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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 $tRNA^{Ala}$, $tRNA^{Thr}$ and $tRNA^{Val}$ were purified from *B. subtilis*, and submodified $tRNA^{Ser}$ and $tRNA^{Val}$ 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* $tRNA^{Val}$ the mo5U was demonstrated to be present in the oligonucleotide that comprises the anticodon. In submethylated *E. coli* $tRNA^{Val}$, 5-hydroxyuridine was detected whereas considerable amounts of cmo5U were lacking.

INTRODUCTION

In serveral *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 (mo^5U), in tRNA of *gram-positive* bacteria (4,5). In *B. subtilis* $tRNA^{Ala}$, $tRNA^{Thr}$ and $tRNA^{Val}$ mo^5U occupies the first position of the anticodon. 5-Methoxyuridine is analogous to uridine-5-oxyacetic acid (cmo^5U, formerly designated as V). This modification also occurs in the wobble position and is present in $tRNA^{Ser}$ and $tRNA^{Val}$ of *E. coli* (6-8).

Previously we have shown that mo^5U (designated in previous communications as '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 mo^5U 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 unpublished).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.: ^{14}C-L-alanine, 173 mCi/mmol; ^{14}C-L-threonine, 232 mCi/mmol; ^{14}C-L-valine, 280 mCi/mmol; ^{14}C-L-serine, 162 mCi/mmol; S-adenosyl-L-(Me-^{14}C)-methionine, 60 mCi/mmol; S-adenosyl-L-(Me-^{3}H)-methionine, 12.2 Ci/mmol.

Sepharose 4B 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 Funacoshi Pharmaceutical Co., Tokyo; Polygram C-300 cellulose thin layer plates (20x20 cm) from Macherey-Nagel Co. Enzymes were from the following sources: RNAase T_1, Worthington Biochemical Co; RNAase T_2, Calbiochem; Nuclease P_1, Yamasa Shoyu Co. Ltd. Tokyo; Nuclease SW, Seikagaku Kogyo Co. Ltd. Tokyo.

For autoradiography Osray T_4 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 58-161 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 NH_4OH (5 : 3); *B:* isopropanol / HCl / H_2O (70 : 15 : 15); *C: tert*butanol / 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 NH_4OH (68 : 20) and *E: tert*amylalcohol methyl ethyl ketone / acetonitril / ethyl acetate / H_2O / 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 ^{14}C-labeled materials and by fluorography for ^{3}H-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-^{14}C)-SAM (10.9 mCi/mmol), or 25 nmol (Me-^{3}H)-SAM (0.91 Ci/mmol), 3 µmol $MgCl_2$ and *B. subtilis* S-100 fractions corresponding to 500-600 µg protein. For preliminary kinetic measurements 6 A_{260} 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 A_{260} units of tRNA were methylated with ^{14}C-labeled SAM at a specific activity of 60 mCi/mmol or ^{3}H-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 A_{260} of untreated tRNA.

Analysis of nucleotides and nucleosides: In vitro methylated tRNA (2-5 A_{260}) or CH_3-labeled oligonucleotides (0.2-1 A_{260}) was incubated with 10 µl of water containing 2-5 units of RNAase T_2 or 1 µg of nuclease P_1 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 ^{3}H-post-labeling (18).

Analysis of CH_3-labeled nucleotide sequences: CH_3-labeled nucleotide sequences were analyzed by the same methods as used for cold analysis (5). In vitro methylated tRNA (20-40 A_{260}) was digested completely with RNAase T_1 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 $tRNA^{Ala}$ or $tRNA^{Thr}$ was purified on an acid DEAE-Sephadex A-25 column. The pentanucleotide con-

taining mo^5U from $tRNA^{Ala}$ and $tRNA^{Thr}$ (approximately 0.5 A_{260}) was hydrolyzed 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 incompletely 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 $tRNA_1^{Val}$ and $tRNA_1^{Ser}$. The puritiy of these tRNAs was over 75% with respect to elution profiles and amino acid acceptor activities. $tRNA_1^{Val}$ fractions contained undermodified $tRNA_1^{Ser}$ which is eluted from BD-cellulose not as mature $tRNA_1^{Ser}$ 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-^{3}H)-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 mo^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,

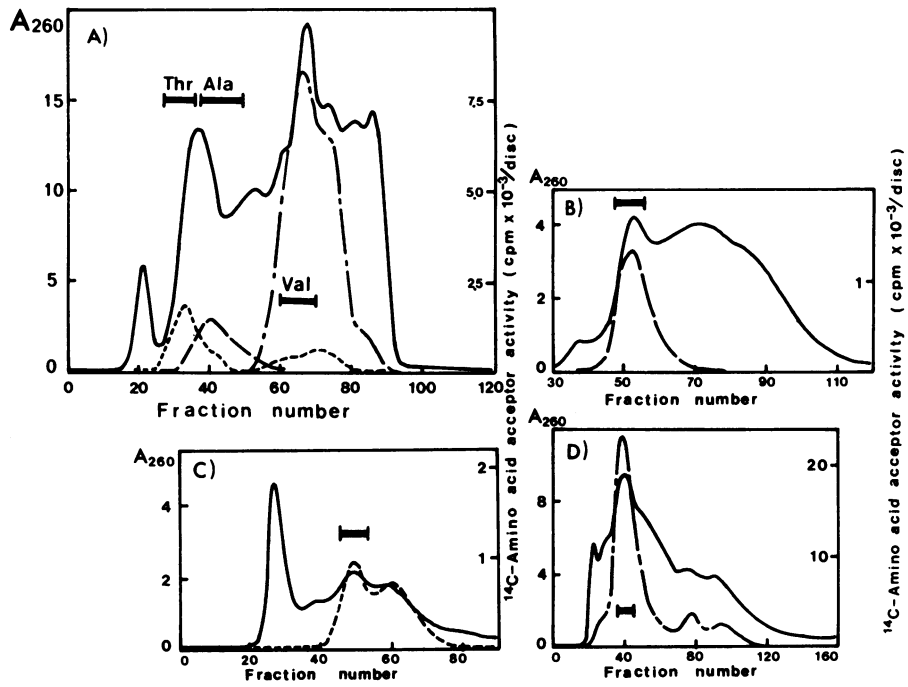


Fig. 1: Purification of $tRNA^{Ala}$, $tRNA^{Thr}$ and $tRNA^{Val}$ from pactamycin treated *B. subtilis*: (*A*) Incompletely modified tRNA, 4000 A_{260}, was applied on a column of Sepharose 4B (2.5x50cm) and eluted with an ammonium sulfate reversed linear gradient from 1.3 M to 0 M (500 mlx2) in the 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 $tRNA^{Ala}$ 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 mlx2) 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 $tRNA^{Thr}$ were further purified on a column of BD-cellulose at the same condition described in *B*. (*D*) Fractions 57-80 enriched with $tRNA^{Val}$ 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 4°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* $tRNA^{Thr}$ and $tRNA^{Val}$ only. In all three tRNA species mo^5U and m^7G

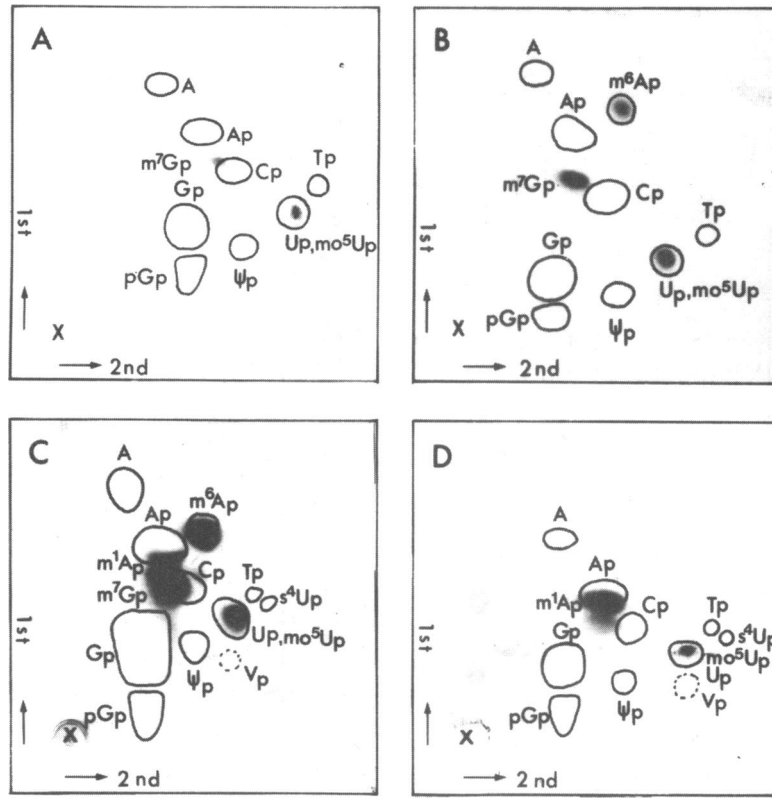


Fig. 2: Two-dimensional thin layer chromatography of RNAase T_2 digests of methylated tRNAs. The solvent systems used were A (1st dimension) and B(2nd dimension). Solid linie UV absorption. Radioactive spots were detected by fluorography (panel A and B) and by autoradiography (panel C and D).
(A) $tRNA^{Thr}$ from *B. subtilis*, (B) $tRNA^{Val}$ from *B. subtilis*,
(C) $tRNA^{Val}$ from *E. coli*, (D) $tRNA^{Ser}$ from *E. coli*,

RNAase T_2 digest of $tRNA^{Ala}$ from *B. subtilis* showed the same pattern as that of *B. subtilis* $tRNA^{Val}$.

are present. In $tRNA^{Ala}$ and $tRNA^{Val}$, an additional methylation product was identified as m^6A. The incorporated radioactivity and the relative distribution of the methylated compounds are shown in Table 1.

Preleminary experiments showed that bulk submethylated tRNA from *E. coli* also served as substrate for the *B. subtilis* enzyme that converts a precursor nucleoside to mo^5U. 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-^{14}C)-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^{5}U, m^{6}A, m^{7}G and small amounts of m^{1}A (Fig. 2, panel C and Table 1).

The main product of tRNA^{Ser} methylation is m^{1}A. *B. subtilis* comprises a specific tRNA (adenine-1)-methyltransferase that converts A (shown as A$^+$) in the sequence A-A$^+$-G-G to m^{1}A 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^{1}A 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^{5}U specific enzyme (Table 1).

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 1: Product analysis of methylated tRNAs.

Source of tRNA	Compounds	Incorporated radioactivity (cpm)	% of total radioactivity recovered
tRNA^{Ala} *B. subtilis*	mo^{5}U	311	5.3
	m^{6}A	689	11.7
	m^{7}G	4885	83.0
tRNA^{Thr} *B. subtilis*	mo^{5}U	3580	50.4
	m^{7}G	3530	49.6
tRNA^{Val} *B. subtilis*	mo^{5}U	1459	20.8
	m^{6}A	1763	25.2
	m^{7}G	3775	54.0
tRNA^{Val} *E. coli*	mo^{5}U	1153	12.1
	m^{6}A	3733	39.1
	m^{7}G	3844	40.3
	m^{1}A$^+$	811	8.5
tRNA^{Ser} *E. coli*	mo^{5}U	130	3.2
	m^{7}G$^+$	770	18.7
	m^{1}A	2238	54.4

$^+$m^{1}A in tRNA^{Val} was derived from contaminating tRNA^{Ser} and m^{7}G in tRNA^{Ser} was also derived from contaminating tRNA(s) (see also Fig. 2).

4. Isolation of mo^{5}U-comprising oligonucleotides.

The incorporation of methyl groups into tRNA *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^{Val} were especially sensitive to *B. subtilis* RNAase(s). Therefore tRNAs were recovered after 30-45 min of *in vitro* methylation and hydrolyzed completely with RNAase T_1. 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 *E. coli* tRNA^{Val} mo^{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 *B. subtilis* tRNA^{Ala} and tRNA^{Thr} and the nonanucleotide from *B. subtilis* tRNA^{Val} contained radioactive mo^{5}U (shaded peaks in Fig. 4). The tRNA^{Val} from *B. subtilis* results in more than one mo^{5}U containing fraction if the incubation time for methylation exceeds 30 min.

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

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

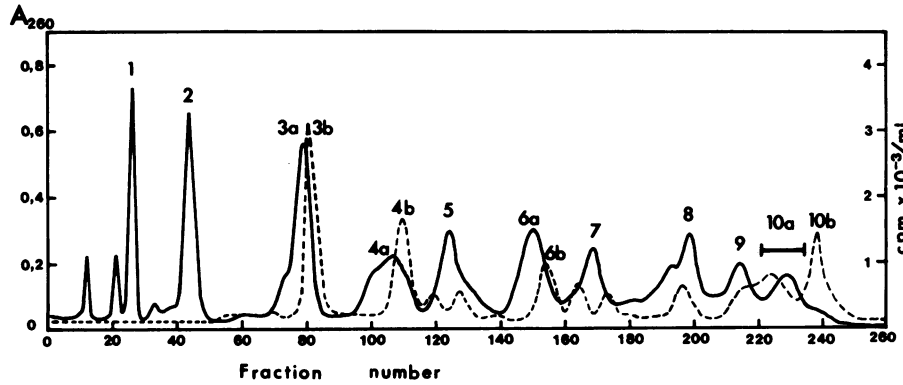


Fig. 3: DEAE-Sephadex A-25 chromatography of RNAase T_1 digest of *E. coli* tRNA^{Val} methylated with *B. subtilis* enzyme and (^{14}C-CH_3)-SAM. Methylated tRNA^{Val} (10 A_{260}) was mixed undermodified tRNA^{Val} (50 A_{260}) and digested with RNAase T_1. The digest was applied on a column of DEAE-Sephadex A-25. Peak 10a was further purified on an acid DEAE-Sephadex A-25 column.
————: Absorbance at 260 nm, ----------: ^{14}C-radioactivity

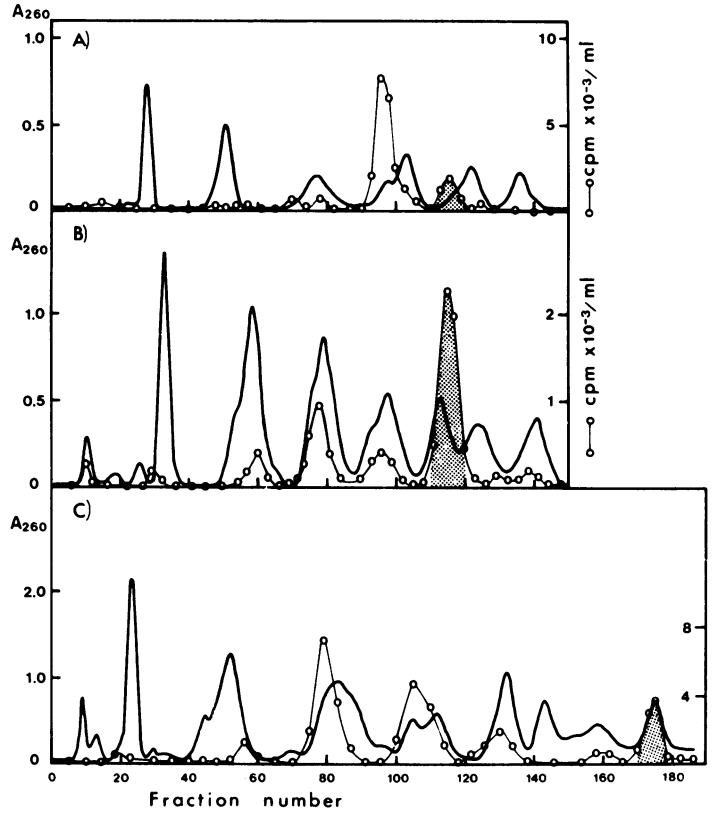


Fig. 4: DEAE-Sephadex A-25 column chromatography at pH 7.5 of (A) RNAase T_1 digest of methylated $tRNA^{Ala}$ and (B) RNAase T_1 digest of methylated $tRNA^{Thr}$ from *B. subtilis*. Each 20 A_{260} was chromatographed and fractions of 2 ml were collected. The shaded pentanucleotides contained mo^5U and were further purified on columns of acid DEAE-Sephadex A-25. (C) RNAase T_1 digest of methylated $tRNA^{Val}$ from *B. subtilis* (40 A_{260} units) was chromatographed and 2ml-fractions were collected. The shaded peak (nonanucleotide) was directly supplied for analysis. ———: Absorbance at 260nm, 0———0: ^3H-radioactivity

pentanucleotides containing radioactive mo^5U 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 T_2. The radioactivity was found to be only in the spot corresponding to mo^5Up (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.

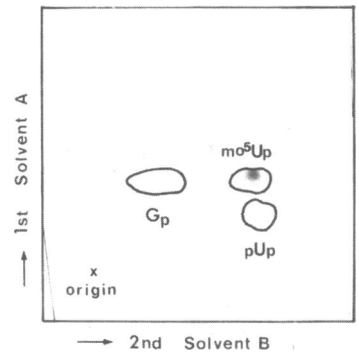
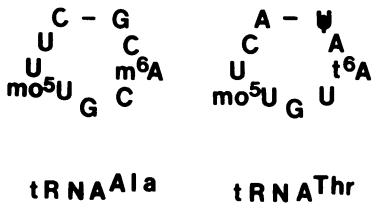


Fig. 5: Fluorography of the RNAase T_2 digest of (Me-^{3}H)-labeled pentanucleotide from *B. subtilis* $tRNA^{Thr}$.



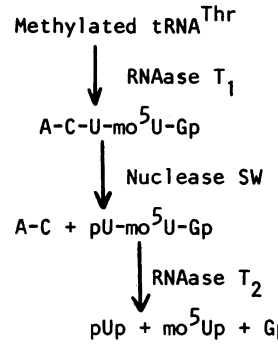
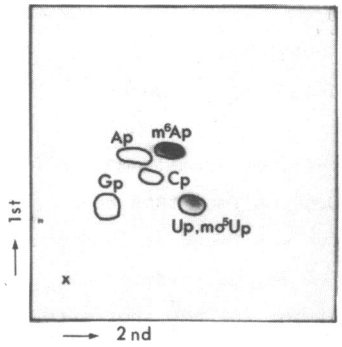


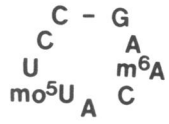
Fig. 6: Sequence analysis of the pentanucleotide containing mo^5U from $tRNA^{Thr}$.

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 $tRNA^{Ala}$, containing mo^5U gave corresponding results. The pentanucleotide from $tRNA^{Ala}$ had the sequence C-U-U-mo^5U-Gp.

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

E. coli submethylated $tRNA^{Val}$ was methylated with *B. subtilis* enzymes *in vitro*. RNAase T_1 digests of this tRNA should contain a pentadecanucleotide with labeled mo^5U and m^6A if the *B. subtilis* enzyme recognizes the proposed precursor of cmo^5U (V) in the wobble position of submodified





tRNA^{Val}

Fig. 7: Fluorography of the RNAase T_2 digest of (Me-^{3}H)-labeled nonanucleotide from *B. subtilis* tRNA^{Val}.

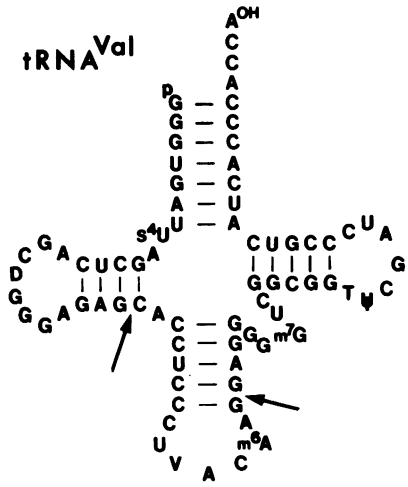


Fig. 8: Nucleotide sequence of *E. coli* tRNA$^{Val}_1$ with pentadecanucleotide obtained upon digestion with RNAase T_1, indicated by arrows.

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

6. The precursor of cmo^{5}U and mo^{5}U.

Since the submodified tRNA^{Val} from *E. coli* is not totally submethylated, it should comprise uridine-5-oxyacetic acid and the common precursor of cmo^{5}U and mo^{5}U. The analysis of submodified *E. coli* tRNA^{Val} by ^{3}H-postlabeling is shown in Fig. 9 A. The derivatives from T, m^{7}G, m^{6}A and cmo^{5}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 ho^{5}U (Fig. 9 B). The identity of the new spot with ho^{5}U-trialcohol was confirmed after elution of the new spot and cochromatography with the same amount of authentic ho^{5}U-trialcohol. Only one spot was detected in the chromatogram.

The pentadecanucleotide from the RNAase T$_1$ digest of *E. coli* submethylated tRNA^{Val} (peak 10a in Fig. 3) was also submitted to cold and ^{3}H-postlabeling analysis. However, ho^{5}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 ho^{5}U was only obtained in detectable amounts when ho^{5}U-containing tRNA was directly analyzed. As the degree of undermodification of tRNA from pactamycin treated *B. subtilis* was rather low, it was unsuccessful to detect ho^{5}U in *B. subtilis* tRNA.

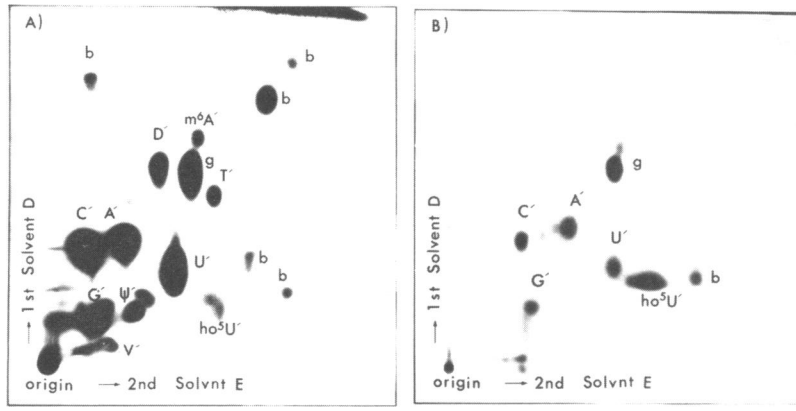


Fig. 9: ^{3}H-postlabeling analysis of *E. coli* undermodified tRNA^{Val} and synthetic ho5U. (A) tRNA^{Val}, 1.0 A260, was hydrolyzed to nucleosides and labeled with 3H-borohydride. (B) Synthetic ho5U and the commercial major four nucleosides, each 0.2 A260 unit were labeled with 3H-borohydride. Chromatography and fluorography were according to ref. 18. Abbreviations show corresponding nucleoside trialcohols.

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 $tRNA^{Ser}$ and $tRNA^{Val}$ 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^5U. We were therefore tempted to speculate that in submethylated $tRNA^{Val}$ and $tRNA^{Ser}$ from *E. coli* 5-hydroxyuridine is present instead of uridine-5-oxyacetic acid and that ho^5U 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^5U, irrespectively of the origin of the tRNA, provided that the tRNA fulfils the structural requirements for recognition by the *B. subtilis* enzyme.

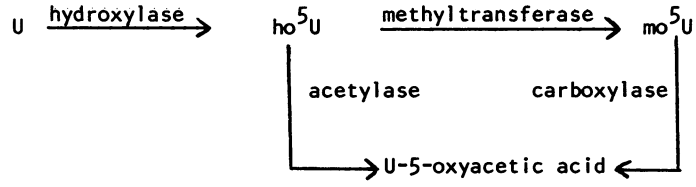
Submethylated $tRNA^{Val}$ from *E. coli* seemed to fulfil these structural requirements for the recognition by the mo^5U-specific *B. subtilis* enzyme much better than submethylated $tRNA^{Ser}$. Since both tRNAs were from the same batch of the methionine starved mutant, *E. coli* met^- rel^- we assume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

The homologous incompletely modified $tRNA^{Ala}$, $tRNA^{Thr}$ and $tRNA^{Val}$ were used as substrates to demonstrate conclusively the specifictiy of the enzyme for the first position of the anticodon under conditions used for the transmethylation reaction *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^5U 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 $tRNA^{Val}$ 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^5U and uridine-5-oxyacetic acid must be derived during their biosynthesis from 5-hydroxyuridine. In

B. subtilis a methyl group is then transfered to ho^5U in specific tRNA. In *E. coli* tRNA ho^5U is either directly acetylated or mo^5U is formed as an intermediate that is further carboxylated to uridine-5-oxyacetic acid as is shown in the following scheme.



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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