

**Chemical synthesis of 5-azacytidine nucleotides and preparation of tRNAs containing 5-azacytidine in its 3'-terminus**

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**ABSTRACT**

5-azacytidine-5'-triphosphate prepared from 5-azacytidine by chemical phosphorylation is a substrate for AMP(CMP)tRNA nucleotidyl transferase from yeast. tRNAs<sup>Phe</sup> from yeast containing 5-azacytidine in their 3'-termini were prepared enzymatically. tRNA<sup>Phe</sup>-Cpn<sup>5</sup>CpA and tRNA<sup>Phe</sup>-n<sup>5</sup>Cpn<sup>5</sup>CpA can be aminoacylated by phenylalanyl-tRNA synthetase from yeast and they are active in the poly(U)-dependent synthesis of poly(Phe) on *E. coli* ribosomes.

The decomposition of 5-azacytidine via hydrolysis of the triazine ring is significantly accelerated by a phosphate group on the 5'-position of the nucleotide. After the incorporation of 5-azacytidine-5'-phosphate into a polynucleotide chain the rate of hydrolysis of the triazine ring decreases considerably.

**INTRODUCTION**

5-Azacytidine (1) is an analogue of cytidine containing an additional nitrogen heteroatom in the position 5 of the pyrimidine ring (2). As a potent inhibitor of the cell growth this drug exhibits pronounced antibacterial activity (3) and is used clinically in the treatment of leukemia (4). Recently it was reported that 5-azacytidine is able to deblock inactive genes (5-8) probably by the inhibition of the methylation of cytosine at specific sites of the DNA.

Inhibition of protein synthesis is probably the main target accounting for the strong cytotoxic activity of 5-azacytidine. Since this effect can be suppressed by actinomycin it was suggested that the incorporation of the analogue into RNA is crucial for inhibition (9,10). It was later demonstrated that 5-azacytidine incorporation into mammalian tRNAs causes an inactivation of tRNA cytosine-5-methyltransferase leading to a marked reduction of 5-methylcytidine content in tRNAs (11).

5-Azacytidine is unstable and it undergoes a rapid hydro-

lytic degradation of its triazine ring at neutral pH (12). Once the analogue is incorporated into the polynucleotide chain such degradation could cause changes in RNA structure and account for inhibitory effects during the protein biosynthesis.

In order to investigate the stability of 5-azacytidine-containing RNA we chemically synthesized 5-azacytidine-5'-triphosphate, and incorporated this nucleotide analogue into the 3'-end of tRNA<sup>Phe</sup> by a ATP(CTP) tRNA nucleotidyl transferase-catalysed reaction. In extension of our previous investigations with tRNA molecules having a modified 3'-terminus (13) we studied the activity of 5-azacytidine-containing tRNAs in in vitro protein biosynthesis.

### MATERIALS AND METHODS

tRNA<sup>Phe</sup>-ApCpCpA and tRNA<sup>Phe</sup>-ApCpC were isolated from yeast bulk tRNA (Boehringer, Mannheim Germany) (14). The shortened tRNAs, tRNA<sup>Phe</sup>-ApC as well as tRNA<sup>Phe</sup>-A were prepared as described previously (15). The aminoacylation capacity of tRNA<sup>Phe</sup>-A<sub>73</sub> in the presence of ATP(CTP) tRNA nucleotidyl transferase, ATP and CTP was 1250 pmol [<sup>14</sup>C] phenylalanine / A<sub>260</sub> unit tRNA. ATP(CTP)tRNA nucleotidyl transferase (E.C. 2.7.7.25) from baker's yeast of a specific activity of 44 000 units/mg protein (16) was a gift of Dr. Hans Sternbach, Göttingen. Phenylalanyl-tRNA synthetase (E.C.6.1.1.20.) from baker's yeast had a specific activity of 1820 units/mg protein (17). 5-azacytidine was a product of Fluka AG (Buchs, Switzerland). [<sup>14</sup>C]Phenylalanine 57 Ci/mol and [<sup>14</sup>C]phenylalanine 521 Ci/mol were obtained from Amersham Buchler (Braunschweig, Germany). Trimethylphosphate was purified by distillation from sodium under reduced pressure. Dimethylformamide was distilled from calcium hydride and stored over 4 Å molecular sieves. <sup>31</sup>P nmr proton-decoupled spectra were recorded on a Bruker-Spectra-Spin spectrometer at 81 MHz, in aqueous solutions containing 0.1 M EDTA, pH 7.0, and 75 % D<sub>2</sub>O. Negative chemical shift values are assigned for compound absorbing at higher field than 85 % H<sub>3</sub>PO<sub>4</sub> external standard. UV-spectra and the kinetics of the hydrolysis of 5-azacytidine and its derivatives were recorded with a Beckman DU-8 spectrophotometer.

Ion exchange thin-layer chromatography (TLC) and thin-layer electrophoresis were performed on Polygram Cel 300 PEI/UV<sub>254</sub> precoated sheets and precoated TLC plates Cel 300-10/UV<sub>254</sub> (both from Macherey-Nagel, Düren, Germany). For ion-exchange TLC the following systems were used: solvent A, 1 M LiCl; solvent B, 1.5 M LiCl; buffer C, 1 M NaOAc pH 6.0. Thin layer electrophoresis was run in 0.1 M citrate buffer pH 5.6 at 23 V/cm.

The poly(U)-dependent translation assay on *E.coli* ribosomes (18) was described previously (19). The hydrolysis of tRNA with ribonuclease T<sub>1</sub> and the conditions for HPLC separation of the digestion products were described in (20).

All evaporations were done below 20°C in vacuum using a rotary evaporator equipped with a dry-ice condenser. The molar extinction coefficient for phosphorylated 5-azacytidine derivatives was assumed to be the same as determined for 5-azacytidine,  $\epsilon_{255} = 2.56 \times 10^3$  at pH 1 (0.1 M HCl),  $\epsilon_{245} = 6.56 \times 10^3$  at pH 7.6 (50 mM TRIS·HCl).

Aminoacylation of tRNA species was performed in a reaction mixture (0.1 ml) containing 150 mM TRIS·HCl, pH 7.5, 100 mM KCl, 50 mM MgCl<sub>2</sub>, 2.5 mM ATP, 2.5 mM 2-mercaptoethanol, 0.02 mM [<sup>14</sup>C]-phenylalanine, specific activity 57 Ci/mol, 0.2 A<sub>260</sub> units tRNA and 5 µg phenylalanyl-tRNA synthetase (17).

Incorporation of 5-azacytidine into tRNA<sup>Phe</sup>-A and tRNA<sup>Phe</sup>-ApC was accomplished as described previously (15). The reaction mixture (0.5 ml) contained 150 mM TRIS·HCl, pH 9.0, 150 mM KCl, 15 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 2 mM n<sup>5</sup>CTP, 10-20 A<sub>260</sub> units of shortened tRNA and 0.2 mg/ml ATP(CTP)tRNA nucleotidyl transferase. After incubation for 4 h at room temperature, the pH was adjusted to 5.0 by addition of 2 M sodium acetate pH 4.0. The reaction mixture was diluted with 0.6 ml water and the solution was applied onto a column of Sephadex A 25 (0.8 x 5 cm) equilibrated with 20 mM sodium acetate, pH 5.2. The column was washed with 400 mM NaCl in 20 mM sodium acetate, pH 5.2 (50 ml) and finally with 1.0 M NaCl in the same buffer. The high salt portion of the eluant contained the modified tRNA. It was isolated by desalting on a Biogel P 2 column (15) and concentrated by evaporation.

Preparation of 5-azacytidine-5'-monophosphate

To a stirred suspension of 5-azacytidine (112 mg, 0.5 mmol) in trimethylphosphate (2 ml), phosphorus oxychloride (70  $\mu$ l, 0.75 mmol) was added at 0°C. The mixture was stirred with exclusion of atmospheric moisture at 0°C for 4 h. The resulting clear solution was added dropwise to 25 ml stirred ice-cold 0.1 M triethylammonium bicarbonate buffer pH 7.6. After 45 min the pH of the mixture was adjusted to about 5 by addition of the same buffer and solvents were removed by evaporation. The residue was dissolved in water (2 ml) and applied on the AG 50 Wx2 (BioRad, München, Germany) ion exchanger (100-200 mesh) column (1.8 x 27 cm) in H<sup>+</sup> form. The column was washed with water and the UV-absorbing fractions of the eluate were collected. The appropriate fractions were pooled. After addition of an equimolar amount of triethylamine, the solution was evaporated. The residue was dissolved in water (1 ml) and freeze-dried. The homogeneous product had R<sub>f</sub>-values of 0.63 (solvent A), 0.80 (solvent B), and 0.47 (buffer C) on TLC. Electrophoretic mobility was equal to CMP. UV-spectrum:  $\lambda_{\max}$  = 255 and 241 nm,  $\lambda_{\min}$  = 231 and 224 nm in 0.1 M HCl, and 50 mM TRIS · HCl (pH 7.6) respectively.  $\epsilon_{255}/\epsilon_{244}$  ratio = 1.22 in 0.1 M HCl, and 0.78 in 50 mM TRIS · HCl (pH 7.6), respectively. <sup>31</sup>P nmr :  $\delta_{\text{H}_3\text{PO}_4} = + 2.312$  ppm.

Preparation of 5-azacytidine-5'-triphosphate

Triethylammonium salt of n<sup>5</sup>CMP (0.215 mmol) was dissolved in 85 % aqueous dimethylformamide and n-tributylamine (0.43 mmol) was added. The resulting clear solution was evaporated, and the residue dried by repeated coevaporation with dry dimethylformamide (6 x 4 ml). The final residue was dissolved in dimethylformamide (3 ml) and carbonyldiimidazole (0.32 mmol, 52 mg) was added with stirring. After 40 min at room temperature the solution was cooled in ice and tetrakis(tributylammonium) pyrophosphate (0.6 mmol) in dimethylformamide was added dropwise with vigorous stirring. After 2 h at room temperature, the reaction mixture was cooled in ice, treated with 30  $\mu$ l methanol and after 15 min evaporated. The residue was dissolved in water (5 ml) and applied onto a Dowex 1 x 4 (200-400 mesh, formate) ion-exchange column (1 x 12 cm). The column was eluted with a linear gradient of am-

monium formate (50 mM - 1.0 M, pH 4.2, 600 ml) and fractions containing the product were pooled, concentrated and desalted on a Sephadex G-10 column (2 x 54 cm). UV-spectrum in 100 mM HCl:

$\lambda_{\max} = 254$ ,  $\lambda_{\min} = 238$ ;  $\epsilon_{238}/\epsilon_{254}$  ratio = 0.71;  $\epsilon_{254}/\epsilon_{270}$  ratio = 2.74. Electrophoretic mobility was equal to CTP.  $R_f$  values: 0.16 (solvent A), 0.25 (solvent B);  $^{31}\text{P}$  nmr ( $\sigma$  in ppm): -10.04 ( $P_\alpha$ ), -21.36 ( $P_\beta$ ) and -8.67 ( $P_\gamma$ );  $I_{\alpha,\beta} = I_{\beta,\gamma} = 19.7$  Hz.

## RESULTS

### Synthesis and stability of 5-azacytidine nucleotides

In order to avoid decomposition of the unstable triazine ring during the synthesis of 5-azacytidine-5'-monophosphate and 5-azacytidine-5'-triphosphate the common phosphorylation procedures (21,22) had to be modified. The reactions and purification steps were performed in the shortest possible time, and extreme pH conditions were avoided. The average yield for the phosphorylation of 5-azacytidine to 5-azacytidine-5'-monophosphate in several experiments was about 70 %. The yield of the triphosphate synthesis was only about 20 %, and could not be improved by variation of the reaction time or of the stoichiometry of the reacting components. Since no further UV-absorbing by-products were detected in the reaction mixture, we assume that the low yield of triphosphate reflects a high reactivity of the triazine ring of the 5-azacytidine with nucleophiles leading to decomposition of the base and loss of UV-absorption.

The 5-azacytidine-5'-triphosphate was characterized by its  $^{31}\text{P}$  nmr spectrum, UV-absorbance and chromatographic properties. It contained about 5 % unidentified impurities and could be stored for several weeks in lyophilized form at  $-20^\circ\text{C}$  without decomposition. As compared to the enzymatic phosphorylation of 5-azacytidine, which was utilized previously (23) the chemical synthesis of 5-azacytidine-nucleotides is convenient especially for a large scale preparation of the compound.

In aqueous solutions 5-azacytidine decomposes rapidly. The kinetics of the decomposition suggests that a nucleophilic attack of a hydroxyl ion on the position 6 of the triazine ring followed by an opening of the ring and the formation of a UV-absorbing aldehyde intermediate takes place during this reaction (12). This

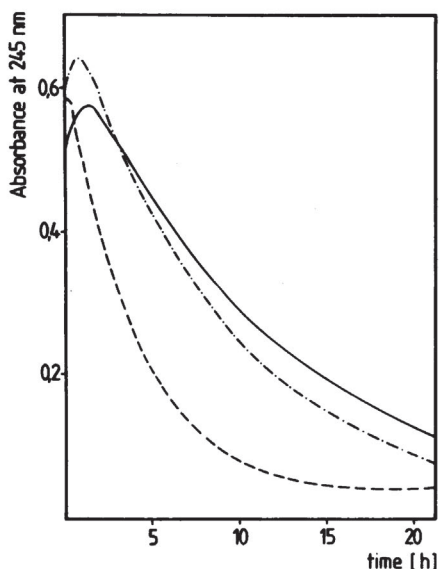


Figure 1: UV-monitored decomposition of 5-azacytidine and its derivatives at 24°C. The compounds were transferred from a cooled stock solutions to quartz cells containing 50 mM TRIS-HCl, pH 7.56. The absorbance was measured at 245 nm. (—) 5-azacytidine, (---) 5-azacytidine-5'-monophosphate, (-.-) 5-azacytidine-5'-triphosphate.

is manifested by a transient increase of the absorbance at 245 nm (fig. 1). The aldehyde is then decomposed to non UV-absorbing products, guanidine and ribose. Measuring the decay of UV-absorbance at 245 nm curves similar to those obtained for the free nucleoside (12) were observed in the case of  $n^5$ CMP and  $n^5$ CTP. By increasing the pH from 7.6 to 8.5 the rate of decomposition is accelerated about three-fold. From the part of the curve reflecting the second, slow reaction, first order rate constants were calculated. The introduction of the phosphate group into 5'-position of the 5-azacytidine increases the rate of decomposition. This effect is

Table 1 First order rate constants and half times of the decomposition of 5-azacytidine and its derivatives a).

| compound  | rate constant [ $h^{-1}$ ] |                 | half time [h]     |                 |
|-----------|----------------------------|-----------------|-------------------|-----------------|
|           | without $Mg^{2+}$          | 15 mM $Mg^{2+}$ | without $Mg^{2+}$ | 15 mM $Mg^{2+}$ |
| $n^5$ C   | 0.083                      | 0.091           | 8.4               | 7.65            |
| $n^5$ CMP | 0.104                      | 0.112           | 6.7               | 6.2             |
| $n^5$ CTP | 0.217                      | 0.136           | 3.2               | 5.1             |

a) Determined in 50 mM TRIS-HCL, pH 7.6 at 24°C. by monitoring the decay of UV-absorbance at 245 nm. The values were calculated for the second, slow reaction.

more pronounced in the case of the 5-azacytidine-5'-triphosphate, which is more labile than the 5-azacytidine-5'-monophosphate. Whereas the  $Mg^{2+}$  has no effect on the stability of 5-azacytidine or 5-azacytidine-5'-monophosphate, the rate of decomposition of the 5-azacytidine-5'-triphosphate decreases considerably upon addition of 15 mM magnesium ions.

#### Synthesis and activity of 5-azacytidine-containing tRNAs

ATP(CTP)tRNA nucleotidyl transferase recognizes 5-azacytidine-5'-triphosphate as a substrate and incorporates this modified nucleotide into the 3'-end of tRNA. Starting from tRNA<sup>Phe</sup>-ApC<sub>74</sub> or tRNA<sup>Phe</sup>-A<sub>73</sub> in the presence of ATP and  $n^5$ CTP, respective tRNAs containing one or two 5-azacytidine residues in the 3'-terminus can be prepared by this reaction. No differences in the rate or extent of incorporation of  $n^5$ CMP as compared to the natural cytidine nucleotide were observed.

In order to identify the  $n^5$ C residues incorporated into the polynucleotide chain, the modified tRNA<sup>Phe</sup> species, tRNA<sup>Phe</sup>-Ap $n^5$ Cpn<sup>5</sup>CpA and tRNA<sup>Phe</sup>-ApCpn<sup>5</sup>CpA were digested by RNase T<sub>1</sub> and the formed oligonucleotides were analyzed by HPLC (20). This analytical approach, however, did not provide clear results. Due to the lability of 5-azacytidine-containing oligonucleotides, several peaks which are not present in the chromatograms of the T<sub>1</sub> digest of native tRNA<sup>Phe</sup> appeared in the chromatograms derived from the modified species. Most probably the expected CpAp $n^5$ Cpn<sup>5</sup>CpA and CpApCpn<sup>5</sup>CpA oligonucleotides decomposed during the incubation with the nuclease. The oligonucleotides CpA<sub>OH</sub> or CpApC<sub>OH</sub> originating from the 3'-end of T<sub>1</sub> ribonuclease digested tRNA<sup>Phe</sup>-A<sub>73</sub> or tRNA<sup>Phe</sup>-ApC<sub>74</sub>, respectively, could not be found in the chromatograms. This demonstrates that the expected incorporation of 5-azacytidine into the shortened tRNAs took place.

More direct evidence showing the incorporation of  $n^5$ CMP into the 3'-terminus of tRNA<sup>Phe</sup> was obtained by enzymatic aminoacylation of shortened tRNAs in the presence of ATP(CTP)tRNA nucleotidyl transferase. The aminoacylation of tRNA<sup>Phe</sup>-A<sub>73</sub> and tRNA<sup>Phe</sup>-ApC<sub>74</sub> takes place only in the presence of the CCA end-regenerating enzyme and is dependent on the presence of CTP.  $n^5$ CTP can substitute the natural substrate in these reactions.

**Table 2** Enzymatic aminoacylation of native and modified tRNAs<sup>Phe</sup> in the presence of ATP(CTP)tRNA nucleotidyl transferase a).

| tRNA <sup>Phe</sup> -                  | Aminoacylation [%] <sup>b)</sup>               |          |                         |
|----------------------------------------|------------------------------------------------|----------|-------------------------|
|                                        | without <sup>5</sup> CTP or n <sup>5</sup> CTP | with CTP | with n <sup>5</sup> CTP |
| -A <sup>b)</sup>                       | 1.6                                            | 100      | 88.9                    |
| -ApC                                   | 1.6                                            | 94.4     | 91.1                    |
| -ApCpn <sup>5</sup> CpA                | 95.1                                           | 93.3     | -                       |
| -Apn <sup>5</sup> Cpn <sup>5</sup> CpA | 92.9                                           | 94.7     | -                       |

a) 0.2 mg/ml ATP(CTP)tRNA nucleotidyl transferase from yeast and 2.5 mM ATP were always present in the aminoacylation mixture, 2.5 mM CTP or n<sup>5</sup>CTP were added to the aminoacylation assay as indicated. b) 100 % corresponds to 1250 pmol Phe/A<sub>260</sub> unit tRNA.

Almost complete aminoacylation of tRNA<sup>Phe</sup>-A<sub>73</sub> and tRNA<sup>Phe</sup>-ApC<sub>74</sub> with ATP(CTP)tRNA nucleotidyl transferase and n<sup>5</sup>CTP present in the aminoacylation mixture, demonstrates the formation of tRNA<sup>Phe</sup>-ApCpn<sup>5</sup>CpA and tRNA<sup>Phe</sup>-Apn<sup>5</sup>Cpn<sup>5</sup>CpA. These modified tRNA species could also be prepared in preparative scale in a reaction mixture containing the shortened tRNAs<sup>Phe</sup>, ATP, n<sup>5</sup>CTP and ATP(CTP)tRNA nucleotidyl transferase. They were aminoacylated to the same extent as the native tRNA<sup>Phe</sup>-CpCpA in a phenylalanyl-tRNA synthetase-catalyzed reaction. The yield of aminoacylation could not be improved by the presence of ATP(CTP)tRNA nucleotidyl transferase and CTP in the aminoacylation mixture (table 2), indicating that the particular positions of the 3'-terminus are completely occupied by n<sup>5</sup>CMP residues.

The rate of hydrolytic decomposition of 5-azacytidine, which

**Table 3** Effect of incubation of native and modified tRNAs<sup>Phe</sup> in 50 mM TRIS-HCL pH 7.6 on their aminoacylation capacity.

| tRNA <sup>Phe</sup> -                  | I N C U B A T I O N     |              |              |
|----------------------------------------|-------------------------|--------------|--------------|
|                                        | 20°C; 4 days            | 20°C; 9 days | 38°C; 4 days |
|                                        | % of remaining activity |              |              |
| -ApCpCpA                               | 98.0                    | 90.5         | 91.4         |
| -ApCpn <sup>5</sup> CpA                | 97.1                    | 93.3         | 87.6         |
| -Apn <sup>5</sup> Cpn <sup>5</sup> CpA | 75.2                    | 59.0         | 58.1         |

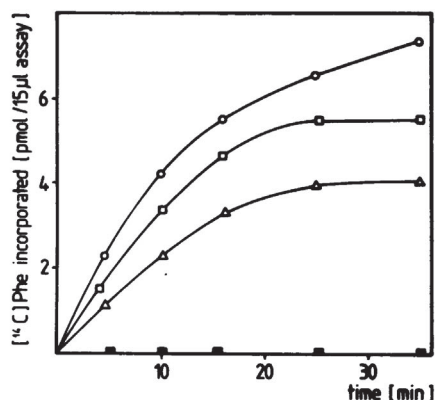


Figure 2: Poly(U) dependent synthesis of poly(Phe) on *E. coli* ribosomes. Experimental conditions are given under Materials and Methods. (—○—) tRNA<sup>Phe</sup>-CpCpA, (—□—) tRNA<sup>Phe</sup>-Cpn<sup>5</sup>CpA. (—Δ—) tRNA<sup>Phe</sup>-n<sup>5</sup>Cpn<sup>5</sup>CpA, (—■—) tRNA<sup>Phe</sup>-CpCpA but without poly(U).

takes place with a half-time of about 8 h at 24°C (s.fig. 1) was compared with the rate of deactivation of 5-azacytidine-containing tRNAs under identical conditions. For this purpose, the effect of prolonged incubation at pH 7.6 on the aminoacylation capacity of the modified tRNAs was measured. The results summarized in the table 3 demonstrate that a prolonged incubation of tRNA<sup>Phe</sup>-ApCpn<sup>5</sup>CpA at room temperature and pH 7.6 does not impair its aminoacylation activity. This tRNA is as stable as the native tRNA<sup>Phe</sup>-CpCpA. If both cytidines, 75 as well as 74, are replaced by 5-azacytidine a significantly higher loss of aminoacylation activity was observed upon incubation. Similar results were obtained when the incubation was performed at pH 7.6 and 38°C. Thus the presence of two neighbouring 5-azacytides in the polynucleotides facilitates the deactivation process. However, even in the unfavourable case of the double-modified tRNA<sup>Phe</sup>-Apn<sup>5</sup>Cpn<sup>5</sup>CpA the rate of deactivation is about 25 times lower than the rate of hydrolytic decomposition of n<sup>5</sup>CMP.

Poly(U)-dependent synthesis of poly(Phe) with 5-azacytidine-containing Phe-tRNAs<sup>Phe</sup> was tested in an *in vitro* system derived from *E. coli*. The results shown in fig. 2 demonstrate an inhibition of the poly(Phe) synthesis, which is dependent on the presence of the 5-azacytidine in the 3'-end of tRNA<sup>Phe</sup>. The inhibition is higher when both C<sub>74</sub> and C<sub>75</sub> are replaced by 5-azacytidine as compared to Phe-tRNA<sup>Phe</sup> in which only the position 75 is occupied by the analogue. Since the amount of the aminoacylated tRNAs as determined by their precipitation in cold aqueous trichloroacetic acid was the same in all three assays, the observed differences reflect the inhibition of the interaction of Phe-

tRNA<sup>Phe</sup>-ApCpn<sup>5</sup>CpA and Phe-tRNA<sup>Phe</sup>-Apn<sup>5</sup>Cpn<sup>5</sup>CpA with the components of the ribosomal elongation system, and are not related to the extent of aminoacylation of the tRNAs. In control experiments the tRNA<sup>Phe</sup>-A<sub>73</sub> and tRNA<sup>Phe</sup>-ApC<sub>74</sub> were regenerated in the presence of the native substrates CTP and ATP and subsequently aminoacylated. These Phe-tRNA<sup>Phe</sup> species showed identical kinetics of poly(Phe) synthesis as native tRNA<sup>Phe</sup>-CpCpA. Thus the inhibition of poly(Phe) synthesis in the case of 5-azacytidine-containing tRNAs is due only to the presence of this cytidine analogue in the CCA-end.

#### DISCUSSION

In the present work 5-azacytidine-5'-monophosphate and 5-azacytidine-5'-triphosphate were synthesized and the rates of their decomposition in aqueous solution were measured. An increase in the rate of decomposition of both nucleotides as compared to the unphosphorylated 5-azacytidine was observed. The introduction of the phosphate group into the 5'-position of 5-azacytidine probably facilitates the nucleophilic attack of water on the carbon 6 atom of the heterocyclic ring presumably by the intramolecular involvement of the phosphate as a general base catalyst. This suggestion is compatible with the fact, that the enhancement of the rate of hydrolysis is more pronounced in the case of 5-azacytidine-5'-triphosphate as compared to 5-azacytidine-5'-monophosphate. Moreover, the binding of Mg<sup>2+</sup> to triphosphate and a partial neutralization of the phosphate ion via a bidentate Mg<sup>2+</sup> complex reverses the hydrolysis-stimulation effect of the  $\beta,\gamma$ -phosphate groups. No such Mg<sup>2+</sup>-effect is observed in the case of the monophosphate.

Based on several investigations of the chemical modification of the CCA-end of tRNA (13,24,25) it can be assumed that a hydrolytic decomposition of the 5-azacytidine in the 3'-end of tRNA would lead to complete loss of its aminoacylation activity. Surprisingly, under conditions under which the n<sup>5</sup>CMP monomer rapidly decomposes, the tRNA containing 5-azacytidine in the position 75 is as stable as the native tRNA<sup>Phe</sup>-ApCpCpA. Most probably the structural elements discussed above, which are facilitating the hydrolytic decomposition of the triazine ring in the free nucleotide, are not existing, or less prominent, if 5-azacytidine is

incorporated into the polynucleotide chain. Moreover, it was reported that stacking interactions inhibit the addition of bisulphite on the 5,6 double bond of the cytidine ring (26,27). Since the residues C<sub>74</sub> and C<sub>75</sub> of tRNA<sup>Phe</sup> from yeast reacted with 240- and 180-times, respectively, more slowly with bisulphite as cytidine-5'-monophosphate (13) it was suggested, that the 3'-end of tRNA possess an ordered, stacked structure. It is likely, that 5-azacytidine in positions 74 and 75 of the tRNA<sup>Phe</sup> is also protected by stacking interactions. Such stabilization, however, does not take place in shorter oligonucleotides. This can be concluded from our observation that the CpApn<sup>5</sup>Cpn<sup>5</sup>CpA and CpApCpn<sup>5</sup>CpA oligonucleotides which should be formed by T<sub>1</sub>-ribonuclease degradation of 5-azacytidine-containing tRNAs are decomposed rapidly at neutral pH and cannot be identified by HPLC.

As one possibility to explain the cytotoxicity of 5-azacytidine, it was suggested, that the incorporation of 5-azacytidine into the 3'-end of tRNA may lead to deactivation of the tRNAs and to a general inhibition of the protein biosynthesis (3). In the present work we could demonstrate, that incorporation of 5-azacytidine into tRNA can indeed take place by the reaction catalyzed by ATP(CTP)tRNA nucleotidyl transferase. We could, however, also show that the incorporation of this modified nucleotide does not lead to inhibition of the aminoacylation of tRNA or to a strong inhibition of protein synthesis. Synthesis of polyphenylalanine on poly(U)-programmed *E. coli* 70 S ribosomes is only slightly slower with modified tRNAs. The consequences of this inhibitory effect of 5-azacytidine incorporation into tRNA under *in vivo* conditions may be, however, significant. Recent experimental data indicate, that the rate of protein biosynthesis and the accuracy of the translation are related (28). A drug leading even to slight inhibition of the rate of protein biosynthesis may cause *in vivo* a formation of abnormal proteins.

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1. Abbreviations:  $n^5C$  = 5-azacytidine = 1-( $\beta$ ,D-ribofuranosyl)-4-amino-5-triazine-2(1H)-one;  $n^5CMP$  and  $n^5CTP$  = 5-azacytidine-5'-monophosphate, and 5-azacytidine-5'-triphosphate, respectively;  $tRNA^{Phe}-ApCpCpA$  =  $tRNA^{Phe}$  = native phenylalanine tRNA from yeast;  $tRNA^{Phe}-ApCpn^5CpA$  and  $tRNA^{Phe}-Apn^5Cpn^5CpA$  are  $tRNA^{Phe}$  analogues, in which the C75 or both the C74 and C75, respectively, are replaced by 5-azacytidine.
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