

Ammonium ions prevent methylation of uridine to ribothymidine in *Azotobacter vinelandii* tRNA

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Abstract. It was shown that tRNA from *Azotobacter vinelandii* grown in the presence of ammonium chloride lacks ribothymidine while that grown in the absence of the ammonium salt contains this modified nucleoside. [³²P]-Labelled tRNA from this organism grown in a medium containing the ammonium salt was digested with RNase T₁ and the pseudouridine-containing tetranucleotide, common to all tRNAs was isolated and analysed for the nucleoside replacing the ribothymidine. It was found to be uridine. Cells previously labelled with [³²P]-phosphate in the ammonium salt medium were washed and incubated in the ammonium salt-free medium to test whether ribothymidine would be formed upon removal of the ammonium ions. Methylation of the uridine did not take place.

Keywords. Ribothymidine; uridine methylation; tRNA modification; *Azotobacter vinelandii* tRNA.

Introduction

The metabolism of some of the modified nucleosides in the tRNA of microorganisms is known to be sensitive to culture conditions. Species whose relative proportions change with the growth conditions include 2-methylthio-N⁶-isopentenyl-adenosine (Buck and Griffiths, 1981; Buu *et al.*, 1981), 2-methylthioribosylzeatin (Ajitkumar and Cherayil, 1985a; Buck and Ames, 1984; Morris *et al.*, 1981), 5-methylaminomethyl-2-thiouridine (Chackalaparampil and Cherayil, 1981) and 4-thiouridine (Ajitkumar and Cherayil, 1985b). We have observed that when the nitrogen fixing bacteria, *Azotobacter vinelandii* and *Rizobium meliloti*, are grown in the presence of an ammonium salt the tRNA from these organisms lacks ribothymidine (T). On the other hand, when *A. vinelandii* is grown in the absence of an ammonium salt its tRNA contains T to the extent of 1 mol/mol (Ajitkumar and Cherayil, 1982). T, which occurs at the T ψ C arm of tRNAs is one of the unique modified nucleosides present in most tRNAs, except the initiator methionine tRNA from many eukaryotes (Gauss and Sprinzl, 1983). There are systems in which the tRNA naturally lacks T. Such tRNAs have been shown to be as good as those which contain T in *in vitro* protein synthesis (Svensson *et al.*, 1971). However, it appears that T

Abbreviations used: T, Ribothymidine; ψ , pseudouridine; 2-DTLC, two dimensional thin-layer chromatography.

plays a subtle role in protein synthesis, although its exact function is not known (Kersten *et al.*, 1981). In certain systems T has been found to be replaced with 2'-O-methylribothymidine or 2-thioribothymidine (Gauss and Sprinzl, 1983). The content of the latter has been reported to increase with temperature in certain thermophilic organisms (Watanabe *et al.*, 1980).

The present study was carried out with the main objective of identifying the nucleoside which replaces T when *A. vinelandii* is cultured in a medium containing an ammonium salt.

Materials and methods

Carrier-free $H_3^{32}PO_4$ was obtained from Bhabha Atomic Research Centre, Bombay. RNases T_1 and T_2 were from Sigma Chemical Co., St. Louis, Missouri, USA. Plastic backed thin layer microcrystalline cellulose plates and DEAE-cellulose paper were purchased from Macherey-Nagel, and Schleicher and Schull, Federal Republic of Germany, respectively.

Growth of bacteria and isolation of tRNA

A. vinelandii OP (Wisconsin) was grown in the absence and in the presence of an ammonium salt. In the first case Burk-modified nitrogen-free medium (Strandberg and Wilson, 1967) was used and in the second case 2.8 mg N/ml or 0.49 mg N/ml as NH_4Cl was added to the nitrogen-free medium. Concentrations of the ammonium salt used were sufficient to completely repress the nitrogenase gene (Gordon and Brill, 1972). Cells were grown in low phosphate medium in the presence of $[^{32}P]$ -phosphate and the labelled tRNA was isolated and purified as described previously (Ajitkumar and Cherayil, 1982).

Analysis of $[^{32}P]$ -labelled products

$[^{32}P]$ -Labelled tRNA was converted to nucleotides by digestion with RNase T_2 and to oligonucleotides by digestion with RNase T_1 . Two dimensional thin-layer chromatography (2-D TLC) of nucleotides was carried out on 10×10 cm cellulose plates, 0.1 mm thickness, using the solvent systems isobutyric acid:0.5 M ammonium hydroxide, 5:3 (v/v) in the first dimension and isopropanol:conc. HCl:water, 70:15:15 (v/v/v) in the second dimension (Nishimura *et al.*, 1967). The plates were dried and subjected to autoradiography. Separation of 3'-phosphates of T, uridine and pseudouridine (ψ) was effected by descending paper chromatography on Whatman 1 mm paper using isopropanol:water:conc. ammonia, 70:30:1 (v/v/v) as the solvent system (Brownlee, 1972). High voltage electrophoresis was carried out on Whatman 3 mm paper or DEAE-cellulose paper in acetic acid-pyridine-EDTA buffer, pH 3.5, at 40-60 V/cm using xylene cyanol and acid fuchsin as dye markers (Smith, 1967). Radioactive samples were recovered from the Whatman paper and the DEAE-cellulose paper by elution with water and 1.0 M triethylammonium acetate, pH 10, respectively (Sanger and Brownlee, 1967).

Results

Effect of ammonium salt on ribothymidine production

In the earlier study, it was found that in *A. vinelandii* cells grown with excess of ammonium chloride (2.8 mg N/ml), the tRNA completely lacked T, while the cells grown in the absence of ammonium chloride contained T to the extent of 1 mol/mol of tRNA (Ajitkumar and Cherayil, 1982). In order to find out whether this was due to any effect of the excess of ammonium ions, the cells were grown with much less ammonium salt (0.49 mg N/ml), only slightly higher than that required to repress the nitrogenase gene (Gordon and Brill, 1972). RNase T₂ digest of [³²P]-labelled tRNA isolated from cells grown with low ammonium salt was subjected to 2-D TLC and subsequent autoradiography. The autoradiogram showed the absence of Tp (figure 1A). This was found to be reversible, as cells grown with ammonium salt, when subcultured in the absence of ammonium salt produced Tp after a few generations. A time dependent appearance of Tp could be noted when ammonium salt-grown cells were washed, incubated with [³²P]-phosphate in a nitrogen-free medium and the tRNA isolated was analysed. It took about 6 h incubation for the detection of Tp under the experimental conditions used. On the other hand, in the case of cells previously grown in the absence of ammonium ions the presence of Tp in the tRNA could be noted in about 2 h (figure 1B).

Escherichia coli is known to contain T in its tRNA. In order to test whether the T content would change with the concentration of ammonium salt, cells were grown in a synthetic medium at two concentrations of ammonium chloride (0.49 mg and 2.8 mg N/ml) and the tRNA was analysed. The T content of the tRNA was not all affected by the ammonium ion concentration (results not presented).

Identification of the nucleotide replacing Tp

The absence of Tp in *A. vinelandii* tRNA could be due to the lack of methylation of the uridine residue at the 54th position or further modification of T to either 2'-O-methylribothymidine or to 2-thioribothymidine or to any other unknown species. In order to establish which of the different possibilities existed in the present case, RNase T₁ digest of [³²P]-labelled tRNA from cells grown under the two conditions were fractionated separately to isolate the tetranucleotide containing pseudouridine common to most tRNAs (Zamir *et al.*, 1965; Gauss and Sprinzyl, 1983). The RNase T₁ digest upon electrophoresis on DEAE-cellulose paper at pH 3.5 showed the presence of 9 bands in both the samples (figure 2A). The bands 2 and 3 in the tetranucleotide region (Sanger and Brownlee, 1967) were eluted out, digested to mononucleotides and analysed by 2-D TLC. Only band 2 from both the samples was found to contain ψ p (data not shown). Contamination of band 2 by other species was obvious. Hence it was further purified by electrophoresis on Whatman 3 mm paper at pH 3.5. The autoradiogram indeed showed contamination (figure 2B). The major band (No. 4) was eluted out and analysed for its nucleotide composition.

The purified band was digested with RNase T₂ and an aliquot was subjected to electrophoresis on Whatman 3 mm paper at pH 3.5. Two spots of equal intensity at the region of Cp and Gp and a third with double the intensity at the region of ψ p were

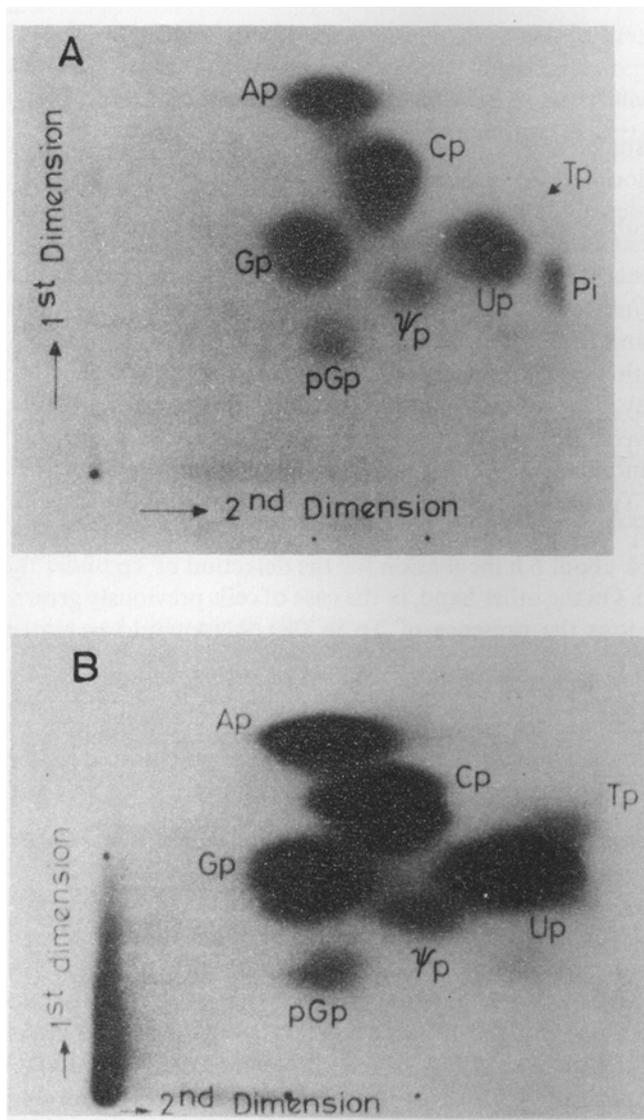


Figure 1. Autoradiogram of 2-D TLC.

RNase T₂ digest of [³²P]-labelled tRNA was subjected to 2-D TLC using isobutyric acid:0.5 M ammonium hydroxide, 5:3 (v/v) in the first dimension and isopropanol:conc. HCl:water, 70:15:15 (v/v/v) in the second dimension.

A. tRNA digest from *A. vinelandii* cells grown with 0.49 mg N/ml of NH₄Cl.

B. Cells grown in the absence of NH₄Cl were washed, incubated with [³²P]-phosphate for 2 h and the labelled tRNA was isolated and analysed.

observed in both the samples (figure 2C). Both samples had a small contamination of Ap. The radioactivity in each of the spots was measured. The relative proportion of the radioactivity in the spots was 1:1:2 (table 1). Tp and p move with a mobility of 0.98 with respect to Up at pH 3.5 (Smith, 1967). Therefore the ratio of radioactivity in the

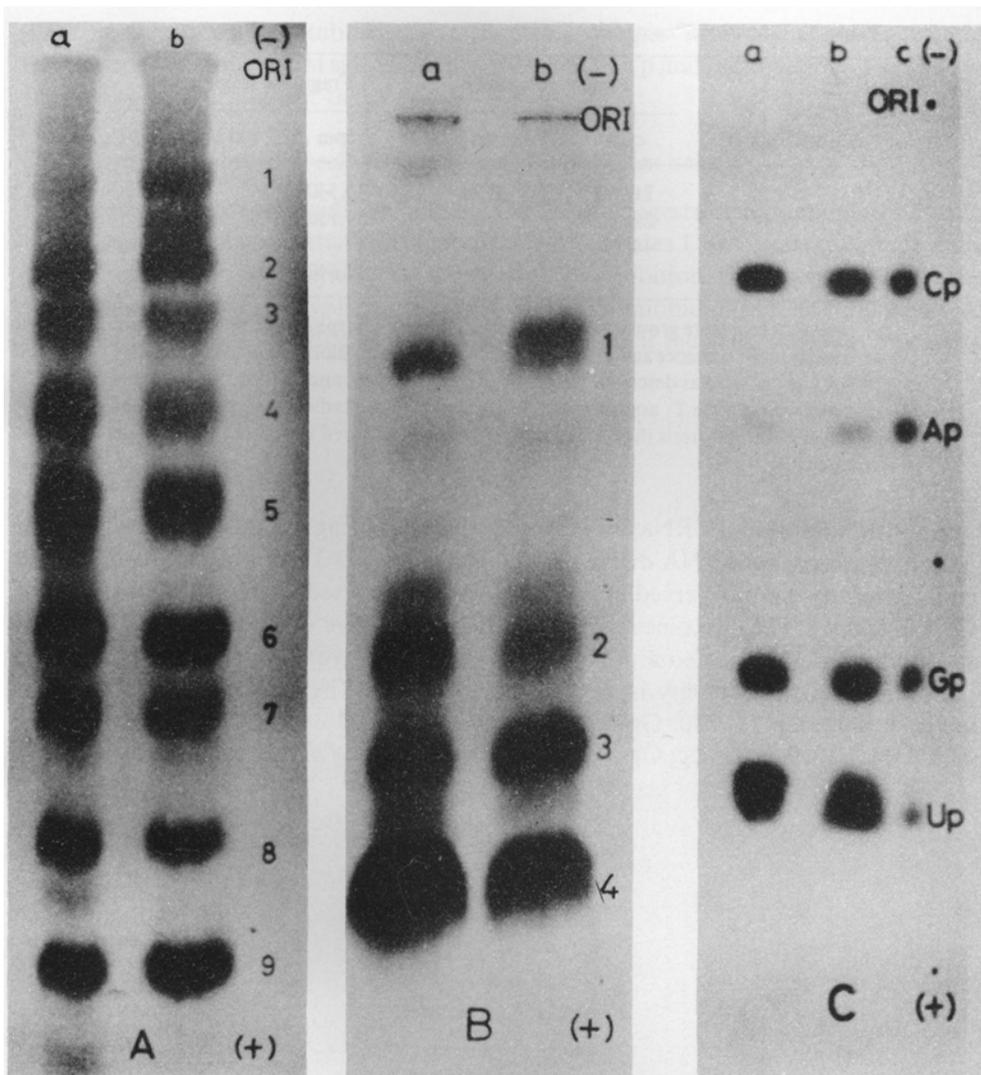


Figure 2. Analysis of the tetranucleotide fragment.

RNase T1 digest of [^{32}P]-labelled tRNA was subjected to electrophoresis on DEAE-cellulose paper (A). The tetranucleotide (band No. 2) was eluted and subjected to re-electrophoresis on Whatman 3 mm paper (B). The purified band (the major band, No. 4) was digested with RNase T2 and an aliquot was subjected to electrophoresis on Whatman 3 mm paper at pH 3.5 (C).

Lane a: tRNA from cells grown in the absence of NH_4^+ ions.

Lane b: tRNA from cells grown in the presence of NH_4^+ ions.

Lane c: Marker nucleotides.

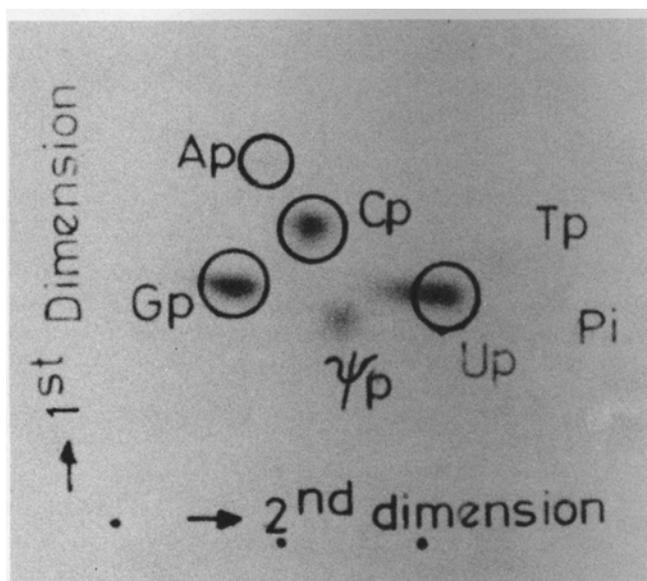
spots (as in table 1) is expected for the tetranucleotide containing Tp. Since the ratio of the mononucleotides in the Tp-lacking tetranucleotide also was 1:1:2 it was evident that the spot due to the mononucleotide that replaced Tp also moved with a mobility similar to that of

Table 1. Nucleotide composition of the tetranucleotide.

Nucleotide spot	Fragment with Tp		Fragment lacking Tp	
	cpm	Ratio (Np:Gp)	cpm	Ratio (Np:Gp)
CP	21,060	0.96	15,540	0.91
Gp	21,960	1.00	17,130	1.00
Tp + ψ p	44,000	2.00	—	—
Unknown + ψ p	—	—	36,640	2.13

A. vinelandii cells were grown with 5 mCi of [32 P] -orthophosphate per 100 ml medium separately in the presence and in the absence of ammonium chloride and the radioactive tRNA was analysed as described in the text. The purified tetranucleotide fragment was digested with RNase T₂ and subjected to electrophoresis. Radioactive spots obtained by paper electrophoresis (figure 2C) were cut out and counted in a liquid scintillation counter.

Another aliquot of the RNase T₂ digest of the Tp-lacking tetranucleotide was mixed with non-radioactive tRNA digest and subjected to 2-D TLC. Four spots of almost equal intensity were observed. Three of them, as expected, were at the positions of Cp, Gp and ψ p. The 4th coincided with the non-radioactive Up spot (figure 3). A minor spot at the region of Tp also could be noted. The radioactive spots were cut out from the plates and the radioactivity in each was determined. The amounts of radioactivity in spots were, Cp 12, 230; Gp 13, 120; Up 14, 370 and p 11, 650, the ratio being 0.93:1.00:1.10:0.89 for Cp, Gp, Up and ψ p. Radioactivity in the region of Tp was only

**Figure 3.** 2-D TLC of RNase T₂ digest of the tetranucleotide.

An aliquot of the RNase T₂ digest of the tetranucleotide (legend to figure 2) was subjected to 2-D TLC on cellulose using the solvent systems given under legend to figure 1.

390 cpm. These results established that the tRNA sample from *A. vinelandii* grown in the presence of ammonium salt had Up in the place of Tp and methylation of uridine at the 54th position had not taken place.

Tests with [³²P]-labelled cells washed free of ammonium salts

Non-methylation of the uridine residue of the tRNA suggested two possibilities. Either the enzyme, uridine-5-methyltransferase which methylates U to T was inhibited or the gene coding for the enzyme was repressed. If inhibition of the enzyme by the ammonium ions was involved, removal of the ammonium salt should result in methylation of the U residue to T. On the other hand, if the gene had been repressed,

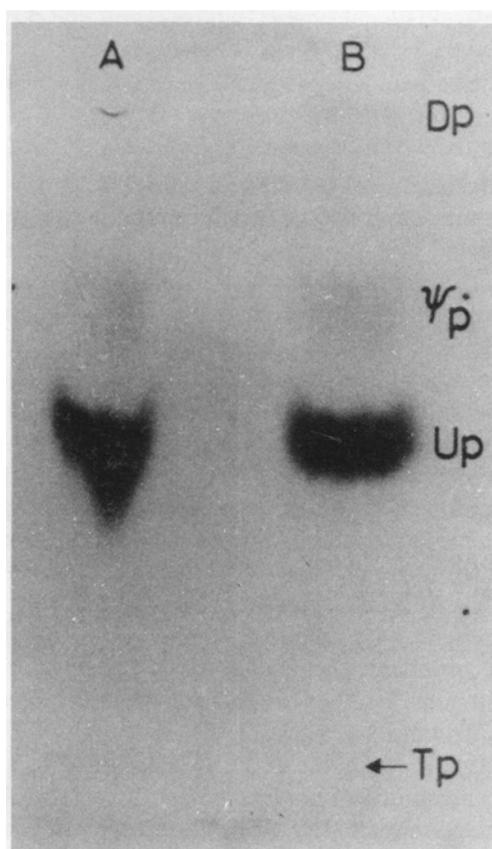


Figure 4. Analysis of RNase T₂ digest of tRNA from cells prelabelled in the presence of ammonium salt.

A. vinelandii cells grown with [³²P]-phosphate in the presence of ammonium salt were washed and incubated in the presence or absence of rifampicin for 2 h. tRNA isolated from the cells was digested with RNase T₂ and subjected to electrophoresis. The radioactivity from the Up region was subjected to paper chromatography in the solvent system isopropanol:water:conc. ammonia, 70:30:1 (v/v/v). **A.** In the presence of rifampicin. **B.** In the absence of rifampicin.

fresh enzyme synthesis after removal of the ammonium ions would be required for the formation of T. The following experiment was carried out to distinguish between these two possibilities. *A. vinelandii* cells grown to mid-log phase with [^{32}P] -phosphate in excess of ammonium salt were washed twice with normal saline and these prelabelled cells were incubated in a nitrogen-free medium containing 100 $\mu\text{g/ml}$ of rifampicin for a period of 2 h. Rifampicin addition was to prevent fresh synthesis of the enzyme. tRNA was isolated and analysed for Tp; it could not be detected (figure 4A). Prolonged incubation with rifampicin resulted in the lysis of the cells. Cells, incubated without rifampicin also did not contain Tp in the tRNA (figure 4B). However it was noticed earlier that cells previously grown in the absence of fixed nitrogen treated in a similar manner, produced Tp in the tRNA in about 2 h (figure 1B). These results suggested that the absence of Tp in the tRNA from cells grown in the presence of an ammonium salt was not due to reversible inhibition of the modifying enzyme by the ammonium ions.

Discussion

It is interesting that the tRNA of *A. vinelandii* lacks T when the organism is grown in media containing an ammonium salt. Lack of T cannot be due to any defect in the genes involved in the synthesis of T as *A. vinelandii* cells produce T when grown in the absence of ammonia. *R. meliloti*, a symbiotic nitrogen fixing bacterium also has been found to lack T in the tRNA when cultured in the presence of ammonium chloride (Ajitkumar and Cherayil, 1982). Being a symbiotic nitrogen fixer *R. meliloti* could not be cultured in the absence of fixed nitrogen. T content of *E. coli* tRNA was not at all affected by the change in the ammonium ion concentration (results not presented). It is tempting to speculate that the phenomenon of the absence of Tp in the tRNA when the organism is grown in the presence of an ammonium salt is characteristic of nitrogen-fixing bacteria. More such bacteria however, have to be studied to establish this.

Most tRNA species contain the tetranucleotide, T⁺~CG (Zamir *et al.*, 1965). Analysis of the tetranucleotide from *A. vinelandii* tRNA has shown that it contains U instead of T when the organism is grown in the presence of ammonium ions (figures 2 and 3). Since the amount of radioactivity in the tetranucleotide was comparable in both cases (see table 1) U CG is the prominent tetranucleotide in the tRNA from cells grown in the presence of ammonium ions. 2-D TLC analysis of the tetranucleotide digest shows the presence of a very small quantity of Tp, approximately 3 % of Gp (see text and figure 3). This suggests that even in the presence of the ammonium salt a small quantity of Tp is synthesized. This amount could not be detected in the total tRNA (figure 1 A) but could be detected when it became enriched by the purification of the tetranucleotide fragment (figure 3). Much larger amount of labelled tRNA than that for 2-D TLC (figure 1) was used for the analysis of the tetranucleotide fragment (figures 2 and 3).

If the enzyme that methylates the U residue was inhibited by the NH_4^+ ions, removal by washing would have prevented inhibition and methylated the U residue. As the tRNA was prelabelled with [^{32}P] -phosphate formation of T would have resulted in the appearance of a radioactive spot due to Tp. Since this was not observed (figure 4) it is to be concluded that the enzyme was not present in the cells or it was irreversibly inhibited. In the case of cells previously grown in the absence of ammonium ions T is produced

under similar conditions of washing and incubation with [32 P] -phosphate (figure 1B). Under the experimental conditions used in the present studies T will be formed only if the enzyme can methylate the processed tRNA. Any precursor of tRNA would be changed to the mature species by the time the cells are harvested and washed (see above). Since formation of T is observed in one case it is to be concluded that the *A. vinelandii* enzyme, uridine-5-methyltransferase, can methylate the mature tRNA. In this respect it appears to be similar to the *E. coli* enzyme which can methylate mature tRNA₁^{Gly} from wheat germ (Marcu *et al.*, 1978).

It is clear from the present results that the ammonium salt is not a reversible inhibitor of uridine-5-methyltransferase of *A. vinelandii*. However it is not clear whether it is an irreversible inhibitor or a repressor. Ammonium salt is known to be a repressor of a number of enzyme systems such as nitrogenase, glutamine synthetase and nitrate reductase (Brill, 1980; Shanmugam and Hennecke, 1979). Probably, like the nitrogenase gene uridine-5-methyltransferase gene of *A. vinelandii*, is repressed by the ammonium salt. The implications of these observations in nitrogen fixation are not clear. Modified nucleotides in tRNA are known to be involved in the expression of operons, the well known example being pseudouridine in histidine tRNA of *Salmonella typhimurium* in the expression of histidine operon (Brenner *et al.*, 1972).

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