On the biosynthesis of 5-methoxyuridine and uridine-5-oxyacetic acid in specific procaryotic transfer RNAs

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ABSTRACT

The uridine-5-O-derivatives, 5-methoxyuridine (mo5U) and uridine-5-oxyacetic acid (cmo5U) occupy the first position of anticodons in certain tRNA species of B. subtilis and E. coli, respectively. Here we present experimental evidence showing that both modifications are derived from a common precursor, 5-hydroxyuridine. Incompletely modified tRNAAla, tRNAThr and tRNAMet were purified from B. subtilis, and submodified tRNASer and tRNAMet from E. coli met- rel-. All five tRNAs accepted methyl groups from S-adenosylmethionine with B. subtilis extracts in vitro and mo5U was formed. In B. subtilis tRNAs the mo5U was proved to be at the specific site; in E. coli tRNAMet the mo5U was demonstrated to be present in the oligonucleotide that comprises the anticodon. In submethylated E. coli tRNAMet, 5-hydroxyuridine was detected whereas considerable amounts of cmo5U were lacking.

INTRODUCTION

In several procaryotic and eucaryotic tRNA species the first position of the anticodon is occupied by a minor nucleoside, frequently modified uridine-derivatives have been found (1). Modified nucleosides in the wobble position might affect codon recognition of specific tRNAs, in suppressor tRNAs they probably give rise to suppressor function (2,3).

We have recently found a new uridine-derivative, 5-methoxyuridine (mo5U), in tRNA of gram-positive bacteria (4,5). In B. subtilis tRNAAla, tRNAThr and tRNAMet mo5U occupies the first position of the anticodon. 5-Methoxyuridine is analogous to uridine-5-oxyacetic acid (cmo5U, formerly designated as V). This modification also occurs in the wobble position and is present in tRNASer and tRNAMet of E. coli (6-8).

Previously we have shown that mo5U (designated in previous communications 'P') can be formed in vitro in an S-adenosylmethionine (SAM) dependent reaction with B. subtilis methylating extract and incompletely modified bulk tRNA from B. subtilis (9). In the heterologous transmethylation reaction mo5U was also produced with submethylated, but not mature...
tRNA from E. coli met^-rel^- (Kersten et al. unpublished). We have therefore suggested that in submethylated E. coli tRNA a precursor is present that is common for cmo^5U and mo^5U and that this precursor is 5-hydroxyuridine.

Here we present experimental evidence showing that specific submodified tRNAs from B. subtilis and E. coli are recognized by the B. subtilis tRNA-methyltransferase that forms mo^5U. The proposed precursor, 5-hydroxyuridine was detected in submethylated tRNA Val from E. coli.

MATERIALS AND METHODS

Chemicals: Radioactive compounds were from Radiochemical Center Amersham, UK.: ^14C-L-alanine, 173 mCi/mmol; ^14C-L-threonine, 232 mCi/mmol; ^14C-L-valine, 280 mCi/mmol; ^14C-L-serine, 162 mCi/mmol; S-adenosyl-L-(Me-^14C)-methionine, 60 mCi/mmol; S-adenosyl-L-(Me-^3H)-methionine, 12.2 Ci/mmol.

Sepharose KB and DEAE-Sephadex A-25 and A-50 were from Pharmacia Uppsala, Sweden; BD-cellulose from Boehringer Mannheim; DEAE-cellulose (DE 23) from Whatman Biochemicals Ltd., UK; Avicel SF-cellulose thin layer plates (10x10 cm) were from Fucacoshi Pharmaceutical Co., Tokyo; Polygram C-300 cellulose thin layer plates (20x20 cm) from Macherey-Nagel Co. Enzymes were from the following sources: RNAase T., Worthington Biochemical Co; RNAase T 2, Calbiochem; Nuclease P., Yamasa Shoyu Co. Ltd. Tokyo; Nuclease S W, Seikagaku Kogyo Co. Ltd. Tokyo.

For autoradiography Osray T₄ film from Agfa-Gavaert, Belgium and for fluorography XR-5 X-ray film from Kodak were used. Pactamycin was a kind gift from Upjohn Co., Mich. 5-Hydroxyuridine was synthesized by the method described by T. Ueda (10).

Growth of bacteria: E. coli K 12 5B-161 met^-spoT^- rel^- (Ikaken) was grown in 10 litre of minimal medium with 4 mg of L-methionine per litre (11). Cells were harvested at a late logarithmic phase. Pactamycin treated B. subtilis W 23 was obtained as described previously (9).

Crude tRNA and S-100 fractions: Crude tRNA was prepared from pactamycin treated B. subtilis and from methionine starved E. coli (12), S-100 fractions as sources of methylating enzymes from exponentially grown B. subtilis (13).

Column and thin layer chromatography: Column chromatographies for the purification of tRNA were performed as described in the following references: DEAE-Sephadex A-50 (14); Sepharose 4B (15); BD-cellulose (16). Oligonucleotides were separated, desalted and purified as reported previously (5). Thin layer chromatography was performed on Avicel SF-thin layer plates (10x10 cm) with the following solvent systems. A: isobutyric
acid / 0.5 N NH₄OH (5 : 3); B: isopropanol / HCl / H₂O (70 : 15 : 15); C: tertbutanol / ammonium formate, pH 3.5 (1 : 1) (17). Postlabeled tRNA was analyzed on polygram C-300 thin layer plates (20x20 cm) (18) with the solvent systems; D: acetonitril / 4 N NH₄OH (68 : 20) and E: tertamylalcohol methyl ethyl ketone / acetonitril / ethyl acetate / H₂O / 88% formic acid (4 : 2 : 1.5 : 2 : 1.5 : 0.18). Spots were detected by UV absorption under an ultraviolet lamp at 253.7 nm for non-labeled samples, by autoradiography for ¹⁴C-labeled materials and by fluorography for ³H-labeled compounds (18).

Methylation of tRNA in vitro: The methylation assay (13) was slightly modified. The reaction mixture contained per ml, 30 μmol triethanolamine-HCl, pH 8.0, 6 μmol 2-mercaptoethanol, 120 nmol (Me-¹⁴C)-SAM (10.9 mCi/ mmol), or 25 nmol (Me-³H)-SAM (0.91 Ci/mmol), 3 μmol MgCl₂ and B. subtilis S-100 fractions corresponding to 500-600 μg protein. For preliminary kinetic measurements 6 A₂₆₀ units of tRNA were withdrawn and acid precipitable radioactivity was measured. The incorporation of methyl groups continued for up to two hours.

For subsequent analysis of nucleotide composition and nucleotide sequence 10 A₂₆₀ units of tRNA were methylated with ¹⁴C-labeled SAM at a specific activity of 60 mCi/mmol or ³H-labeled SAM at a specific activity of 12.2 Ci/ mmol for 45 min. Before RNAase digestion, the labeled tRNA was mixed with 10-30 A₂₆₀ of untreated tRNA.

Analysis of nucleotides and nucleosides: In vitro methylated tRNA (2-5 A₂₆₀) or CH₃-labeled oligonucleotides (0.2-1 A₂₆₀) was incubated with 10 μl of water containing 2-5 units of RNAse T₂ or 1 μg of nuclease P₁ in a capillary tube at 37°C for 1 hr and chromatographed two-dimensionally with solvent A and solvent B. tRNA and oligonucleotides were also analyzed by ³H-post-labeling (18).

Analysis of CH₃-labeled nucleotide sequences: CH₃-labeled nucleotide sequences were analyzed by the same methods as used for cold analysis (5). In vitro methylated tRNA (20-40 A₂₆₀) was digested completely with RNAase T₁ and fractionated on a column of DEAE-Sephadex A-25 (0.5x120 cm) with a linear gradient of sodium chloride from 0.14 to 0.7 M (500 ml x 2) in the presence of 7 M urea and 0.02 M Tris-HCl, pH 7.5. The radioactivity in 0.2 ml of appropriate fractions was counted in 10 ml of dioxane scintillation fluid. The relevant oligonucleotide from tRNAAla or tRNA Thr was purified on an acid DEAE-Sephadex A-25 column. The pentanucleotide con-
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containing m^5U from tRNA^Ala and tRNA^Thr (approximately 0.5 A^260) was hydro-
lyzed with 1 unit of nuclease SW (silkworm endonuclease) (19) at 37°C for
1 hr. The digest was chromatographed on a thin layer plate with solvents
A and C. The radioactive spot was scraped from the thin layer plate and
extracted with water. The extract was further hydrolyzed with 1 unit of
RNAase T_2 and analyzed by two-dimensional thin layer chromatography with
solvents A and B.

RESULTS

1. Purification of B. subtilis tRNA^Ala, tRNA^Thr and tRNA^Val.

B. subtilis cultures were treated with pactamycin to accumulate in-
completely modified tRNA (20). From 90 g wet weight cells four thousand
A^260 units of tRNA was obtained, chromatographed on Sepharose 4B and amino
acid acceptor activities were measured (21). The tRNA fractions with acceptor
activity for alanine, threonine and valine, were pooled and purified further
on BD-cellulose (Fig. 1). These tRNAs were more than 75% pure as judged from
the elution profile, from the amino acid acceptor activity and from the
chromatographic pattern of the RNAase T_1 digest.

2. Purification of tRNA^Val and tRNA^Ser from E. coli.

E. coli met^- rel^- was cultivated at a low concentration of methionine
to accumulate submethylated tRNA. From 45 g (wet weight) cells 4600 A^260
of crude tRNA was isolated. tRNA^Val and tRNA^Ser were prepared as described
by Nishimura (14) for normal tRNA^Val and tRNA^Ser. The purity of these tRNAs
was over 75% with respect to elution profiles and amino acid acceptor
activities. tRNA^Val fractions contained undermodified tRNA^Ser which is
eluted from BD-cellulose not as mature tRNA^Ser with alcohol but already at
increasing salt concentrations (data are not shown).

3. Methylation of specific tRNAs with B. subtilis enzymes and analysis of
the products.

Each specific tRNA was methylated in vitro with labeled SAM and
B. subtilis extracts. As described previously the extent of in vitro
methylation of bulk tRNA from pactamycin treated B. subtilis was found to
be rather low (9). Therefore (Me^-3H)-SAM with high specific activity was
used to methylate B. subtilis tRNA. In vitro methylation of unfractionated
tRNA from pactamycin treated B. subtilis with homologous enzymes and SAM
has been shown to result the following products: an uracil-derivative 'P'
(later identified as m^5U), methylated adenosines (m^1A, m^2A or m^6A), m^2G
and m^7G (9).

The methylation products found in tRNA^Ala and tRNA^Val were identical,
Fig. 1: Purification of tRNA$^{\text{Al}}$, tRNA$^{\text{Thr}}$, and tRNA$^{\text{Val}}$ from pactamycin treated B. subtilis: (A) Incompletely modified tRNA, 4000 A$_{260}$, was applied on a column of Sepharose 4B (2.5x50cm) and eluted with an ammonium sulfate reversed linear gradient from 1.3 M to 0 M (500 ml x 2) in the presence of 0.006 M 2-mercaptoethanol, 0.01 M MgCl$_2$, 0.001 M EDTA and 0.02 M sodium acetate, pH 4.5 at 4°C. Fractions of 4.4 ml were collected. (B) Fractions 37-50 enriched with tRNA$^{\text{Al}}$ were further separated at pH 7.5 on a column of BD-cellulose (1.5x90 cm) with a linear gradient of NaCl from 0.5 to 1.5 M (500 ml x 2) in the presence of 0.02 M Tris-HCl, pH 7.5 and 0.01 M MgCl$_2$ at room temperature. Each 2 ml-fraction was collected. (C) Fractions 27-36 enriched with tRNA$^{\text{Thr}}$ were further purified on a column of BD-cellulose at the same condition described in B. (D) Fractions 57-80 enriched with tRNA$^{\text{Val}}$ were chromatographed at pH 6.0 on a column of BD-cellulose (1.5x90cm), equilibrated with 0.4 M NaCl containing 0.02 M sodium acetate, pH 6.0. Elution was performed at 40°C with a linear gradient of NaCl from 0.5 to 1.5 M in the presence of 0.02 M sodium acetate, pH 6.0. Fractions of 2.5 ml were collected.

---: Absorbance at 260nm, ---: $^{14}$C-Alanine acceptor activity, ------: $^{14}$C-Threonine acceptor activity, ----: $^{14}$C-Valine acceptor activity.

Therefore in Fig. 2 (A and B) the labeled nucleotides are shown for B. subtilis tRNA$^{\text{Thr}}$ and tRNA$^{\text{Val}}$ only. In all three tRNA species m$^{5}$U and m$^{7}$G
Fig. 2: Two-dimensional thin layer chromatography of RNAase T2 digests of methylated tRNAs. The solvent systems used were A (1st dimension) and B (2nd dimension). Solid line UV absorption. Radioactive spots were detected by fluorography (panel A and B) and by autoradiography (panel C and D).

(A) tRNA\textsuperscript{Thr} from B. subtilis, (B) tRNA\textsuperscript{Val} from B. subtilis, (C) tRNA\textsuperscript{Val} from E. coli, (D) tRNA\textsuperscript{Ser} from E. coli.

RNAase T2 digest of tRNA\textsuperscript{Ala} from B. subtilis showed the same pattern as that of B. subtilis tRNA\textsuperscript{Val}.

are present. In tRNA\textsuperscript{Ala} and tRNA\textsuperscript{Val}, an additional methylation product was identified as m\textsuperscript{5}A. The incorporated radioactivity and the relative distribution of the methylated compounds are shown in Table 1.

Prefermimentary experiments showed that bulk submethylated tRNA from E. coli also served as substrate for the B. subtilis enzyme that converts a precursor nucleoside to m\textsuperscript{5}U. The extent of methylation of bulk submethylated tRNA from E. coli was found to be ten times higher as with the homo-
logous substrates. Therefore (Me-^1H^-)-SAM with low specific activity was used as donor of methyl groups. The products found in tRNA^Val of E. coli upon methylation with B. subtilis enzymes were mainly mo^5U, m^6A, m^7G and small amounts of m^1A (Fig. 2, panel C and Table I).

The main product of tRNA methylation is m^1A. B. subtilis comprises a specific tRNA (adenine-1)-methyltransferase that converts A (shown as A^+) in the sequence A-A^-G-G to m^1A in the D-loop, D-stem region (13). In E. coli tRNA^Ser the sequence A-A^-G-G is present in this specific region (8). Thus the internal A^+ can be converted to m^1A by the B. subtilis enzyme. tRNA^Ser was not used for further nucleotide analysis, because the yield was rather low and the tRNA proved to be a poor substrate for the mo^5U specific enzyme (Table I).

5-Methyluridine, which is missing in submethylated tRNA from E. coli is not produced, because the B. subtilis tRNA-(uracil-5)-methyltransferase is tetrahydrofolate-dependent (9,22).

Table I: Product analysis of methylated tRNAs.

<table>
<thead>
<tr>
<th>Source of tRNA</th>
<th>Compounds</th>
<th>Incorporated radioactivity (cpm)</th>
<th>% of total radioactivity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA^Ala</td>
<td>mo^5U</td>
<td>311</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>m^6A</td>
<td>689</td>
<td>11.7</td>
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<tr>
<td></td>
<td>m^7G</td>
<td>4885</td>
<td>83.0</td>
</tr>
<tr>
<td>tRNA^Thr</td>
<td>mo^5U</td>
<td>3580</td>
<td>50.4</td>
</tr>
<tr>
<td></td>
<td>m^7G</td>
<td>3530</td>
<td>45.6</td>
</tr>
<tr>
<td>tRNA^Val</td>
<td>mo^5U</td>
<td>1459</td>
<td>20.8</td>
</tr>
<tr>
<td></td>
<td>m^6A</td>
<td>1763</td>
<td>25.2</td>
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<tr>
<td></td>
<td>m^7G</td>
<td>3775</td>
<td>54.0</td>
</tr>
<tr>
<td>tRNA^Val</td>
<td>mo^5U</td>
<td>1153</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>m^6A</td>
<td>3733</td>
<td>39.1</td>
</tr>
<tr>
<td></td>
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<td>3844</td>
<td>40.3</td>
</tr>
<tr>
<td></td>
<td>m^1A</td>
<td>811</td>
<td>8.5</td>
</tr>
<tr>
<td>tRNA^Ser</td>
<td>mo^5U</td>
<td>130</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>m^7G</td>
<td>770</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>m^1A</td>
<td>2238</td>
<td>54.4</td>
</tr>
</tbody>
</table>

*mo^1A in tRNA^Val was derived from contaminating tRNA^Ser and m^6A in tRNA^Val was also derived from contaminating tRNA(s) (see also Fig. 2).*
4. Isolation of mo\textsuperscript{5}U-comprising oligonucleotides.

The incorporation of methyl groups into tRNA \textit{in vitro} continued for up to 2 hrs. During incubation for more than 1 hr tRNAs were partially degraded into halves or smaller molecules as judged from gel-electrophoresis of tRNA samples removed from the methylation assay at different intervals. Both tRNAs\textsubscript{Val} were especially sensitive to \textit{B. subtilis} RNAase(s). Therefore tRNAs were recovered after 30-45 min of \textit{in vitro} methylation and hydrolyzed completely with RNAase T\textsubscript{I}. The chromatographic patterns of the tRNA digests agreed well with those obtained from the corresponding normal tRNA, described previously (Fig. 3 and 4) (5,6).

In the case of \textit{E. coli} tRNA\textsuperscript{Val}\textsubscript{mo\textsuperscript{5}U} was found in more than one oligonucleotide fraction (Fig. 3). A scission in the anticodon loop during incubation for methylation might give rise to such extra radioactive peaks.

The pentanucleotides from \textit{B. subtilis} tRNA\textsuperscript{Ala} and tRNA\textsuperscript{Thr} and the nonanucleotide from \textit{B. subtilis} tRNA\textsuperscript{Val} contained radioactive mo\textsuperscript{5}U (shaded peaks in Fig. 4). The tRNA\textsuperscript{Val} from \textit{B. subtilis} results in more than one mo\textsuperscript{5}U containing fraction if the incubation time for methylation exceeds 30 min.

5. Sequence analysis and characterization of mo\textsuperscript{5}U comprising oligonucleotides.

The pentanucleotides from tRNA\textsuperscript{Ala} and tRNA\textsuperscript{Thr} were further purified on acid DEAE-Sephadex A-25 and served for nucleotide sequence analysis. The

![Figure 3: DEAE-Sephadex A-25 chromatography of RNAase T\textsubscript{I} digest of \textit{E. coli} tRNA\textsuperscript{Val} methylated with \textit{B. subtilis} enzyme and (\textsuperscript{14}C-CH\textsubscript{3})-SAM. Methylation tRNA\textsuperscript{Val} (10 A\textsubscript{260}) was mixed undermodified tRNAVal (50 A\textsubscript{260}) and digested with RNAase T\textsubscript{I}. The digest was applied on a column of DEAE-Sephadex A-25. Peak 10a was further purified on an acid DEAE-Sephadex A-25 column.](image)

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- Absorbance at 260 nm
- $1^4\text{C}$-radioactivity

1280
Fig. 4: DEAE-Sephadex A-25 column chromatography at pH 7.5 of (A) RNAase T₁ digest of methylated tRNA Ala and (B) RNAase T₁ digest of methylated tRNA Thr from B. subtilis. Each 20 A₂₆₀ was chromatographed and fractions of 2 ml were collected. The shaded pentanucleotides contained mo₅U and were further purified on columns of acid DEAE-Sephadex A-25. (C) RNAase T₁ digest of methylated tRNA Val from B. subtilis (40 A₂₆₀ units) was chromatographed and 2ml-fractions were collected. The shaded peak (nonanucleotide) was directly supplied for analysis.

<table>
<thead>
<tr>
<th>Absorbance at 260nm</th>
<th>0: 3H-radioactivity</th>
</tr>
</thead>
</table>

pentanucleotides containing radioactive mo₅U from tRNA Ala and tRNA Thr were split with nuclease SW and the products were separated. In both analyses two UV absorbing spots were detected, one of which was radioactive. The labeled spots were eluted and hydrolyzed with RNAase T₂. The radioactivity was found to be only in the spot corresponding to mo₅Up (Fig. 5).

The procedure of the whole sequence analysis is summarized in Fig. 6 for tRNA Thr as representative, in addition the proposed structures of the anticodons of tRNA Ala and tRNA Thr from B. subtilis are shown.
Fig. 5: Fluorography of the RNAase $T_2$ digest of (Me-3H)-labeled pentanucleotide from $B.\text{subtilis}$ tRNA$\text{Thr}$.

Fig. 6: Sequence analysis of the pentanucleotide containing mo$^5$U from tRNA$\text{Thr}$.

It is evident that the methyl group is attached to the specific uridine residue in the wobble position of the anticodon. The analysis of the pentanucleotide from tRNA$\text{Ala}$, containing mo$^5$U gave corresponding results. The pentanucleotide from tRNA$\text{Ala}$ had the sequence C-U-mo$^5$U-Gp.

The nonanucleotide obtained after RNAase $T_1$ digestion of $B.\text{subtilis}$ tRNA$\text{Val}$ has radioactive mo$^5$U together with $m^6$A (Fig. 7). Since mo$^5$U and $m^6$A are present together in the nonanucleotide (see structure of the anticodon-loop of tRNA$\text{Val}$), sequence analysis of the oligonucleotide of tRNA$\text{Val}$ was omitted.

E. coli submethylated tRNA$\text{Val}$ was methylated with $B.\text{subtilis}$ enzymes in vitro. RNAase $T_1$ digests of this tRNA should contain a pentadecanucleotide with labeled mo$^5$U and $m^6$A if the $B.\text{subtilis}$ enzyme recognizes the proposed precursor of cmo$^5$U (V) in the wobble position of submodified
Fig. 7: Fluorography of the RNAase T2 digest of (Me-3H)-labeled nonanucleotide from B. subtilis tRNAVal.

Fig. 8: Nucleotide sequence of E. coli tRNAVal with penta-decanucleotide obtained upon digestion with RNAase T1, indicated by arrows.

E. coli tRNAVal (Fig. 8). An extra minus charge derived from carboxylic acid of cmo5U might make the complicated elution profile at the longest oligonucleotide region (Fig. 3). An oligonucleotide was obtained from the region 10a that contained radioactive mo5U and m6A. The radioactive products in peak 10b were almost all m6A.
6. The precursor of \( \text{cmo}^5\text{U} \) and \( \text{mo}^5\text{U} \).

Since the submodified \( \text{tRNA}^{\text{Val}} \) from \( E. \text{coli} \) is not totally submethylated, it should comprise uridine-5'-oxygenic acid and the common precursor of \( \text{cmo}^5\text{U} \) and \( \text{mo}^5\text{U} \). The analysis of submodified \( E. \text{coli} \) \( \text{tRNA}^{\text{Val}} \) by \( ^3\text{H}\)-postlabeling is shown in Fig. 9A. The derivatives from \( \text{T}, \text{m}^7\text{G}, \text{m}^6\text{A} \) and \( \text{cmo}^5\text{U} \) were detected at 0.34, 0.61, 0.35 and 0.54 mol/mol tRNA respectively. In addition a new nucleoside derivative was found at less than 0.05 mol/mol tRNA in the same position as that of oxidized-reduced derivative from synthetic \( \text{ho}^5\text{U} \) (Fig. 9B). The identity of the new spot with \( \text{ho}^5\text{U}-\text{trialcohol} \) was confirmed after elution of the new spot and cochromatography with the same amount of authentic \( \text{ho}^5\text{U}-\text{trialcohol} \). Only one spot was detected in the chromatogram.

The pentadecanucleotide from the RNAase \( T_1 \) digest of \( E. \text{coli} \) submethylated \( \text{tRNA}^{\text{Val}} \) (peak 10a in Fig. 3) was also submitted to cold and \( ^3\text{H}\)-postlabeling analysis. However, \( \text{ho}^5\text{U} \) is rather unstable during the processes of column and thin layer chromatographies (23) and desalting procedure and also oxidation-reduction during postlabeling analysis. Therefore \( \text{ho}^5\text{U} \) was only obtained in detectable amounts when \( \text{ho}^5\text{U} \)-containing tRNA was directly analyzed. As the degree of undermodification of tRNA from pactamycin treated \( B. \text{subtilis} \) was rather low, it was unsuccessful to detect \( \text{ho}^5\text{U} \) in \( B. \text{subtilis} \) tRNA.

![Fig. 9: \( ^3\text{H}\)-postlabeling analysis of \( E. \text{coli} \) undermodified \( \text{tRNA}^{\text{Val}} \) and synthetic \( \text{ho}^5\text{U} \). (A) \( \text{tRNA}^{\text{Val}} \), 1.0 A\text{260}, was hydrolyzed to nucleosides and labeled with \( ^3\text{H}\)-borohydride. (B) Synthetic \( \text{ho}^5\text{U} \) and the commercial major four nucleosides, each 0.2 A\text{260} unit were labeled with \( ^3\text{H}\)-borohydride. Chromatography and fluorography were according to ref. 18. Abbreviations show corresponding nucleoside triolcohols.](Image)
DISCUSSION

The modifications of tRNA of gram-positive and gram-negative microorganisms show characteristic differences (24, 22, 9, 4, 5, 25). One of these refers to the modification of an uridine-residue, present in the first position of the anticodon in certain tRNA species. 5'-Methoxyuridine was found in tRNAs of Bacillaceae, B. brevis, B. coagulans and B. subtilis (4, 5). The chemically analogous uridine-5'-oxyacetic acid occurs at the same specific site in tRNA\textsubscript{Ser} and tRNA\textsubscript{Val} of E. coli.

In preliminary experiments we had observed, that submethylated but not mature tRNA from E. coli served as substrate for a SAM-dependent tRNA methyltransferase from B. subtilis, the product of which was mo\textsuperscript{5}U. We were therefore tempted to speculate that in submethylated tRNA\textsubscript{Val} and tRNA\textsubscript{Ser} from E. coli 5'-hydroxyuridine is present instead of uridine-5'-oxyacetic acid and that ho\textsuperscript{5}U is the precursor in the biosynthesis of both uridine-derivatives. The B. subtilis enzyme could then convert the precursor at the specific site to mo\textsuperscript{5}U, irrespectively of the origin of the tRNA, provided that the tRNA fulfils the structural requirements for recognition by the B. subtilis enzyme.

Submethylated tRNA\textsubscript{Val} from E. coli seemed to fulfill these structural requirements for the recognition by the mo\textsuperscript{5}U-specific B. subtilis enzyme much better than submethylated tRNA\textsubscript{Ser}. Since both tRNAs were from the same batch of the methionine starved mutant, E. coli \textsuperscript{met \textasciitilde} we assume that the amount of precursor was about the same in both tRNAs.

The homologous incompletely modified tRNA\textsubscript{Ala}, tRNA\textsubscript{Thr} and tRNA\textsubscript{Val} were used as substrates to demonstrate conclusively the specificity of the enzyme for the first position of the anticodon under conditions used for the transmethylation reaction \textit{in vitro}.

Trace amounts of 5'-hydroxyuridine were observed in bulk tRNA from B. subtilis under certain growth conditions (25). Although we could not detect ho\textsuperscript{5}U in incompletely modified B. subtilis tRNAs, this can be explained because the degree of submethylation in specific tRNAs of pactamycin treated B. subtilis is rather low (9). In addition 5'-hydroxyuridine is rather unstable during tRNA isolation and analysis. Even in submethylated tRNA\textsubscript{Val} from E. coli in which we have demonstrated and identified 5'-hydroxyuridine it must have been degraded in part during the process of nucleoside-analysis of tRNA.

The results presented here indicate, that mo\textsuperscript{5}U and uridine-5'-oxyacetic acid must be derived during their biosynthesis from 5'-hydroxyuridine. In
B. subtilis a methyl group is then transferred to ho$^5$U in specific tRNA. In E. coli tRNA ho$^5$U is either directly acetylated or mo$^5$U is formed as an intermediate that is further carboxylated to uridine-5-oxyacetic acid as shown in the following scheme.

\[
\begin{align*}
U & \xrightarrow{\text{hydroxylase}} \rightarrow ho^5U & \xrightarrow{\text{methyltransferase}} & \rightarrow mo^5U \\
& \quad \downarrow \text{acetylase} & \quad \downarrow \text{carboxylase} & \rightarrow \text{U-5-oxyacetic acid}
\end{align*}
\]

The two alternatives in the biosynthesis of uridine-5-oxyacetic acid in E. coli tRNAs are currently being investigated to understand in more detail the development of tRNA molecules and their modifying enzymes during evolution.

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