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Composition and Characterization of tRNA from *Methanococcus vannielii*

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Purified bulk tRNA from *Methanococcus vannielii* (carbon source, formate) showed variation in the modified nucleoside pattern reported for *Escherichia coli* as analyzed by both ion-exchange and thin-layer chromatography. Ribothymidine and 7-methylguanosine were absent; 1-methyladenosine, 1-methylguanosine, N^2-methylguanosine, N^2,N^2-dimethylguanosine, thiolated nucleosides, pseudouridine, dihydrouridine, and $O^{2'}$-methylcytidine were quantitated. In vitro methylation by *M. vannielii* extracts with *S*-adenosylmethionine and undermethylated *E. coli* tRNA revealed active tRNA methyltransferases for formation of methylated residues found in native *M. vannielii* tRNA, but none for the formation of 7-methylguanosine or ribothymidine. The native *M. vannielii* tRNA became methylated in the 7-methylguanosine position by *E. coli* extracts, but ribothymidine was not formed. Both *M. vannielii* and *E. coli* tRNA methyltransferases produced unidentified methylated residues in tRNA's lacking or deficient in ribothymidine.

The pattern of the types or degrees of modification of the nucleosides of tRNA has been studied in a limited number of bacteria, e.g., *Escherichia coli, Bacillus subtilis*, and *Bacillus stearothermophilus* (2, 4, 6, 7, 32). Most analytical studies of nucleosides have concentrated on purified species from *E. coli* rather than on bulk tRNA (15). In this study, bulk tRNA from a methanogenic bacterium, *Methanococcus vannielii* (28), was analyzed for its nucleoside components by two different analytical techniques. The methylated nucleosides found in *M. vannielii* tRNA were different from those reported for *E. coli*. To characterize the methyltransferase activities of this organism, in vitro methylations were performed for an extended time period, 18 h, using supernatant extracts from both this bacterium and *E. coli*, *S*-adenosylmethionine (AdoMet) as the methyl donor, and several tRNA acceptor substrates. Analysis of the ^{14}C-methylated nucleosides produced in this experiment showed that the methyltransferase activities of both organisms can be related to the composition of their native tRNA's. However, several nucleosides could not be identified. The relationship between the tRNA nucleoside composition of *M. vannielii* and its effectiveness as an acceptor substrate for *E. coli* methyltransferases is discussed.

MATERIALS AND METHODS

Materials. *S*-adenosyl-L-[*methyl*-^{14}C]methionine, 56 mCi/mmol, and KB^3H_4, 5 to 20 Ci/mmol, were purchased from Amersham/Searle. N^5,N^{10}-[*methenyl*-^{14}C]methenyltetrahydrofolate, 56 mCi/mmol, was the gift of L. Shugart. Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] and the ribonuclease (RNase) enzymes T1 and T2 were from Calbiochem; pancreatic RNase, snake venom diesterase, and alkaline phosphatase were from Worthington Biochemical Corp. Nucleosides were from Calbiochem; P-L Biochemicals, Inc.; Sigma Chemical Co.; or gifts from several members of this laboratory. Eastman cellulose chromatogram sheets, product no. 13255, 20 by 20 cm, were used for thin-layer chromatography. All other chemicals were of the best biochemical or reagent grade. The one-letter symbols for nucleosides have been used, following the recommended abbreviations of the IUPAC-IUB Commission on Biochemical Nomenclature (5).

Bacterial strains. *M. vannielii* was the generous gift of T. R. Stadtman, who described the original isolate in 1951 (28). Methane and CO_2 were produced from an anaerobic culture on a simple 1.5% sodium formate-0.1% NH_4Cl-mineral salts medium. One 400-liter fermentor culture (30°C) produced 298 g of cells (wet weight) in 64 h. *Methanosarcina barkeri* was obtained from T. R. Stadtman.

tRNA preparations. Bulk tRNA from *E. coli* B that was purified by two passages over G-100 Sephadex (Pharmacia Fine Chemicals, Inc.) was the generous gift of L. C. Waters (35). Methyl-deficient or undermethylated bulk tRNA (UMtRNA) from *E. coli* RC^{rel} (strain χ 402, *met* λ^-) was a gift from L. Shugart (24). Nucleoside analyses of these tRNA's have been reported from this laboratory (23, 35). The extractions of bulk tRNA from frozen cells of *M. vannielii* were patterned after procedures used by Waters et al. for *E. coli* B (35), with one or two purifications over G-100 Sephadex columns (1.6 by 86 cm, 0.65 M

NaCl-10 mM sodium acetate [pH 5.1]-10 mM $MgCl_2$-1 mM dithiothreitol). Variation in procedure is described in Results (see Table 2). Analysis of the tRNA 3' termini was made by tritium labeling in a procedure similar to that of Leppla et al. (12), and the radioactive nucleoside derivatives were separated with the thin-layer chromatographic (TLC) system of the Randeraths (18). Gel electrophoresis to check for the presence of large-molecular-weight nucleic acids followed the procedure of Loening (13), and the analysis was done by Beth C. Mullin.

One A_{260} unit of tRNA is the amount of tRNA in 1 ml of 10 mM $MgCl_2$ that has an absorbance of 1.0 at 260 nm when measured with a 1.0-cm optical path.

Enzyme preparation. Crude extracts from *M. vannielii* and *E. coli* contained tRNA methyltransferases. Alumina grinding of frozen *M. vannielii* cells (24), followed by high-speed centrifugation (4 h at 100,000 × *g*), overnight dialysis of the supernatant fraction, and repeated 100,000 × *g* centrifugation to remove ribosomal particles, resulted in an active preparation. *E. coli* extracts from L. Shugart were prepared in a similar manner.

Nucleoside analysis of bulk tRNA's. Two methods were used to determine the nucleoside content of tRNA from enzyme digests. One procedure was the technique described by the Randeraths (18), in which in vitro tritium-labeled nucleoside trialcohol derivatives are separated by TLC. Radioactive spots were located by a fluorographic procedure with exposure to Kodak RP Royal X-Omat film.

The other procedure involved chromatography on A-6 Aminex (Bio-Rad Laboratories) columns of nucleosides obtained from tRNA by a different digestion procedure. Initial experiments were performed under conditions of anion exclusion, at pH 9.8 (25), and cation exchange, at pH 4.65 (29-31), with the eluted nucleosides monitored at 254 and 280 nm by a model 230 Chromatronix dual-channel absorbance detector. For more accurate estimation of the major and minor nucleoside components, the enzyme digest of 9 A_{260} units of *M. vannielii* tRNA was chromatographed by the same cation-exchange conditions, at pH 4.65, on a longer A-6 Aminex column, 0.5 by 75 cm. The eluted material was measured at 260 and 280 nm by use of a Beckman DB spectrophotometer with a log-scale recorder, which permitted measurement of 2 to 10 nmol of nucleoside (30). Direct spectrophotometric readings of the collected samples were used to quantitate the major nucleosides and pseudouridine.

Analysis of in vitro methylation. Enzymatic incorporation of ^{14}C-labled methyl groups into 3 to 6 A_{260} units of several tRNA's with [*methyl*-^{14}C]AdoMet was measured by the method of Shugart et al. (23). Experimental details are given in Table 4. Approximately one-half of each ^{14}C-methylated sample was digested to the nucleoside level with RNase, snake venom diesterase, alkaline phosphatase, and then RNase T1 (19). Radioactive nucleosides were separated by cation-exchange chromatography on A-6 Aminex, pH 4.65, and by ascending two-way TLC with solvent mixtures A (*n*-butanol-isobutyric acid-concentrated NH_4OH-water [75:37.5:2.5:25], first dimension, 17 cm) and B [saturated $(NH_4)_2SO_4$ solution-0.1 M sodium acetate (pH 6)-isopropanol (79:19:2), second dimension, 16.5 cm], designated TLC system I (19). A one-dimensional thin-layer system with pH 9 borate buffer was used to identify ribose-methylated nucleosides (2).

The remaining half of each sample was digested to the 3'-nucleotide level with RNase T2 (8), and resulting digests were separated by TLC system II, with solvents C (isobutyric acid-0.5 M NH_4OH [5:3], first dimension, 17 cm) and D (isopropanol-concentrated HCl-water [70:15:15], second dimension, 12 cm) (8, 15, 16).

Radioactive spots from the thin-layer chromatograms were located by exposure to Kodak RP Royal X-Omat film for 4 to 12 days. The areas showing radioactivity were removed from the thin-layer backing and measured by scintillation counting. Fractions eluting from cation-exchange columns and other liquid samples were measured for radioactivity in a liquid scintillation mixture.

RESULTS

Nucleoside composition. Bulk tRNA's of *M. vannielii* and *E. coli* B showed different patterns of modification in the nucleosides mea-

TABLE 1. *Nucleoside content of bulk tRNA's as determined by the Randerath procedure with two digestion conditions*

Nucleoside	tRNA nucleoside content (mol %) of:			
	E. coli B		*M. vannielii*	
	I[a]	II[b]	I[a]	II[b]
Major				
A	19.22	19.55	16.98	16.48
C(+s^2C)	30.62	30.73	31.31	31.28
G	29.89	29.36	27.28	28.31
U(+s^4U)	15.35	15.63	18.87	18.34
Modified				
m^1A			0.86	0.91
m^2A	0.26	0.27		
tc^6A[c]	0.13	0.17		
m^1G	0.23	0.18	0.22	0.17
m^2G			0.36	0.28
m_2^2G			0.60	0.58
m^7G	0.23	0.20		
ψ	1.78	1.77	1.95	2.00
hU	0.72	0.65	0.91	0.90
T	1.12	1.08		
cmo^5U[c]	0.20	0.21		
nbt^3U[c]	0.12	0.11		
mnm^5s^2U[c]	0.13	0.11		
Unknown			0.66	0.50

[a] Condition I: 2 h at 50°C, 0.12 M ammonium acetate-0.014 M Mg^{2+}, pH 8.8 (30).

[b] Condition II: 6 h at 37°C, 0.03 M Bicine-0.01 M Mg^{2+}, pH 8 (18).

[c] tc^6A, N^6-Threoninocarbonyladenosine; cmo^5U, 5-carboxymethoxyuridine; nbt^3U, 3-(3-amino-3-carboxypropyl)uridine; mnm^5s^2U, 5-(methylaminomethyl)-2-thiouridine (5).

sured by the Randerath technique (Table 1 and Fig. 1), with 1-methylguanosine as the only common methylated nucleoside. *M. vannielii* tRNA did not have ribothymidine, 7-methylguanosine, 2-methyladenosine, or certain hypermodified nucleosides found in *E. coli* B tRNA (4, 15, 17, 35), but it did have approximately 1% 1-methyl adenosine, small amounts of N^2-methyl- and N^2,N^2-dimethylguanosine, and, possibly, an unknown nucleoside that was not identified. The variations of time, temperature, and buffer did not affect the results of the enzymatic digestion of both tRNA's by pancreatic RNase, snake venom diesterase, and alkaline phosphatase. Condition I (30) was used for tRNA digestion before chromatographic analyses on A-6 Aminex columns, whereas condition II (18) was used in the standard Randerath procedure. This agreement in results of nucleoside content by enzymatic digestion with two digestion conditions is significant in relating the results obtained with this tritium-labeling technique to those achieved by cation-exchange chromatography.

M. vannielii cells lyse easily (28), and phenolic extracts have large amounts (85%) of high-molecular-weight nucleic acids, which are separated with difficulty from the desired 4S material. Two tRNA preparations from different cultures are compared in Table 2. Preparation 1 followed the Zubay procedure (36) with the precipitation

TABLE 2. *Nucleoside composition of bulk tRNA's from different cultures of M. vannielii as determined by the Randerath procedure*[a]

Nucleoside	tRNA nucleoside content (mol %) of:	
	Prepn 1	Prepn 2
A	17.19	17.36
C(+s^2C)	29.88	29.98
G	29.06	28.91
U(+s^4U)	18.48	18.45
m^1A	1.09	0.91
m^1G	0.23	0.23
m^2G	0.46	0.38
m_2^2G	0.77	0.74
ψ	1.89	2.09
hU	0.95	0.95

[a] Preparation 1, 20 liters, 37°C, 48-h culture; preparation 2, 400-liter fermentor, 37°C, 32-h culture. Details of tRNA purifications are described in the text. The unknown spot seen in Fig. 1 appeared in all *M. vannielii* tRNA preparations.

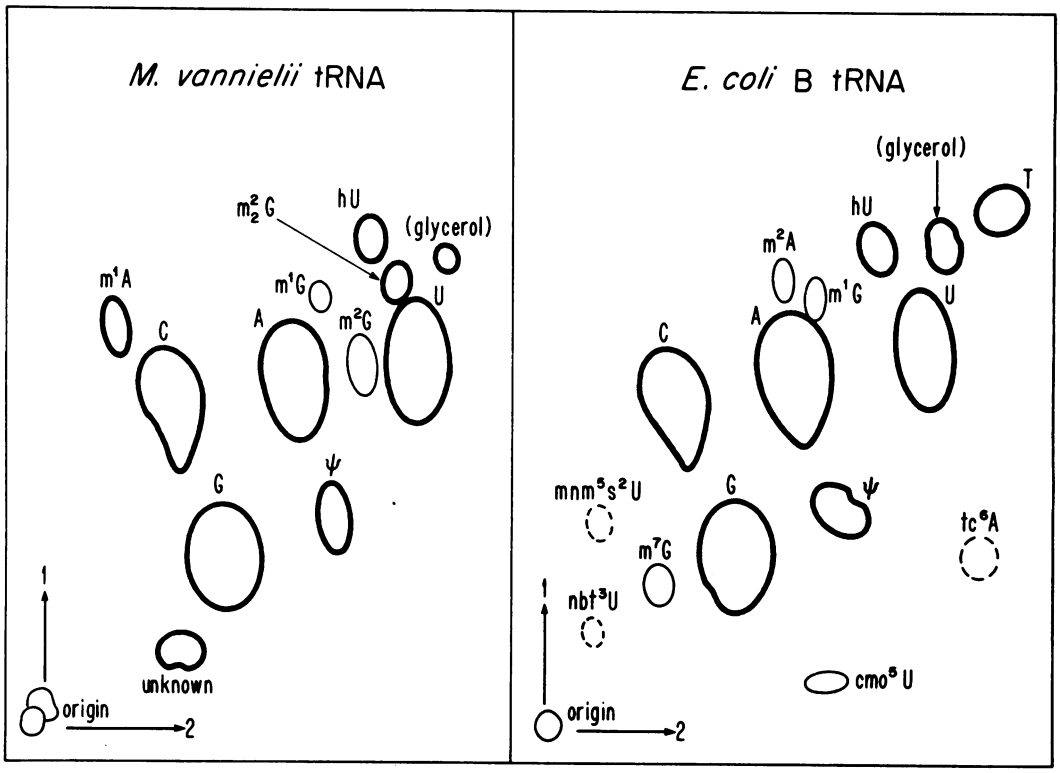

FIG. 1. *Schematic fluorographs of chromatograms of 5 μCi of ^{3}H-labeled nucleoside trialcohol derivatives from enzyme digests of M. vannielii and E. coli B tRNA's (0.03 A_{260}-unit equivalent). Total exposure time, 5 days. Relative densities are: >0.5% (——); 0.2 to 0.5% (——); <0.2% (- - -).*

by NaCl and isopropanol to remove the high-molecular-weight contaminants before chromatography with diethylaminoethyl-cellulose and G-100 Sephadex. In the larger preparation, 2, the procedure followed the method of Waters et al. (35) for preparation of highly purified bulk *E. coli* tRNA. The isopropanol precipitation step before diethylaminoethyl-cellulose chromatography was eliminated, and two successive G-100 Sephadex chromatographic steps removed high-molecular-weight nucleic acids. Previous experience in this laboratory has shown that bulk tRNA could be lost in the isopropanol precipitation step. The results in Table 2 show that the nucleoside composition, as measured by the Randerath tritium-labeling technique, was relatively constant in these preparations of *M. vannielii* tRNA. Gel electrophoresis analysis showed that both tRNA preparations compared favorably for uniformity of molecular size. Comparative analyses with the purified *E. coli* B tRNA preparations (35) indicated that all appeared to be of comparable purity, each containing small amounts of 5S material. The 3′ termini of preparation 2 were somewhat degraded; analysis showed these ends to be 80% adenosine and 20% cytidine. Apparently, the degradation did not proceed beyond the CCA terminus. Both preparations showed amino acid acceptance activity (A. N. Best, unpublished data), which would indicate that functional tRNA's were present in these preparations.

A-6 chromatography of nucleosides from enzyme digests of 1.5 A_{260} units showed several unidentified minor peaks (Fig. 2) under conditions of anion exclusion, 20 mM $(NH_4)_2CO_4$ (pH 9.8),

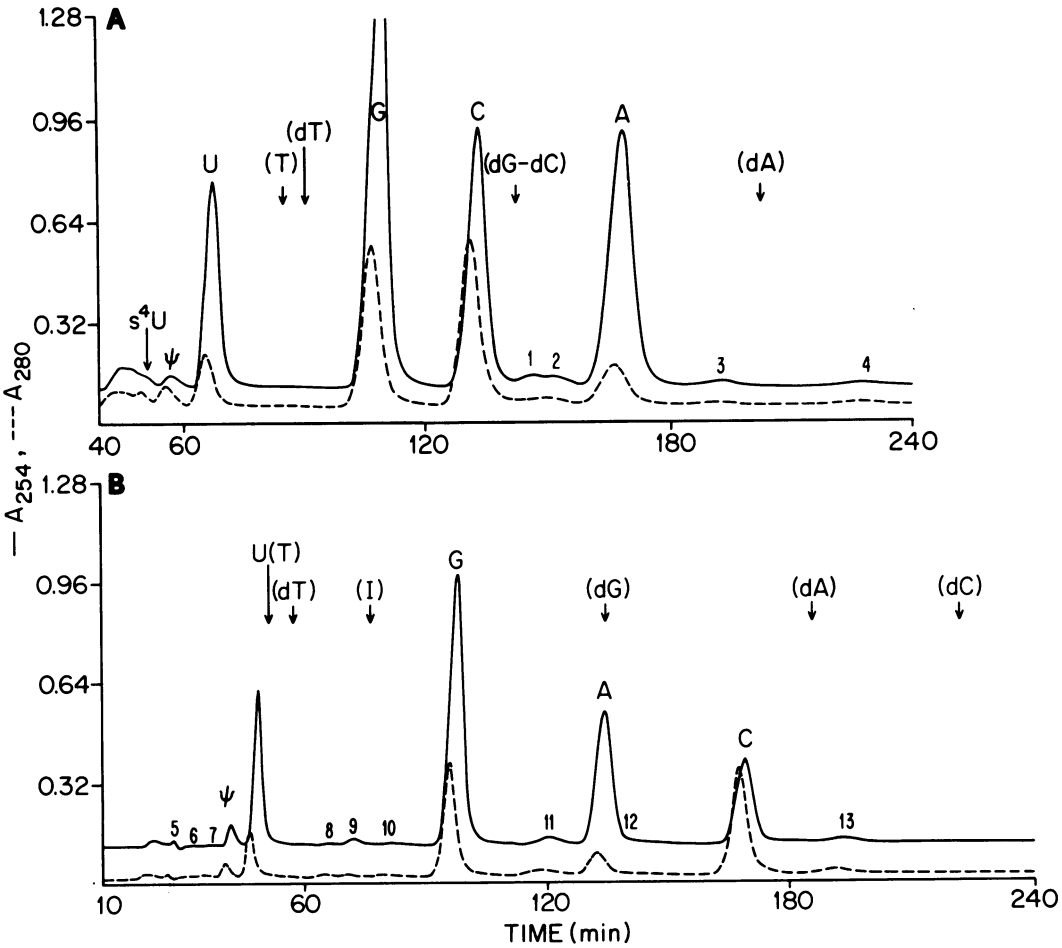

FIG. 2. *Chromatographic profiles of nucleosides from M. vannielii tRNA on A-6 Aminex columns, 0.6 by 46 cm, 50°C, by (A) anion exclusion (pH 9.8) and (B) cation exchange (pH 4.65). Unknown minor nucleotides (verified by monitoring A_{254} readings at eightfold sensitivity) are numbered, and the following are identified: 9, not I or m^1I: 10, s^4U; 11, $m_2^2G+s_2C$; 13, Cm.*

and cation exchange, 0.44 M ammonium formate (pH 4.65). None of these unidentified peaks could be attributed to residues from fragmented DNA. Both chromatographic conditions confirmed the absence of ribothymidine in tRNA preparations 1 and 2. A longer column served to identify and quantitate some of the minor components seen in Fig. 2B, and the results of nucleoside analysis by cation exchange are summarized in Table 3. The presence of a high concentration of $O^{2'}$-methylcytidine, as well as of thiolated uridine and cytidine, was established. Comparison of results obtained by the two methods, tritium labeling and anion exchange, is complicated, since each has its limitations (18, 30) and since it is impossible to compensate for the missing data. The pseudouridine content was higher by cation exchange than by tritium labeling (Table 2), but 1-methyladenosine was about the same. With the large amount of tRNA analyzed (9 A_{260} units), there is no doubt about the absence of 7-methylguanosine, ribothymidine, and isopentenyl derivatives. One interesting unidentified minor component (see peak 9, Fig. 2B) co-chromatographed with inosine and 1-methylinosine in this analytical column. However, the A_{280}/A_{260} ratio is not that of inosine (a nucleoside produced by in vitro methylation and eluting in this position is reported in the following section). The existence of ribose-methylated adenosine or guanosine could not be detected by the experimental procedures used in this study.

TABLE 3. *Nucleoside composition of M. vannielii as obtained by cation-exchange chromatography*[a]

Nucleoside	Mol %
Major	
A	17.35
C	26.95
G	29.59
U	15.56
Minor	
m^1A	0.95
i^6A	0.00
ms^2i^6A	0.00
Cm[b]	1.62
s^2C	0.60
m_2^2G	0.36
m^7G	0.00
ψ	2.77
T, Um	0.00
s^4U	0.71
Unknown[c]	3.54

[a] A-6 Aminex column, 0.5 by 75 cm, pH 4.65 (see text). Experimental conditions did not separate Am from C, m^1G from G, or m^2G or Gm from A; T and Um co-chromatograph. Dihydrouridine (hU) does not absorb at 260 nm.

[b] Cm and m^5C co-chromatograph in A-6 systems. Cm was identified by its absorbance ratio. Randerath analyses (Tables 1 and 2) did not reveal the presence of m^5C.

[c] Unknown includes 3.06%, eluting in 1 to 1.6 void volumes, and 0.35%, eluting in the I, m^1I position.

Methylation of tRNA. Preliminary in vitro experiments with labeled AdoMet and *M. vannielii* extracts showed good enzymatic incorporation of radioactive methyl groups into low levels (0.1 to 0.3 A_{260} units) of *E. coli* UMtRNA, heterologous acceptor, and very low incorporation into homologous *M. vannielii* tRNA, 1,078 and 44 pmol/A_{260} unit per h, respectively. The *E. coli* UMtRNA does serve as a methyl acceptor by *E. coli* extracts (11, 23), 1,200 to 1,400 pmol/A_{260} unit per h. The normal or completely methylated *E. coli* B tRNA, as expected (23, 35), is a poor acceptor of methyl groups by tRNA methyltransferases from *E. coli* extracts. *M. vannielii* tRNA preparations could serve as methyl acceptors with the *E. coli* tRNA methyltransferases, usually to the extent of about 300 to 400 pmol/A_{260} unit per h. It was important to know if the methylation pattern with heterologous tRNA by *M. vannielii* extracts with AdoMet as donor reflected the composition of its native bulk tRNA. Of equal significance was the identification of methylated nucleosides acquired by *M. vannielii* tRNA after in vitro methylation with active *E. coli* methyltransferases.

An attempt to answer these questions was made by an 18-h in vitro methylation experiment with both *M. vannielii* and *E. coli* extracts containing *E. coli* methyltransferases, labeled AdoMet as the methyl donor, and selected tRNA acceptor substrates (Table 4). The picomoles of *methyl*-^{14}C-labeled groups incorporated are good, considering the high levels of tRNA and inhibition of the reaction by formation of *S*-adenosyl-L-homocysteine (10).

TABLE 4. *In vitro methylation into acceptor tRNA's by tRNA-transmethylating extracts of M. vannielii and E. coli*[a]

Sample	tRNA-transmethylating extract source	Acceptor tRNA, source (A_{260} units)	*methyl*-^{14}C incorporation (pmol/18 h)
1	*M. vannielii*	*E. coli* UMtRNA (2.9)	2,837
2	*M. vannielii*	*E. coli* B tRNA (6.2)	3,176
3	*E. coli*	*M. vannielii* tRNA (3.0)	2,886
4	*E. coli*	*E. coli* UMtRNA (2.9)	2,792

[a] Samples were incubated for 18 h at 30°C, under the conditions of Shugart et al. (23), with [*methyl*-^{14}C]AdoMet, 56 mCi/mmol, as the methyl donor and saturating amounts of enzyme extracts. At the conclusion of the reaction, each sample was extracted with phenol several times at pH 4.5, and the labeled tRNA was collected by alcohol precipitation. Details of nucleoside and nucleotide analysis of these labeled tRNA's are described in the text.

The chromatographic profiles of the *methyl*-^{14}C-labeled nucleosides obtained from the enzymatic digests of these in vitro-methylated tRNA's are shown in Fig. 3. In Fig. 3A, *M. vannielii* extract was found to contain tRNA methyltransferases that function to form 1-methylguanosine, N^2-methyl- and N^2,N^2-dimethylguanosine, $O^{2'}$-methylcytidine, and 1-methyladenosine in the heterologous tRNA's, *E. coli* UMtRNA and the normal tRNA of *E. coli* B (Fig. 3A). The above-mentioned nucleosides were reported in native *M. vannielii* tRNA (Tables 1 through 3). However, no ribothymidine or 7-methylguanosine was formed in *E. coli* UMtRNA by the *M. vannielii* extract. These two nucleosides were not found in analyses of native *M. vannielii* tRNA (Tables 1 through 3).

An interesting feature of *M. vannielii* methylation is the significant amount of an unidentified methylated nucleoside obtained from labeled *E. coli* UMtRNA that elutes in the pseudouridine position (fraction 13, sample 1). This methylated nucleoside was not found in the in vitro-methylated *E. coli* B tRNA (sample 2). By comparison with nucleoside markers, the unknown in fraction 13 could not be ribothymidine, $O^{2'}$-methyluridine, or 3-methyluridine. Another unidentified methylated product in fraction 23, formed by *M. vannielii* extracts in both tRNA's co-chromatographs on cation exchange with 1-methylinosine. (The coincidence of this elution pattern with an unidentified minor nucleoside from *M. vannielii* tRNA was mentioned in the previous section.) The small amounts of N^6-methyladenosine might have originated from the labile 1-methyladenosine (18). The identity of the methylated residue in fraction 97 as N^6,N^6-dimethyladenosine is not certain.

In Fig. 3B, *E. coli* extracts showed active tRNA methyltransferases for the formation of ribothymidine and 7-methylguanosine, the major methylated residues (UMtRNA, sample 4). With *M. vannielii* tRNA as the acceptor substrate (sample 3), *E. coli* extract showed tRNA methyltransferase activity for the formation of 7-methylguanosine but not ribothymidine, two nucleosides that are absent in *M. vannielii* tRNA (Tables 1 through 3). Since the results reported above with UMtRNA as acceptor substrate demonstrate the presence of an active tRNA methyltransferase in this *E. coli* extract for the formation of ribothymidine, it appears that *M. vannielii* tRNA cannot serve as an acceptor substrate for this tRNA methyltransferase. However, some guanosine residues of *M. vannielii* tRNA could be methylated to form 7-methylguanosine by *E. coli* enzyme. *E. coli* extracts also formed with acceptor *M. vannielii* tRNA the methylated nucleosides $O^{2'}$-methylcytidine, N^6-methyladenosine, some 1-methylguanosine, and another unknown methylated nucleoside, eluting in fraction 13 in the pseudouridine position. It was somewhat surprising that there is additional methylation of cytidine ribose, as the native *M. vannielii* tRNA contained more than 1% of this residue (Table 3). Borate TLC identified fraction 82 as ribose-methylated cytidine and did not reveal ribose methylation of the other major nucleosides.

The relative amounts of these *methyl*-^{14}C-labeled nucleosides obtained by cation-exchange chromatography (Fig. 3) are compared with those achieved by separation of a portion of the enzymatic tRNA digests with TLC system I in Table 5. In addition, to be certain of the correlation between these two systems, radioactive areas from the thin-layer chromatograms were eluted and subjected to analysis by the cation-exchange system. The results, in percent radioactivity, compare well in these two separation systems. It was definitely established that the unknown nucleosides eluting in fraction 13 (pseudouridine position, cation exchange) from samples 1 and 3 were not ribothymidine. Moreover, these unknowns in fraction 13 are not the same, for thin-layer separation (Table 5) showed a methylated nucleoside migrating near uridine in sample 1, whereas sample 3 had no radioactive nucleoside in that position. In cation-exchange chromatography, at pH 4.65, pseudouridine elutes in about 2 column volumes, near the breakthrough material containing other negatively charged residues. Thin-layer separation showed that radioactive nucleosides migrating near 1-methylinosine corresponded to fraction 23 (1-methylinosine portion, cation exchange) in samples 1 and 2. The identities of other *methyl*-^{14}C-labeled nucleosides separated by cation exchange were confirmed. The N^6-methyladenosine formed in sample 3 (*E. coli* extract with *M. vannielii* tRNA) was, evidently, not formed from labile 1-methyladenosine (18), as the latter nucleoside in samples 1 and 2 was stable under these separation conditions.

Identification of unknown *methyl*-^{14}C-labeled nucleosides. Further experiments were performed in attempts to identify the two unidentified methylated nucleosides (Table 5) from samples 1 and 3, known not to be ribothymidine, that eluted in fraction 13, pseudouridine position, by the cation-exchange analyses. These unknown nucleosides were formed in the in vitro methylations of *E. coli* UMtRNA by *M. vannielii* extract (sample 1) and *M. vannielii* tRNA by *E. coli* extract (sample 3). Ribothymidine was formed only in sample 4, by the in vitro methylation of *E. coli* UMtRNA by *E. coli* extract.

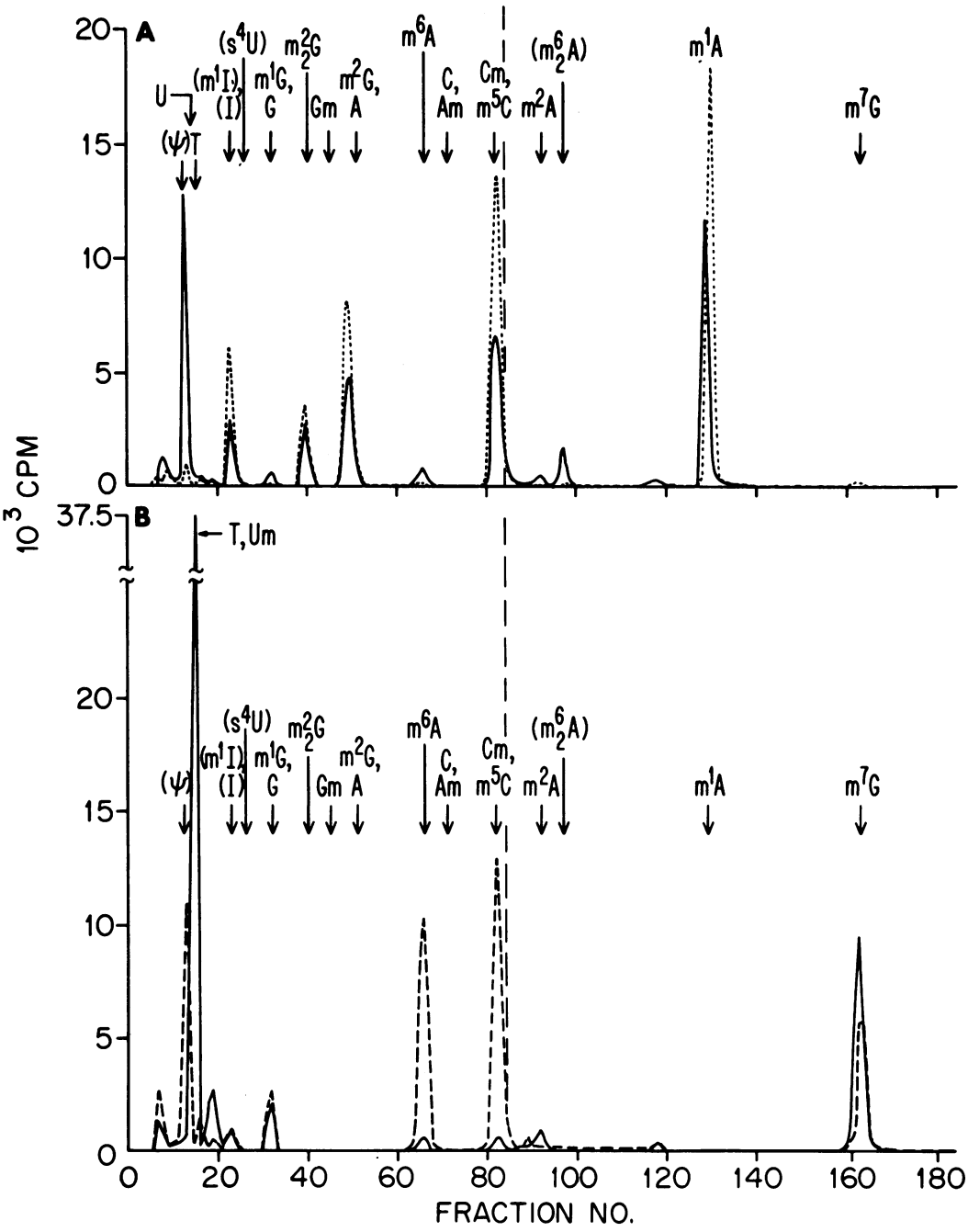

FIG. 3. *Cation-exchange chromatographic profiles of methyl-^{14}C-labeled nucleosides obtained from in vitro methylated tRNA's. (A) Labeled nucleosides from E. coli UMtRNA (——) and normal E. coli B tRNA (- - -) methylated by M. vannielii extract, samples 1 and 2. (B) Labeled nucleosides from M. vannielii tRNA (— —) and E. coli UMtRNA (——) methylated by E. coli extract, samples 3 and 4. In vitro methylation conditions and total incorporation are described in Table 4. From each sample, 31.25% of the radioactivity was used for cation exchange analysis (0.44 M ammonium formate at pH 4.65, 0.6 by 92-cm A-6 Aminex column, 50° C). Fractions 1 through 84 are 1.5 ml each; fractions 85 through 185 are 4 ml each.*

TABLE 5. *Comparative separation by cation exchange and TLC of methyl-^{14}C-labeled nucleosides from in vitro-methylated tRNA's*[a]

Peak fraction by cation exchange	Nucleoside	Radioactivity (%)							
		M. vannielii extract with:				*E. coli* extract with:			
		E. coli UMtRNA (sample 1)		*E. coli* BtRNA (sample 2)		*M. vannielii* tRNA (sample 3)		*E. coli* UMtRNA (sample 4)	
		Cation exchange	TLC I	Cation exchange	TLC I	Cation exchange	TLC I	Cation exchange	TLC I
7	(Vo)[b]	3.65		1.44		4.08		2.54	
13	(ψ)[b]	18.71	15.88[c]	1.05	1.04	17.89[c]		0.0	
15	T	0.0		0.0		0.0		54.35	58.20[c]
23	(m^1I)[b]	5.02	5.37[c]	7.52	8.0[c]	1.75		1.74	
32	m^1G	1.24		0.16		4.14	18.48[c]	3.99	4.53[c]
40	m_2^2G	6.14	3.75[c]	7.24	2.83[c]	0.0		0.0	
50	m^2G	13.49	10.94[c]	17.16	12.74[c]	0.0		0.0	
66	m^6A	2.00		0.30		23.39	25.35	1.37	
82	Cm	20.54	25.28	30.11	32.94	28.40	30.63	1.85	
92	m^2A	1.06		0.0		0.28		1.78	
97	(m_2^6A)[b]	3.62		0.19		0.0		0.0	
129	m^1A	22.99	30.67	31.91	37.78	0.0		0.0	
163	m^7G	0.40		0.0		15.38	19.84[c]	24.39	31.22[c]
	Unidentified	1.14		2.92		4.69		7.99	
	Unidentified		8.11		4.69		3.67		6.06

[a] In vitro methylation conditions are listed in Table 4; cation exchange results are shown in profile in Fig. 3; TLC system I for nucleoside separation is described in the text.

[b] Elution peak of a standard nucleoside, not necessarily identification of a fraction. Vo is breakthrough position of undigested material.

[c] Correlation of these nucleosides was confirmed by re-chromatography with cation exchange.

The portion of methylated tRNA that was digested by RNase T2 and chromatographed on thin-layer cellulose by TLC system II showed that samples 1 and 3 each had (among other radioactive nucleotides) an unidentified radioactive spot that had not migrated to the position of ribothymidine 3'-phosphate in sample 4 (Table 6). They could be at least dinucleotides, since RNase T2 does not cleave phosphodiester linkages adjacent to ribose-methylated nucleosides or other modified residues, such as N^2,N^2-dimethylaguanosine (9, 20). However, they are not in the chromatographic region associated with ribose-methylated dinucleosides (9). By comparison with known nucleotides as markers and previous experience with TLC system II, spots *A* and *B* were probably the phosphorylated forms of the unknowns in fraction 13, Table 5. Accordingly, these two unidentified radioactive spots (referred to as spot *A* from sample 1 and spot *B* from sample 3) and the comparison spot, ribothymidine 3'-phosphate (referred to as spot *C* from sample 4), were eluted with water and completely digested to the nucleoside level by the four-enzyme procedure (19), and this digest was subjected to the same separation techniques described for nucleosides in Table 5, cation exchange at pH 4.65 and TLC system I.

Table 6 shows the original chromatogram position of these spots as nucleotides after separation by TLC system II as well as the separation achieved by TLC system I and cation exchange of the eluates *A*, *B*, and *C* after digestion to nucleosides. The spot *A* methylated residue, which moved near uridine 3'-phosphate with TLC system II after T2 digestion, chromatographed near uridine (TLC system I) as a nucleoside. It eluted in the pseudouridine position with cation exchange and, presumably, was the methylated residue in fraction 13 (Table 5). However, spot *B* was a fragment of several nucleotides. After digestion to nucleosides, several radioactive spots were found by thin-layer separation (*B*-1 and -2) that were in the area of methylated guanosines. (The methylated guanosines, except for 7-methylguanosine, were not well separated on the 20-cm chromatograms.) Fragment *B*-1 had radioactive material eluting with pseudouridine and 1-methylguanosine on cation exchange.

It is apparent from these results that the methylated nucleotide 3'-phosphates *A* and *B*, which, after digestion to nucleosides, chromatographed by cation exchange with pseudouridine (*B* slightly ahead of of *A*), are not the same compound. By all results, *A* is a single nucleoside, whereas *B* is a mixture. The conclusion from these

TABLE 6. *Characterization of certain nucleotides obtained from RNase T2 digests of methyl-^{14}C-labeled tRNA's by TLC and cation-exchange chromatography*[a]

Sample	Nucleoside 3'-phosphates			Nucleoside 3'-phosphates digested to nucleosides			
	Spot	TLC II (R_f)		Spot	TLC I (R_f)		A-6 cation exchange (elution position)
		Solvent C	Solvent D		Solvent A	Solvent B	
1	*A*	0.33	0.67	*A*	0.21	0.55	ψ
3	*B*	0.56	1.00	*B*-1	0.28	0.22	ψ, m^1G
				B-2	0.39	0.17	Not tested
4	*C*	0.41	0.90	*C*	0.42	0.44	T
	Up	0.41	0.77	U	0.24	0.57	
	ψp	0.32	0.56	ψ	0.10	0.56	
	Tp	0.44	0.90	T	0.41	0.46	
	dTp	0.50	0.94	$m^1G + m^2G$	0.27	0.22	

[a] See Table 4 for identification of bacterial and tRNA extracts. Description of digestion of the nucleotides to nucleosides and subsequent identification is described in the text.

data is that *M. vannielii* can methylate some residue of a tRNA that has incomplete ribothymidine methylation (*E. coli* UMtRNA) but is unable to form this methylated nucleoside in ribothymidine-sufficient (i.e., normal) tRNA from *E. coli* B. This unknown methylated nucleoside is not ribothymidine. In turn, ribothymidine-deficient *M. vannielii* tRNA can serve as a substrate for the formation by *E. coli* extract of an unidentified methylated nucleotide(s) that does not contain ribothymidine. It seems possible that spot *A* from sample 1 is a methylated uridine. Spot *B* from sample 2 was a composite of two or three nucleosides. The chromatogram positions of both spot *A* and spot *B* in TLC system II are not the same as those reported for the 3'-phosphates of 5-methyl-2-thiouridine and 5-methoxyuridine (14, 34). Other chromatographic evidence eliminates several modified residues found in *E. coli* tRNA, such as N^6-threoninocarbonyladenosine, 5-carboxymethoxyuridine, and 5-(methylaminomethyl)-2-thiouridine (Table 1).

DISCUSSION

This investigation was originally centered on the nucleoside composition of tRNA in the methanogenic bacterium *M. vannielii*. Most research on related bacteria of this class has been focused on the details of methane production (27). Two excellent analytical methods, tritium labeling and cation exchange, which complement each other to some degree, have been used in this study. Thiolated nucleosides and at least one ribose-methylated nucleoside, along with several other unknown minor components, were detected by the A-6 cation-exchange system. The tritium-labeled nucleoside trialcohol derivatives of the digested *M. vannielii* tRNA revealed the existence of four methylated residues, pseudouridine, and dihydrouridine. The results agree fairly well, when consideration is given to technical limitations (18, 30, 31). The use of the two methods of analysis gives a more comprehensive survey of modified nucleosides in bulk tRNA's.

The purified bulk tRNA of *M. vannielii* showed a complete absence of two commonly occurring methylated nucleosides, ribothymdine and 7-methylguanosine. The latter nucleoside is very alkali labile, and reported values are often low (18). However, the in vitro methylation studies demonstrated that this bulk tRNA from *M. vannielii* can serve as substrate for AdoMet-mediated *E. coli* tRNA (guanine-7)-methyltransferase but not tRNA (uracil-5)-methyltransferase. *M. vannielii* extracts did not contain AdoMet-mediated tRNA methyltransferases for formation of 7-methylguanosine or ribothymidine (5-methyluridine) in a suitable tRNA acceptor, *E. coli* UMtRNA. The absence of 7-methylguanosine in bulk tRNA could not be attributed to loss during purification or to the nucleoside analytical conditions. The *E. coli* tRNA (uracil-5)-methyltransferase has been shown by Shugart and Stulberg to be very labile and subject to great loss in activity during enzyme purification (24). For this reason, the in vitro methylations were made with fresh, ribosome-free supernatant extracts from both *M. vannielii* and *E. coli*.

The combined results from these in vitro methylation studies would indicate that the deficiency in *M. vannielii* in both ribothymidine and 7-methylguanosine can be attributed to the absence of the specific AdoMet-mediated tRNA methyltransferases in this organism. It would appear that the donor, AdoMet, is present in sufficient amounts for tRNA methylation, since *M. vannielii* tRNA compares favorably with

bulk *E. coli* tRNA in total content of methylated residues and should not be considered an "undermethylated" tRNA. The methylation experiments indicate that bulk tRNA from *M. vannielii* differs from many ribothymidine-deficient tRNA's in a way that prevents its acceptance of a methyl group for ribothymidine formation (3, 21, 22, 23, 33). It is not known if this is due to a modification of the usual uridine acceptor residue in the so-called GTψC region in loop IV of tRNA (26). The possible existence of this modified uridine can be inferred from the formation during in vitro methylation experiments of a sizeable quantity of an unknown methylated residue migrating near uridine as a nucleoside with thin-layer chromatography and near uridine 3'-phosphate as a nucleotide from RNase T2 digestion. This residue was formed by *M. vannielii* extracts only in the tRNA substrate that was deficient in ribothymidine. The nucleoside analyses of bulk tRNA (Tables 1 through 3) did not reveal the existence of this hypothetical modified uridine.

The methylated nucleosides found in bulk *M. vannielii* tRNA, 1-methyladenosine, $O^{2'}$-methylcytidine, N^2-methyl- and N^2,N^2-diethylguanosine, and 1-methylguanosine, are of interest as they are more characteristic of eucaryotes than of procaryotes (7, 26). Some of these nucleosides have been reported by Vold for *B. subtilis* tRNA; however, 1% 1-methyladenosine indicates that *M. vannielii* tRNA has an unusually high content of this nucleoside, 0.8 mol per 80 residues as compared with 0.15 for *B. subtilis* (32). The $O^{2'}$-methylcytidine content is also high (1.62%), despite efforts to remove contaminating high-molecular-weight RNAs. Appropriate AdoMet-mediated tRNA methyltransferase activity for the formation of all of these nucleosides was found in *M. vannielii* crude extract. This extract also methylated some residue in *E. coli* tRNA's that migrated with or near 1-methylinosine with several chromatography systems. An unknown that was not inosine or 1-methylinosine was seen in bulk *M. vannielii* tRNA. Further experiments would be needed to see if *M. vannielii* can methylate inosine.

With the exception of the nucleosides in or near the anticodon region, the role of modified nucleosides in tRNA remains something of an enigma, and differences observed in this study between *M. vannielii* and other bacterial tRNA's cannot be evaluated. *M. vannielii* tRNA appears to contain none of the hypermodified nucleosides of *E. coli* that are associated with loop II of the tRNA molecule (15, 26). Of the modified nucleosides found in bulk *M. vannielii* tRNA, the frequency of their positional occurrence in individual species is not known. The tRNA of another methanogenic bacterium, *M. barkeri*, has been analyzed, and it showed lower frequency and variety in nucleoside modification than did *M. vannielii* tRNA (Best, unpublished data).

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