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Evidence for the Utilization of Host tRNA(m^5U)Methylase to Modify tRNA Coded by Phage T4

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Mutants of *Escherichia coli* defective only in the biosynthesis of 5-methyluridine (ribothymidine) in their transfer ribonucleic acid (tRNA) were employed to investigate whether phage T4 induces its own tRNA(m^5U)methylase and whether T4-specific tRNA contains 5-methyluridine. The amounts of other methylated derivatives in the T4-specific tRNA were also determined. The results establish that T4-specific tRNA contains 5-methyluridine, and that there is an absolute requirement for a functional host tRNA(m^5U)methylase to undertake this modification. Comparison of several physical properties of the host enzyme before and after phage T4 infection did not suggest any phage-directed alteration of the enzyme. The distribution of the methylated constituents in T4-specific tRNA is distinguishable from that in host tRNA. This change, however, may simply reflect a different population of tRNA chains produced by the T4 phage, rather than some change in the tRNA methylase activity of the host.

INTRODUCTION

Several reports from different laboratories have established that after infection of *Escherichia coli* with T-even bacteriophages transfer ribonucleic acid (tRNA) is synthesized, and that the newly formed tRNA can hybridize with the phage deoxyribonucleic acid (DNA) (Daniel *et al.*, 1968 a + b; Weiss *et al.*, 1968). Recently it was shown that bacteriophage T5 also codes for phage-specific tRNA (Scherberg and Weiss, 1970). In the case of T4, at least five species of tRNA have been shown to hybridize to T4 DNA, while as many as 14 different tRNA species are coded for by the phage T5 genome (Littauer and Daniel, 1969; Scherberg and Weiss, 1970). The phage-specific tRNA has been shown to contain pseudouridine and thiolated bases (Daniel *et al.*, 1968a; Weiss *et al.*, 1968). tRNA labeled *in vivo* with methyl groups after phage T4 infection contains labeled 5-methyluridine (m^5U),[2] but whether the labeled methyl group is present in the phage-specific tRNA has not been shown (Boezi *et al.*, 1967).

The synthesis of T4-specific tRNA is an early phage function (Scherberg *et al.*, 1970). Infection carried out in the presence of chloramphenicol influences neither the synthesis of T4-specific tRNA nor the ability of this tRNA to accept amino acids *in vitro*

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[2] Abbreviations used in this paper are those recommended by the Commission on Biochemical Nomenclature (CBN-70): m^5U, 5-methyluridine (ribothymidine), $m_2{}^6Ade$, N^6-dimethyladenine, m^7Gua, 7-methylguanine, m^5Ura, 5-methyluracil (thymine), and so forth. The Fischer numbering convention, which assigns the same number to the homologous parts of the purine and pyrimidine rings, has been used. In addition, tRNA(m^5U) methylase represents the tRNA methylase producing 5-methyluridine, and *trm* and Trm^- designate the genotype and phenotype, respectively, of a mutant defective in methylating tRNA. Transfer RNA which is isolated from *Escherichia coli* cells infected with bacteriophage T4 is called T4 tRNA; that portion which hybridizes with T4 DNA is called T4-specific tRNA. SSC is a buffer of the following composition: 0.15 *M* NaCl and 0.015 *M* sodium citrate, pH 7.0; 1/100 SSC, 2 × SSC and 6 × SSC are buffers of 1/100, two times, and six times this concentration.

(Scherberg *et al.*, 1970). Therefore, either the amino acid charging of the phage tRNA is independent of chemical modification, or phage tRNA does require modification for its charging, but host enzymes are employed for this function. Several arguments for each possibility have been discussed by Scherberg *et al.* (1970). Amino acid charging *in vitro* of *E. coli* tRNA completely lacking m^5U seems to proceed normally and, therefore, the first alternative is certainly possible with respect to that specific modification.

Wainfan *et al.* (1965) reported changes in the relative activities of the different base-specific tRNA methylases after T2 infection. To our knowledge, however, no critical experiments have been reported which answer the question of whether T4 uses host enzymes or induces its own enzymes to modify the phage-specific tRNA.

Our approach in investigating this question has been to use mutants of *Escherichia coli* which lack, both *in vivo* and *in vitro*, all detectable capacity to produce m^5U in their tRNA (Björk and Isaksson, 1970). Infection by phage T4 in such a mutant should reveal whether or not T4 induces its own tRNA (m^5U)methylase, and also whether or not the T4 tRNA made in such a mutant contains m^5U. In this paper three kinds of experiments utilizing such a mutant are reported. It was found that T4-specific tRNA could be methylated *in vitro* only when it was synthesized in a *Trm*$^-$ host; that T4 tRNA contains m^5U only when the T4 tRNA was made in the wild-type host; and finally that no tRNA (m^5U)methylase activity could be found after phage T4-infection in the mutant. These results show that phage T4-specific tRNA contains m^5U and that a functional host enzyme is necessary for this modification.

MATERIALS AND METHODS

a. *Strains and media.* Two pairs of strains were constructed that differ in the methylating function but are otherwise as isogenic as possible. Two *metA*$^+$ and two *argH*$^+$ transductants of *E. coli* K12 AB1932(*metA*, *argH*) have been used. Strain AB1932-5-41(*metA*$^+$, *argH*, *trmA5*$^+$) has received only the *metA*$^+$ gene from the donor IB5(*metA*$^+$, *argH*$^+$, *trmA5*) while strain AB1932-5-39-(*metA*$^+$, *argH*, *trmA5*) in addition to *metA*$^+$ has received the *trmA5* allele. The tRNA of the latter strain lacks detectable m^5U. Strain AB1932-5-466(*metA*, *argH*$^+$, *trmA5*$^+$) has received only the *argH*$^+$ allele, while strain AB1932-5-451(*metA*, *argH*$^+$, *trmA5*) has received *argH*$^+$ and the *trmA5* allele. The distribution *in vivo* of the methylated bases in the tRNA of these organisms has been determined, and no differences have been seen other than the complete lack of m^5U in the tRNA of Trm$^-$ derivatives (Björk and Neidhardt, to be published).

The phage strain used was T4Bc$^+$; it has no cofactor requirement for adsorption, and was obtained from Dr. S. P. Champe.

For [^{32}P]PO_4 labeling the cells were grown in TG low-phosphate medium (Echols *et al.*, 1961); otherwise the salt medium solution P (Fraenkel and Neidhardt, 1961) was used supplemented with 0.2% ammonium sulfate and 0.2% glucose. Amino acids were supplemented to a final concentration of 50 μg/ml of the L-epimer, if not otherwise stated. The cells were grown aerobically on rotary shakers at 37°C. Optical density at 420 nm (determined in a Zeiss PMQII spectrophotometer with a 1-cm light path) was used to measure the growth of liquid cultures. Under these conditions an OD_{420} of 1.00 corresponds to a culture containing 150 μg total dry weight/ml (Williams and Neidhardt, 1969).

b. *Preparation of T4Bc*$^+$ *DNA.* Phage particles were separated from cell debris by filtration through Celite. The remaining Celite and cell debris were removed by three centrifugations at 5000 rpm in a Sorvall centrifuge. The lysate, at a pH of 7.4 and containing 10 m*M* Mg^{2+}, was incubated with DNase (10 μg/ml) and RNase (10 μg/ml) for 1 hr at 30°C. The solution was centrifuged for 2 hr at 17,000 rpm in a Sorvall centrifuge and the phage pellet slowly resuspended in 10 m*M* Tris–HCl, pH 7.4. The DNA preparation was obtained essentially by the method described by Daniel *et al.* (1968a), except that a pronase treatment (incubation for 2 hr at 37°C with about 66 μg/ml of pronase which had previously been self-digested for 2 hr at 37°C) was included

to reduce RNase activity. The DNA preparation was dissolved in 1/100 × SSC and dialyzed against the same buffer. Incubation of the final T4 DNA preparation for 20 hr at 37°C in 2 × SSC together with ^{14}C-labeled tRNA gave no evidence of RNase activity.

c. *[^{32}P]PO_4 labeling of T4 tRNA.* This labeling was performed essentially according to the procedures described by Daniel *et al.* (1968a). Cells were grown in TG-media with 10^{-4} *M* phosphate to OD_{420} = 0.9 (about 2 × 10^8 cells/ml), and the culture was infected with phage T4Bc$^+$ at a multiplicity of 10. The suspension was incubated for 2 min without shaking and then carrier-free [^{32}P]PO_4 was added (0.5 μCi/ml). The culture was shaken for another 10 min; to inhibit lysis more phage was then added to a final multiplicity of 20, and nonradioactive potassium phosphate buffer, pH 7.0, was added to a final concentration of 0.01 *M*. Survival of bacteria 2 min after the first infection was about 0.2–1.0%. The culture was shaken for another 60 min and the infected cells were rapidly cooled and harvested by centrifugation.

d. *Methylation in vivo of T4 tRNA.* Methionine auxotrophs were grown in the minimal medium described above, but supplemented with L-methionine (6.9 μg/ml), adenine (30 μg/ml), and thiamine (1 μg/ml). The methionine concentration was chosen to support growth to about OD_{420} = 2.6. Adenine was added to repress purine biosynthesis. At OD_{420} = 2.0 (about 4 × 10^8 cells/ml), phage T4Bc$^+$ was added to multiplicity of 6.5 and 2 min later 91 μCi/ml of L-[*methyl*-^{3}H]methionine (5.2 Ci/mmole) was added. After 10 min of infection more phage was added to a final multiplicity of 13 and the infected cells were harvested 12 min after infection by pouring the culture onto ice and collecting the cells by centrifugation. Bacterial survival after 3 min of infection was 0.4–0.7%.

e. *tRNA preparations.* After three phenol extractions of [^{32}P]PO_4-labeled cells, the nucleic acids were precipitated with ethanol. The DNA was collected on a glass rod and the RNA precipitate was collected by centrifugation. The pellet was extracted with 2 ml of 2 *M* LiCl in triethylammonium acetate buffer, pH 5.2, to dissolve the tRNA and 5S rRNA (Avital and Elson, 1969). These RNAs were then reprecipitated by ethanol. The tRNA precipitate was dissolved in 1 ml of 10 m*M* Tris–HCl, pH 7.4 (containing 10 m*M* magnesium acetate) and 0.1 ml of 1% Duponol and 1 ml of 90% phenol were added. The mixture was shaken for about 5 min at room temperature to reduce RNase contamination, and then the RNA was collected from the water phase by ethanol precipitation. The tRNA pellet was washed twice with cold 96% ethanol and then dissolved in 1 ml of the same Tris–HCl buffer as above. Electrophoretically purified DNase (50 μg) was added and the solution was incubated at 37°C for 1 hr. The incubation was stopped by phenol extraction followed by an ethanol precipitation. The pellet was again extracted with 2 *M* LiCl in triethylammonium acetate buffer, pH 5.2, and dialyzed against water for 19 hr.

The tRNA labeled *in vivo* with methyl groups was prepared by resuspending the infected cells in 1 ml of 10 m*M* Tris–HCl, pH 8.0, containing 10 m*M* magnesium acetate. Lysozyme (200 μg/ml) and electrophoretically purified DNase (25 μg/ml) were added, and the suspension was frozen and thawed three times and then incubated at 37°C for 10 min. Three phenol extractions were made, then 1 *M* Tris was added to the combined water phases in order to increase the pH to about 9–9.5 and the RNA solution was incubated at 37°C for 30 min. This treatment should strip most of the radioactive methionine from the tRNA. The RNA solution was then applied to a Sephadex G200 column equilibrated with 0.05 triethylammonium acetate buffer (pH 5.2). This procedure separates ribosomal RNA and tRNA as well as low molecular-weight materials (Boman and Hjerten, 1962). The tRNA was pooled and concentrated with *n*-butanol as described before (Björk and Isaksson, 1970).

f. *Analysis of the distribution of methylated bases in phage-infected cells.* The concentrated tRNA solution was transferred to a small Pyrex tube. The RNA was dried in a stream of air; 20 μl of 1 *M* HCl was added and the tube was sealed. The RNA was hydrolyzed at 100°C for 30 min; this pro-

cedure degrades the RNA to purine bases and pyrimidine nucleotides. Synthetic methylated bases were added as markers, and a portion was subjected to two-dimensional thin-layer chromatography in *n*-butanol–water (86:14, v/v, ammonia in gas phase) (Solvent 1) and isopropanol-concentrated HCl (170:41, water to 250) (Solvent 2). This procedure has been described before (Björk and Svensson, 1967) but some modifications have been used here. Instead of glass plates coated with cellulose, Eastman chromatosheets without fluorescent indicator (Nr 6064) were used. They were developed in Solvent 1 for 3–4 hr and for 6–7 hr in Solvent 2. The separation was as effective as that described before for glass plates (Björk and Svensson, 1967). The different methylated bases were located under ultraviolet light. Instead of scraping off the cellulose as described previously, the chromatograms were sprayed with protective lacquer spray to avoid flaking of cellulose, and the spots were cut out for determination of radioactivity. Since the methylated pyrimidine nucleotides do not migrate in the first solvent they appear together with other charged substances between the origin and the front of the second solvent. This area was divided into segments, each being 0.5 cm wide in the direction of the migration of the second solvent and a histogram of this part of a chromatogram is drawn as shown in Fig. 1. The amount of lacquer is not critical for the efficiency of counting for either ^{3}H or ^{14}C. For ^{3}H this efficiency was 10%. This procedure separates all the methylated purines indicated in Table 2; it is convenient and reproducible, as judged both from the distribution of spots revealed by ultraviolet light and by the distribution of radioactivity in the methylated compounds.

To degrade the tRNA to bases, perchloric acid hydrolysis was employed. The concentrated and carefully dried RNA was hydrolyzed in 20 μl of 70% perchloric acid for 2 hr at 100°C together with 100 μg of *E. coli* tRNA, added as carrier. The hydrolysis was stopped by diluting with 50 μl of water and neutralizing with 10 *N* KOH. The neutralized hydrolyzate was concentrated under a stream of air. It was then dissolved, together with methylated bases as markers, and subjected to two-dimensional thin-layer chromatography in the same solvent as described above. This procedure separates all methylated purines as discussed above, as well as m^5Ura, m^1Ura, m^5Cyt, and m^1Cyt. The method of measuring the radioactivity in the different spots was as described above.

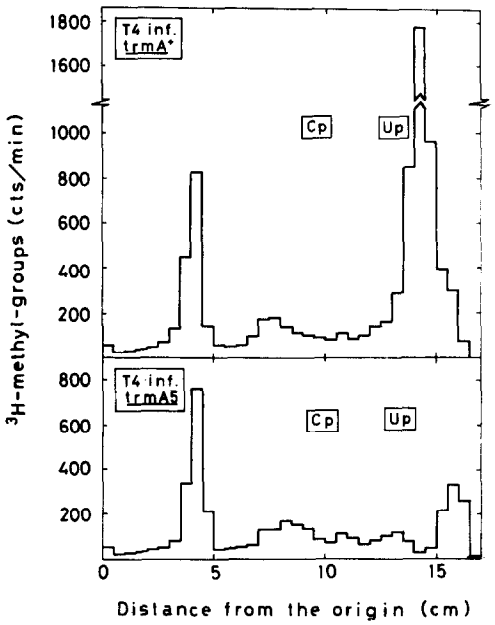

FIG. 1. Thin-layer chromatography of hydrolyzed tRNA labeled *in vivo* with ^{3}H-methyl-groups following infection of phage T4 in *Trm*$^{+}$ and *Trm*$^{-}$ hosts. The radioactive tRNA was hydrolyzed with 1 M HCl and subjected to two-dimensional thin-layer chromatography. The histograms show the compounds migrating only in the second direction (solvent: isopropanol–HCl–water) for tRNA originating from phage T4-infected *E. coli* strains AB1932-5-466 (*trmA*$^{+}$, upper panel) and AB1932-5-451 (*trmA5*, lower panel). After spraying with a plastic lacquer, segments (0.5 × 2.5 cm) were cut out from the chromatosheet and assayed for radioactivity. The activities for each segment are shown. The positions of cytidylic acid (Cp) and uridylic acid (Up) were determined by ultraviolet absorbance. "Unknown 1" in Table 2 migrates after Cp (around 8 cm); "Unknown 2" migrates between mCp and mUp, (around 13 cm); the latter two migrate immediately ahead of Cp and Up, respectively; "Unknown 3" migrates faster than mUp (around 16 cm), and is easily detected in the infected, as well as the uninfected, *Trm*$^{-}$ cells. The peak around 4 cm is part of m^7Gua.

g. *tRNA methylase preparations.* Enzyme

was prepared from spheroplasts to reduce the RNase level. The technique has been described before (Björk and Isaksson, 1970) but the ribosomes were here removed by centrifugation at 105,000*g* for 2.5 hr in 0.01 *M* magnesium concentration. To the supernatant liquid streptomycin sulfate was added to a final concentration of 20 mg/ml; the precipitate was removed by centrifugation. After a 40–70% ammonium sulfate fractionation the enzyme preparation was dialyzed for 16 hr. The preparation had a A_{280}/A_{260} ratio of about 1.0 which indicates a nucleic acid content of about 3%.

h. *Methylation of tRNA in vitro and isolation of the methyl-labeled product.* The incubation mixture was the same as described before (Björk and Isaksson, 1970) except for a higher concentration of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (7.8 nmoles/ml, 8.5 Ci/mmole). The reaction mixture contained 2.2 mg protein/ml for the two first 4 hr and then more enzyme (0.7 mg/ml) and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (to a final concentration of 9.5 nmoles/ml) were added and the incubation was continued for another 2 hr. The incubation temperature was 37°C. Samples withdrawn at 4 hr and 6 hr of incubation suggested that the methylation reaction had virtually terminated after 4 hr of incubation.

The reaction was stopped by phenol extraction and the tRNA was precipitated with ethanol. The tRNA was dissolved in 1 ml of 10 m*M* Tris–HCl, pH 7.5, containing 10 m*M* magnesium acetate. Fifty micrograms of electrophoretically purified DNase was added and the solution was incubated for 1 hr at 37°C. Preincubated pronase (100 μg) was added and the incubation was continued for another 4 hr at 37°C. The solution was made 0.02 *M* in EDTA and then extracted twice with phenol. The tRNA was precipitated with ethanol, washed three times with 2 ml of cold 67% ethanol, and finally dissolved in 6 × SSC. These tRNA preparations had less than 0.1% of alkali-stable material.

i. *Hybridization.* The procedure used was that described by Gillespie and Gillespie (1971) or by Gillespie and Spiegelman (1965), including a 1-hr treatment at room temperature with pancreatic RNase which had been incubated for 10 min at 90°C at pH 5.4. The hybridization was performed at 35°C for 20 hr in 3 × SSC and 50% in formamide, or at 65°C for 18 hr in 2 × SSC.

j. *Other methods.* Protein was determined by the method of Lowry *et al.* (1951) and RNA concentration was determined by measuring the absorption at 260 mμ in a Zeiss PMQII spectrophotometer. A solution of 1 mg RNA/ml gives an absorption of $A_{260} = 25$ (1-cm light path).

k. *Materials.* Pronase (Protease VI, from *Streptomyces griseus*), lysozyme, and protective lacquer spray were obtained from Sigma Chemical Co., St. Louis, Missouri. Electrophoretically purified deoxyribonuclease was from Worthington Biochemical Corp., Freehold, NJ. Methylated bases were purchased from Cyclo Chemical, Los Angeles, CA. Carrier-free $[^{32}P](PO_4)^{3-}$, L-[*methyl*-^{3}H]methionine (5.2 Ci/mmole), *S*-adenosyl-L-[*methyl*-^{14}C]methionine (58 mCi/mmole), and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (8.5 Ci/mmole) were obtained from Amersham/Searle.

RESULTS

We have chosen three different approaches to study whether or not T4-specific tRNA normally contains m^{5}U, and if so, whether or not the host enzyme is used for the modification: (a) we compared the T4-specific tRNA (hybridized to T4 DNA) made in a Trm$^+$ host with that made in a Trm$^-$ host with respect to its ability to be methylated *in vitro*, (b) we compared the distribution of the methylated bases in T4 tRNA originating from a Trm$^+$ or a Trm$^-$ host labeled *in vivo*, and finally (c) we looked for a possible phage-induced tRNA (m^{5}U)methylase in a host completely lacking such an enzymatic activity.

a. *Methylation in vitro of T4-specific tRNA.* The cells, *E. coli* AB1932-5-41(trmA$^+$) and *E. coli* AB1932-5-39(*trmA5*), were grown in TG media and infected with T4Bc$^+$ and 2 min later incubated with $[^{32}P]PO_4$ for a 10-min period. To minimize the label in mRNA, the cells were superinfected with T4Bc$^+$ and incubated for 60 min with nonradioactive phosphate after the 10-min labeling period. The cells were then harvested and the tRNA was prepared from them as

described in Materials and Methods. The two tRNA preparations, were then methylated *in vitro*, under conditions which saturate all added tRNA with ^{3}H-methyl groups. The tRNA was reisolated with phenol extraction and then treated with DNase and pronase to minimize the contamination of DNA and proteins. Radioactivity of the tRNA was measured at this stage. The T4 tRNA from this mutant had a ^{3}H/^{32}P ratio of 3.2 while that from the wild type had a ratio of 0.05. A saturation level of about 0.5 methyl groups per tRNA chain has been obtained and this is of the same magnitude as has been reported before for tRNA from Trm$^-$ cells (Björk and Isaksson, 1970). This suggests that phage T4 does not induce a T4-specific tRNA (m^{5}U) methylase which is able to methylate the methyl-deficient tRNA in the Trm$^-$ host. The tRNA was then hybridized to T4Bc$^+$ DNA as described in Materials and Methods and the results are shown in Table 1.

It is apparent that the ^{3}H/^{32}P ratio is different between tRNA originating from Trm$^+$ and Trm$^-$ cells. Only the T4-specific tRNA which had been synthesized in a Trm$^-$ mutant could be methylated *in vitro*, and, therefore, it seems that T4-specific tRNA normally contains m^{5}U, and that a functional host enzyme is used to make this m^{5}U. Since the ^{3}H counts in the hybrid could be the result of unspecifically bound host tRNA methylated *in vitro*, it was important to show that host tRNA did not significantly bind to the filters. As shown in Table 1, less than 0.002% hybridization could be demonstrated between *E. coli* tRNA and phage

TABLE 1

HYBRIDIZATION BETWEEN T4 DNA AND T4 tRNA FROM Trm$^+$ AND Trm$^-$ HOSTS METHYLATED *in Vitro* WITH ^{3}H-METHYL GROUPS[a]

Exp. No.	Source of tRNA	T4 DNA per filter (μg)	^{32}P (cpm)	^{3}H (cpm)	^{3}H ^{32}P	% Hybridization using sp act based on (μg RNA/100 μg T4 DNA)	
						^{32}P data	^{3}H data
1	From T4-infected AB1932-5-41(Trm$^+$)	90	91	5	0.06	0.03	—
2	From T4-infected AB1932-5-41(Trm$^+$)	45	61	1	0.02	0.04	—
3	From T4-infected AB1932-5-41(Trm$^+$)	23	36	0	0	0.04	—
4	From T4-infected AB1932-5-39(Trm$^-$)	90	126	141	1.1	0.04	0.01
5	From T4-infected AB1932-5-39(Trm$^-$)	45	56	61	1.1	0.03	0.01
6	From T4-infected AB1932-5-39(Trm$^-$)	23	24	51	2.1	0.03	0.02
7	From uninfected AB1932-5-41	45	3	—	—	<0.002	—

[a] Values are the average of close quadruples and they have been corrected for radioactivity bound to filters containing no DNA, amounting to 52 ^{32}P-cpm and 62 ^{32}P-cpm, when tRNA originated from T4-infected AB1932-5-41(*Trm*$^+$) and AB1932-5-39(Trm$^-$), respectively. ^{3}H-counts per minute has been corrected for blanks (21 cpm and 26 cpm, respectively) and appearance of ^{32}P counts in the ^{3}H channel (1.6% of the ^{32}P-cpm). Input for each filter was 35,600 ^{32}P-cpm (10.4 μg) and 2000 ^{3}H-cpm for tRNA from T4-infected AB1932-5-41 (*Trm*$^+$), and 43,300 ^{32}P-cpm (11.2 μg) and 162,000 ^{3}H-cpm for tRNA from AB1932-5-39(*Trm*$^-$). In the case of tRNA labeled *in vivo* with [^{32}P]PO_4 from uninfected *E. coli* (Exp. 7), the values are the average of close triplicates and the input was 514,000 cpm (137 μg) for each filter. These values have been corrected for radioactivity bound to filters containing no DNA (95 cpm). Hybridization was performed in 2 ml of 6 × SSC-formamide (1:1) for 20 hr at 37°C (Gillespie and Gillespie, 1971) and all samples are counted in a liquid scintillation counter for 40 min or 10,000 counts.

DNA, in agreement with the 0.001% previously reported (Daniel *et al.*, 1968a). When the calculations are based on ^3H counts the percentage of hybridization between T4 tRNA and T4 DNA found was about 5–50 times higher (0.01–0.02%) than the unspecific binding. If this latter hybridization was the result of unspecifically bound host tRNA, 330 cpm [^{32}P] would be expected in Experiment 7 (see Table I). This is not the case, and therefore it is likely that the ^3H counts bound to the filter in Exp. 4–6 with the T4 tRNA synthesized in the Trm$^-$ host represent T4-specific tRNA, that has been methylated *in vitro*.

This experiment has been repeated using the hybridization technique previously used by Daniel *et al.* (1968a); that is, in 2 × SSC at 65°C for 18 hr. (Results not shown). Our percentage of hybridization between T4 tRNA and T4 DNA agreed well with those reported by Daniel *et al.* and also this time radioactive methyl groups were bound to T4 DNA only when the T4 tRNA originated from the Trm$^-$ host. The percentage of hybridization calculated from the amount of radioactive methyl groups bound to the filter in this experiment was about 5–10 times higher than the level of unspecific binding of *E. coli* tRNA.

The above data suggest that T4-specific tRNA contains m^{5}U and that the synthesis of m^{5}U on the preformed polymer requires a functional host tRNA (m^{5}U)methylase.

b. *Distribution of methylated compounds in T4 tRNA labeled in vivo.* If phage T4 induces a tRNA(m^{5}U) methylase, m^{5}U should be found in T4 tRNA even in a host which completely lacks this enzyme. The use of radioactive methionine of high specific activity in an *in vivo* labeling experiment should reveal even a small amount of m^{5}U.

Two *E. coli* strains AB1932-5-466(*metA*,-*trmA*$^+$) and AB1932-5-451(*metA*,*trmA5*), were grown in 25 ml of minimal medium containing sufficient L-methionine (6.9 μg/ml) to support growth to OD_{420} = 2.6. At OD_{420} = 2.0, T4Bc$^+$ was added at a multiplicity of infection of 6.5, and 2 min later L-[*methyl*-^3H]methionine (5.2 Ci/mmole, 91 μCi/ml) was added. Since the amount of cold methionine left at the time of infection was known, the final specific activity could be calculated, and was found to be 3,210 mCi/mmole. After 3 min of infection the bacterial survival was 0.4% for strain 5-466 and 0.7% for strain 5-451. After 10 min of labeling the cells were harvested and tRNA was prepared and analyzed as described in Materials and Methods. The results are shown in Table 2, together with the distribution of methylated compounds in uninfected cells. A very low amount of mUp was found in the infected mutant and, as seen in Fig. 1, this low amount is not distributed as a peak, but consists of radioactivity scattered between the region for "Unknown 2" and "Unknown 3" in the area where mUp should migrate. Figure 1 strongly suggests that no mUp is present in tRNA in the phage-infected mutant and since m^{5}Up is the most abundant compound in the mUp region, this then suggests that no m^{5}Up is formed in the mutant. This was verified by chromatography of perchloric acid hydrolyzate of the tRNA under conditions that separate m^{5}Ura and m^{1}Ura. When tRNA from strain AB1932-5-466 (Trm$^+$) was analyzed this way, 6,020 cpm were recovered and 53% of this was found in m^{5}Ura, and no radioactivity was found in m^{1}Ura. When tRNA from the phage-infected mutant (Trm$^-$) was analyzed in a similar way, 3350 cpm were recovered of which none was found in either m^{5}Ura or m^{1}Ura. Therefore, if the mUp value is corrected for "Unknown 3" contaminants, it is a direct measurement of m^{5}Up, since no m^{1}Ura was found in the two cases. The only difference between the distribution of methylated compounds in infected Trm$^+$ and infected Trm$^-$ was in the level of m^{5}Ura. As seen in Table 2, the two independent analyses after T4 infection show good agreement except for this compound. The small amount of m^{2}Gua and m_2^6Ade might originate from small pieces of degraded rRNA originating from uninfected survivors, since these bases are normally found in the rRNA fraction. These results clearly show that no m^{5}U (i.e., less than 0.2% of the level found in Trm$^+$ host) is found in tRNA from phage-infected Trm$^-$ cells, strongly suggesting that T4 does not induce its own tRNA(m^{5}U)methylase, but requires a functional host enzyme to synthesize m^{5}U in its own tRNA. From the bac-

TABLE 2

DISTRIBUTION OF METHYLATED CONSTITUENTS IN tRNA OF Trm$^+$ AND Trm$^-$ CELLS LABELED *in Vivo* BEFORE AND AFTER PHAGE T4 INFECTION[a]

Compound	trmA$^+$ Cells		trmA5 Cells	
	−T4	+T4	−T4	+T4
m_2^6Ade	<0.1	0.6	<0.1	0.5
m^6Ade	3.0	1.7	3.0	1.8
m^2Ade	11	9.0	11	9.0
Ade + m^1Ade	<0.1	<0.1	<0.1	<0.1
m_2^2Gua	<0.1	<0.1	<0.1	<0.1
m^2Gua	<0.1	0.6	<0.1	0.9
m^7Gua	29	98	29	101
m^1Gua	6.5	11	5.9	12
"Unknown 1"	11	44	12	43
mCp	6.7	7.0	5.5	7.5
"Unknown 2"	4.9	17	6.1	19
mUp	49	197	0.8[b]	3.5[b]
"Unknown 3"	1.4	—	4.1	41

[a] All of the values in the table are expressed as picomoles per microgram tRNA from −T4 and per 100 μg from +T4. Data for uninfected *trmA*$^+$ and *trmA5* cells are averages of three and two independent determinations, respectively, of the distribution *in vivo* of methylated compounds from tRNA. (Data from Björk and Neidhardt, to be published.) Cells were grown in medium containing L-[methyl-^{14}C]methionine for several generations, total RNA was extracted and tRNA purified by Sephadex G200 molecular sieve chromatography. tRNA was hydrolyzed with 1 *M* HCl and the products were subjected to thin-layer chromatography. This procedure separates the methylated purines indicated in the table (Björk and Svensson, 1967). The radioactivity in the different areas of the chromatogram was determined and calculated as picomoles/μg tRNA applied using m^2Ade as an internal standard.

The analyses of methylated constituents from T4Bc$^+$-infected *trmA*$^+$ and *trmA5* cells (strain AB1932-5-466(*trmA*$^+$*argH*$^+$,metA) and AB1932-5-451(*trmA5*,*argH*$^+$,*metA*), were performed as described in Materials and Methods. The value of m^2Ade is the average value of the two independent determinations, 9.9×10^{-2} pmoles/μg tRNA for AB1932-5-466 and 8.1×10^{-2} pmoles/μg tRNA for AB1932-5-451. Each analysis is an average of three repeated analyses; between 6,000 and 15,000 cpm (corresponding to 2.6–4.8 μg tRNA) was applied to each chromatogram and the recovery was 78–100%.

Compounds labeled "Unknowns" all migrate only in the second solvent. A histogram of that part of a chromatogram is shown in Fig. 1, and in the text of this figure the different "Unknowns" mCp and mUp are defined.

[b] Values significantly different from the corresponding value in Trm$^+$.

terial survival (0.4% for Trm$^+$), the generation time (50 min), the labeling time, the density of methyl groups per tRNA chain in *E. coli*, the amount of tRNA in each cell (39 μg/mg dry weight; Forchhammar and Lindahl, 1971), the specific activity of the radioactive methionine, and the total radioactivity in the tRNA preparation (3.12×10^6 dpm), the amount of radioactivity attributed to the tRNA from the survivors could be calculated and was found to be about 6% of the total radioactivity in the tRNA. Although host RNA synthesis does not cease immediately after T4 infection, most of the RNA synthesized is T4 specific (Landy and Spiegelman, 1968). It is known that T4 mRNA does not contain any methylated bases (Moore, 1966), and since, furthermore, the tRNA was purified by Sephadex G200 molecular sieve chromatography, we think that most of the radioactivity in the tRNA preparations must be associated with T4-specific tRNA.

According to Moore (1966), the amount of methyl groups incorporated into 4S RNA relative to the incorporation of uridine during a 10-min labeling period after T4 infection (from the 3rd to the 13th minute) was the same as that in a similar experiment with uninfected cells. This suggests that the T4-specific tRNA has about the same density of methyl groups as tRNA from uninfected *E. coli*. Assuming this is true, the amount of T4-specific tRNA made between 2 min and 12 min after infection is about 3% of total tRNA. This is in agreement with the calculated value of less than 5% reported for T2-infected cells (Smith and Russel, 1970). Using this estimated amount of T4-specific tRNA, the absolute values for the distribution of methylated compounds in T4-specific tRNA can be calculated, and the result of such a calculation is found in Table 3. These values have been corrected for the small contribution from the methylated compounds originating from the tRNA of the survivors.

The results in Table 3 indicate that a lower absolute level of both m^6Ade and m^2Ade is found in T4-specific tRNA than in *E. coli* tRNA. However, the absolute level of m^5U is about the same, and the values correspond to about 1.1 and 1.2 m^5U per tRNA chain for uninfected and infected *E. coli*, respectively. This would suggest that T4-specific tRNA contains one m^5U per tRNA chain, as has been suggested for *E. coli* tRNA. Since m^6Ade and m^2Ade and other minor methylated compounds do not exist in every tRNA chain, their values expressed as picomoles/microgram tRNA will depend heavily on which tRNA molecules are synthesized by T4. This might explain why the level of some methylated constituents deviate from the value found in *E. coli* tRNA; in other words, the values may reflect a change in the substrate for the methylases rather than in the enzymes themselves.

c. *tRNA(m^5U)methylase activity after T4 infection in Trm$^+$ and Trm$^-$ hosts.* As shown before, *trmA*-mutants lack m^5U in their tRNA, and enzyme extracts from such mutants are unable to methylate tRNA from other *trmA*-mutants; suggesting that the *trmA* gene is probably the structural gene for the tRNA(m^5U)methylase (Björk and Isaksson, 1970). Since there is no interfering host activity, such mutants were used in investigating whether or not T4 induces tRNA(m^5U)methylase. Using tRNA from a *trmA5* mutant, a T4-induced enzyme was looked for in strain AB1932-5-39(*trmA5*) at various times after T4Bc$^+$ infection. As seen in Table 4, no activity could be demonstrated. Since it is known that some tRNA methylases (but not host tRNA(m^5U)-methylase) migrate with the ribosome fraction when centrifuged at high speed (Sarkar and Comb, 1966), both crude extracts and ribosome-free extracts were examined. In this way we would have detected a phage-induced enzyme if it were similar to other tRNA methylases. The residual activity seen in crude extracts from the mutant, both uninfected and infected, is the normal noise

TABLE 3

COMPARISON OF *in Vivo* METHYLATED CONSTITUENTS IN *Escherichia coli* tRNA AND CALCULATED VALUES FOR PHAGE T4-SPECIFIC tRNA

Compound	Methylated constituents (pmoles/μg tRNA)	
	E. coli tRNA[a]	T4-specific tRNA[b]
m^6Ade	3.0	0.4
m^2Ade	11	2.2
m^7Gua	29	30
m^1Gua	6.5	3.2
"Unknown 1"	11	14
mCp	6.7	1.9
"Unknown 2"	5.0	5.2
m^5Up	45	48
"Unknown 3"	4.6	13

[a] Values for *E. coli* tRNA have been taken from Table 2.

[b] Values for T4-specific tRNA have been calculated from data in Table 2 as described in the text. m^5Up values are mUp corrected for the amount of "Unknown 3" (cf. Fig. 1) found in *trmA5* cells.

TABLE 4

SPECIFIC ACTIVITY OF tRNA(m^5U)METHYLASE IN AB1932-5-41(*Trm$^+$*) AND AB1932-5-39(Trm$^-$) BEFORE AND AFTER T4 INFECTION[a]

Enzyme extract from	tRNA(m^5U)methylase activity (units/g protein)	
	Crude enzyme extract	Crude enzyme extract with ribosomes removed
AB1932-5-41(Trm$^+$),		
Uninfected	276	394
30 min after T4 inf.	255	361
AB1932-5-39(Trm$^-$),		
Uninfected	13–35	—
16 min after T4 inf.	14	—
30 min after T4 inf.	—	0–23
70 min after T4 inf.	21	—

[a] The amount of enzyme which transfers 1 nmole of methyl groups to tRNA from *trmA5* cells in 10 min at 37°C is defined as one unit. The enzyme was prepared from cells grown in minimal medium as described earlier (Björk and Isaksson, 1970), but 10% ethylene glycol was included in the buffer and neither spheroplast formation nor ammonium sulfate fractionation was performed. When ribosomes were removed the extract was centrifuged for 1 hr at between 192,000 and 275,000*g*.

level and is attributed to other tRNA methylases since it is conceivable that the tRNA preparation used contained small amounts of generally methyl-deficient tRNA, which other methylases can use as substrate. Therefore, these results indicate that T4 does not induce a tRNA-(m^5U)methylase activity with similar specificity as the host enzyme.

It is known that T4 alters the *E. coli* valyl-tRNA synthetase (Neidhardt and Earhart, 1966), and, therefore, some experiments were performed to see if a similar alteration might occur in the tRNA(m^5U)-methylase. No major differences between this methylase from infected and uninfected wild-type cells were seen in sedimentation behavior in a 5–20% sucrose gradient, specific activity after phage infection (see Table 4), K_m for *S*-adenosyl-L-methionine, heat stability at 50°C, or stability in 2 *M* or 4 *M* urea. Stability during storage at +4°C was greatly increased after T4 infection, but it is not certain that this reflects a change in the tRNA(m^5U)methylase molecule. A set of control experiments were performed by mixing uninfected wild-type cells with T4-infected mutant cells and vice versa. Extracts were prepared from these mixed cultures and the results indicated that some T4 proteins unspecifically stabilize the tRNA-(m^5U)methylase during storage at +4°C. Although analysis of these several physical properties gave no indication for a phage alteration of the host tRNA(m^5U)methylase, such a phenomenon has not been ruled out by these findings.

DISCUSSION

Several modifications in tRNA have been shown to occur during phage infection (see review, Daniel *et al.*, 1970). It was not at first known whether these changes involved synthesis of T4-coded tRNA or modification of preexisting host tRNA. Some of these changes have subsequently been shown to be due to modification of host tRNA (Kano-Sueoka and Sueoka, 1968) and others to the synthesis of T4 coded tRNA (Daniel *et al.*, 1968a; Waters and Novelli, 1967; Weiss *et al.*, 1968). It seems as if the phages have utilized both possibilites to change the cellular environment to their own specific needs.

One way to change the preexisting tRNA is by inducing a new tRNA-modifying enzyme, or altering such a host enzyme. Changes in the activity and of physical properties of methylating enzyme have been reported after infection or induction of phages (Wainfan *et al.*, 1965 and 1966; Wainfan, 1968). Gold *et al.* (1964), could not see any change in tRNA methylase activity after T2 or T4 infection, and their finding is in agreement with our results that the specific activity of tRNA(m^5U)methylase did not change after phage infection more than could be attributed to the production of phage proteins. An economical way for the phage to develop a suitable enzyme is by altering an existing one. Such a phenomenon has been described for valyl-tRNA synthetase after phage T-even infection (Neidhardt and Earhart, 1966), but no effect was detected for the phenylalanine-, glycine-, and histidine-activating enzymes (Earhart and Neidhardt, 1967). Although our results suggest that no tRNA(m^5U)methylase is induced by T4, a phage-directed alteration of the host enzyme, as suggested for tRNA methylases by the results of others, might occur. However, the constancy of a number of investigated physical properties suggests, but does not rule out, that no such phage-directed alteration occurs in the case of the tRNA(m^5U)methylase.

Minor nucleosides from T4-coded tRNA have not been extensively analyzed, but the presence of thiolated nucleosides and pseudouridine has been established (Daniel *et al.*, 1968a; Weiss *et al.*, 1968). Of the methylated nucleosides, only m^5U has been characterized and shown to be synthesized *in vivo* after T4 infection, but it was not ruled out that this m^5U was part of host tRNA (Boezi *et al.*, 1967). Smith and Russel (1970) studied the methylation of T2 tRNA but they did not analyze the distribution of the different methylated nucleosides. We have presented here two different sets of results which support the conclusion that T4-specific tRNA contains m^5U. The first is the ability of T4-specific tRNA to accept methyl groups *in vitro* only when it originates from a Trm^- host and not when it originates from wild-type cells (Table 1). This tRNA was measured as RNA-T4 DNA hybrids, and the

results indicate that T4-coded tRNA contains m^5U. In the hybridization experiment, 0.01–0.02% hybridization based on ^3H counts (methyl groups) was found and since only less than 0.002% hybridization with host tRNA occurred, this result suggests that the T4-specific tRNA had accepted methyl groups rather than that unspecifically bound host tRNA contributed these ^3H-counts. The magnitude of the nonspecific hybridization between T4 DNA preparation and host tRNA found is in agreement with a reported result of 0.001% (Daniel *et al.*, 1968a). Therefore, it is concluded that T4-specific tRNA can be methylated *in vitro* only when it has been synthesized in a Trm^- hosts.

The second result which supports the above conclusion comes from the analysis of T4 tRNA labeled *in vivo* with [^3H]methyl groups both in Trm^+ and Trm^- hosts. The results in Fig. 1 and Table 2 clearly demonstrate that m^5U was not formed in the Trm^- host, but was formed in the Trm^+ host. Together with the hybridization data discussed above, these results show that T4-specific tRNA normally contains m^5U and that a functional tRNA(m^5U)methylase of the host is necessary for its modification. This is consistent with our enzymatic assays, which failed to demonstrate any phage-induced tRNA(m^5U)methylase activity in the Trm^- mutant after phage infection.

This conclusion is based on the assumption that there is no interference between a product of the *trmA5* mutation and a hypothetical T4-induced tRNA(m^5U)methylase. This assumption is strengthened by the fact that there is no interference *in vitro* between enzyme extracts from Trm^+ and Trm^- cells. Furthermore, since we have shown that T4-specific tRNA can be methylated *in vitro* by host enzyme (Table 1), it is likely that a hypothetical T4-induced tRNA(m^5U)methylase should be able to use a tRNA from Trm^- cells as substrate. Using such substrate no phage-induced increase in the activity of tRNA(m^5U)methylase in Trm^+ cells was seen (Table 4). Thus, there is no indication of such phage-induced enzyme, irrespective of the validity of the assumption mentioned above.

Since the contribution of radioactivity by the bacterial survivors could be estimated, we were also able to calculate the absolute amount of different methylated compounds in T4-specific tRNA. This calculation was based on the assumption that T4-specific tRNA has about the same methyl group density as *E. coli* tRNA (Moore, 1966). Since host RNA synthesis in phage-infected cells does not stop immediately, there might be some host tRNA synthesized even in phage-infected cells. From the known distribution of methylated compounds in tRNA from *E. coli* and the fact that the lowest level of a methylated constituent in T4-specific tRNA is zero, it is possible to calculate that the *maximum* amount of radioactive host tRNA in our tRNA preparation was about 25% of total radioactivity. It should be pointed out that this value most probably is an overestimation since it implies that one of the most common methylated nucleosides (m^2A) in *E. coli* tRNA is completely absent in T4-specific tRNA. Landy and Spiegelman (1968) have shown that in a 2-min pulse from 3–5 min after T4 infection, between 13 and 16% of the RNA synthesized is host specific, but that after this time of phage infection no host-specific RNA is synthesized. A correction for the host tRNA synthesized in infected cells only in the beginning of the labeling period does not greatly influence our estimation. Therefore, we believe that the results in Table 3 give a good estimation of the amount of each of the different methylated compounds in T4 tRNA. From these calculations the amount of m^5U per T4-specific tRNA chain was found to be about 1.2, strongly suggesting that T4-specific tRNA, like *E. coli* tRNA, has one m^5U per chain. A comparison of the level of other methylated nucleosides after phage infection revealed that some stayed about the same (m^7Gua, and m^5Up), while others either decreased (m^6Ade, m^1Gua and m^2Ade) or increased ("Unknown 3"). Such a result could easily be caused by a change in the species of tRNA produced after T4 infection, rather than a change in the tRNA methylases themselves. These results do not exclude a possible modification of host enzymes or induction of new enzymes, but, as discussed above, in the case of the tRNA(m^5U)methylase a functional host enzyme is necessary

for the production of m^5U in the T4-specific tRNA.

It has been reported that T4-specific tRNA, which accepts amino acids *in vitro*, is synthesized even in the presence of chloramphenicol (Scherberg *et al.*, 1970). This would imply that either a posttranscriptional modification is not essential for amino acid activation, or that host enzymes are involved in these modifications. In the case of m^5U both possibilities seem to be true, since the level of m^5U in tRNA from *E. coli* is not critical for the amino acid charging *in vitro* (Svensson *et al.*, 1971) and the functional host enzyme is necessary for the modification to m^5U. Whether a similar situation exists for the other modified nucleosides and corresponding modifying enzymes awaits further investigation.

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