figs. 5 and 6). It has been demonstrated directly that ribothymidine is made under the assay conditions (Fig. 5) and that added tetrahydrofolate is capable of mediating the transfer of a 1-carbon unit from some component present in crude extracts to tRNA(UΨC) (Table I and Figs. 5 and 6). Formaldehyde and carbon 3 of serine can serve as a source of the 1-carbon unit, presumably following nonenzymatic (10) and enzymatic (6), respectively, synthesis of 5,10-methylene-tetrahydrofolate (Fig. 6 and Table II, Experiments 4 and 5).

Through use of the [³H]release assay and experiments with 5,10-[¹⁴C]methylene-tetrahydrofolate and 5-[¹⁴C]methyltetrahydrofolate, it was shown that 5,10-methylene-tetrahydrofolate itself is the folate derivative involved in ribothymidine formation (Table II, Experiments 5 and 7, and Fig. 6). The use of 5,10-methylene-tetrahydrofolate, and not 5-methyl-tetrahydrofolate, as the 1-carbon donor is consistent with data obtained in other laboratories (32, 33) which indicate that *S. faecalis* does not contain 5-methyl-tetrahydrofolate nor the enzyme, 5,10-methylene-tetrahydrofolate reductase, that synthesizes it and that this organism cannot metabolize 5-methyl-tetrahydrofolate.

A reducing agent is required for the reduction of the methylene moiety (Table II, Experiments 1 and 6). Although FADH₂ stimulates ribothymidine formation, it cannot be concluded that FADH₂ itself is serving as the direct reducing agent. The involvement of an intermediary electron carrier is likely. Through an *in vivo* experiment with [⁶⁺H]tetrahydrofolate (Fig. 7) it was shown that the mechanism of the reduction does not involve transfer of a hydride from position 6 of tetrahydrofolate, as it does in thymidylate synthesis (25). This conclusion is based upon three assumptions—that at least one-tenth of the tRNA molecules were modified with respect to ribothymidine, that tRNA and DNA were present in the cells in approximately equal amounts (34), and that the specific activity of ribothymidine synthesized *in vivo* would be at least equal to that of thymidine. Under these conditions, we would have been able to detect incorporation of label into ribothymidine had it occurred. No incorporation of label *in vitro* from [⁶⁺H]tetrahydrofolate into ribothymidine has been observed.

Both the mono- and triglutamate derivatives of tetrahydropteroylglutamic acid are active in the *in vitro* system, showing approximately equal activities at 0.4 mM with respect to the (1R)-diastereoisomer.

In a preliminary experiment with an extract from *B. subtilis* and [⁶⁺H]tRNA(UWC) from *S. faecalis*, 5,10-methylene-tetrahydrofolate, but not 5-methyl-tetrahydrofolate, was effective in ribothymidine formation (data not presented). *S. faecalis* tRNA(UWC) is also a substrate for the enzyme from *Escherichia coli* that makes ribothymidine with Ado-Met (data not presented).

It is concluded that ribothymidine biosynthesis occurs at the polynucleotide level and that the methyl moiety of ribothymidine of the tRNA of *S. faecalis*, and probably *B. subtilis* and *B. cereus*, is derived from 5,10-methylene-tetrahydrofolate with the essential reducing hydride coming from FADH₂ or, more likely, an unidentified component in the extract or on the enzyme. Efforts are underway to purify this folate-dependent RNA methylating enzyme from *S. faecalis*, to identify all of the components involved in the reaction, and to define the reaction mechanism.

Acknowledgment—We thank Dr. Daniel V. Santi for [⁶⁺H]tetrahydrofolate and valuable suggestions.

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