# Favored Incorporation of Tubercidin in Poly(adenylic,7-deazaadenylic acids) and Their Function as Messenger Ribonucleic Acids in Protein Synthesis†

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ABSTRACT: The following polynucleotides containing the antibiotic tubercidin (Tu; 4-amino-7-β-D-ribofuranosylpyrrolo-[2,3-*d*]pyrimidine) were enzymatically synthesized by polymerization of adenosine 5′-diphosphate–tubercidin 5′-diphosphate mixtures with polynucleotide phosphorylase: poly($A_2$,Tu), poly(A,$Tu_2$), and poly(Tu). The incorporation of the antibiotic was favored by the enzyme. The polymers are compared to poly(adenylic acid) [poly(A)] with respect to their structure, conformation, and ability to direct polylysine synthesis in a ribosome-dependent protein synthesis system. From physical data (thermal melting, NMR, and circular dichroism) it is concluded that tubercidin destabilizes the structure of the polynucleotide chain and that this may be due to an altered polarization of the nucleobases and their enhanced rotation around the N-glycosylic bond. Since there is an apparent correlation between thermal unfolding of the polymers and their ability to mediate polylysine synthesis, it is suggested that partial destacking of the messenger ribonucleic acid favors its binding to the ribosome and/or its ability to enhance codon–anticodon-specific protein synthesis.

The isolation of 7-deazanucleosides (Suhadolnik, 1979) such as the nucleoside Q[1] (Ohgi et al., 1979) from naturally occurring polynucleotides (Kasai et al., 1975) has focused our interest toward the structural and biological implications of the replacement of the purine moiety by the pyrrolo[2,3-*d*]-pyrimidine aglycone in polynucleotides. Before the isolation of this nucleoside was achieved, 7-deazanucleosides such as tubercidin (Anzai et al., 1957), sangivamycin, or toyocamycin were only found as monomeric nucleoside antibiotics in the culture filtrates of microorganisms. With the discovery of the nucleoside Q in the first position of the anticodon of *Escherichia coli* tRNAs arose the speculation that structural modification of the base moiety might cause changes in the triplet recognition of nucleic acids. Furthermore, the occurrence of a 7-deazaguanosine derivative in polymeric material implies that 7-deazaadenosine derivatives such as tubercidin (Suzuki & Marumo, 1960) or aratubercidin (Winkeler & Seela, 1980) may also act on the polymer level. Unfortunately, very little is known about 7-deazapurine-containing polynucleotides and their function as substrates in polymerization and base recognition in translation (Acs et al., 1964; Nishimura et al., 1966). This is true for both naturally occurring tRNAs and synthetic oligo- or polynucleotides containing 7-deazanucleosides as monomeric units (Torrence & Witkop, 1975; Torrence et al., 1974; Ikehara & Ohtsuka, 1965).

A deeper understanding of these phenomena may be obtained from the structural and functional parameters of such polynucleotides. Therefore, we synthesized tubercidin-containing polynucleotides and studied their structural properties by UV and CD. Furthermore, the incorporation rate of the antibiotic in synthetic mRNAs and their efficiency in ribosome-dependent polylysine synthesis was tested.

## Materials and Methods

The $^{31}P$ NMR spectra were measured in $D_2O$ (external $H_3PO_4$) with a Bruker HX-60 spectrometer, and chemical shifts are reported in δ values. UV spectra were measured with a Zeiss PMQ 3 or Shimadzu UV-200 spectrophotometer and CD spectra with a Mark V UV–vis auto-dichrograph (Instruments SA, France). $^{14}C$ activity was counted in a Beckmann LS 8000 scintillation spectrometer. Temperature was controlled in the melting experiments by a digital R 40/2 thermometer connected with a Pt resistor (MGW Lauda, Germany).

Column chromatography was performed on DE-52 cellulose, Dowex 1-X2, or Merck I ion-exchange resin and gel chromatography on Ultrogel AcA 34 with an LKB Uvicord II as the detection unit. Thin-layer chromatography (TLC) was performed on silica gel plates F-254 (Woelm, Germany), cellulose plates G 1440/LS 254 (Schleicher & Schüll, Germany), and PEI-cellulose plates, Polygram CEL 300 PEI/$UV_{254}$ (Macherey-Nagel & Co., Germany). Thin-layer electrophoresis (TLE) was carried out on silica gel in a TLE double chamber (Desaga, Germany).

Solvent systems were as follows: A, 0.9 M triethylammonium bicarbonate; B, 0.5 M triethylammonium bicarbonate; C, 0.1 M sodium citrate, pH 6.5; D, 1 M LiCl; E, 1-propanol/concentrated $NH_3/H_2O$ (55:10:35 v/v/v); F, 0.04 M ammonium bicarbonate. The phosphate reagent was prepared as follows: One aliquot (0.95 mL) of a freshly prepared 1:6 mixture of 10% aqueous L-ascorbic acid and 0.42% ammonium molybdate tetrahydrate in 0.5 M $H_2SO_4$ was added to a solution (0.05 mL) of unknown phosphate. The mixture was kept at 37 °C for 1 h, and the absorbance was read at 820 nm (Chen et al., 1956).

Polynucleotide phosphorylase (EC 2.7.7.8) (*Micrococcus luteus*) was purchased from P-L Biochemicals (Milwaukee, WI). Snake venom phosphodiesterase (EC 3.1.4.1) was a product of Boehringer (Mannheim, Germany). Poly(adenylic

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[1] Abbreviations used: A, adenosine; Tu, tubercidin, 4-amino-7-β-D-ribofuranosylpyrrolo[2,3-*d*]pyrimidine; nucleoside Q, 2-amino-5-[(*1S,4R,5S*)-4,5-[(dihydroxycyclopent-2-en-1-yl)amino]methyl]-7-β-D-ribofuranosyl-3*H*-pyrrolo[2,3-*d*]pyrimidin-4-one; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; TuMP, tubercidin 5′-monophosphate; TuDP, tubercidin 5′-diphosphate; PNPase, polynucleotide phosphorylase; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; CD, circular dichroism; DMF, dimethylformamide; tRNA, transfer ribonucleic acid; mRNA, messenger ribonucleic acid.

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acid) [poly(A)] was purchased from Sigma Chemical Co. (St. Louis, MO) and trisodium adenosine diphosphate from Pharma-Waldhof (Düsseldorf, Germany).

*Tubercidin 5′-Monophosphate.* A total of 80 mg (0.3 mmol) of tubercidin in 1 mL of trimethyl phosphate was treated for 5 h with 53 μL (0.6 mmol) of phosphorus oxychloride at 4 °C. The mixture was hydrolyzed with ice and neutralized with solution A, and the solvent was evaporated in vacuo. The residue was then dissolved in water, applied to a 45 × 3.5 cm ion-exchange column containing DE-52 cellulose ($HCO_3^-$ form), and chromatographed with a linear gradient of 1000 mL of solution B/1000 mL of water, and triethylammonium tubercidin 5′-monophosphate eluted at ~0.3 M. The fractions were pooled, and 2635 $A_{270}$ units (72%, with $\epsilon$ = 12200 at 270 nm) of amorphous material was obtained after evaporation: TLE (silica gel, solution C) $R_e$ = 1.0 (+) relative to tubercidin at 1.0 (−); TLC (PEI-cellulose, solution D) $R_f$ = 0.4; $\lambda_{max}$ ($H_2O$) 271 nm; $^{31}P$ NMR ($D_2O$) −2.28 ppm; ($D_2O$/NaOD, pD 12.0) −3.85 ppm.

*Tubercidin 5′-Diphosphate.* A total of 1220 $A_{270}$ units (0.1 mmol) of triethylammonium tubercidin 5′-monophosphate was applied to a 25 × 2 cm cation-exchange column (Merck, type I, pyridinium form) and eluted with 500 mL of water. The solvent was evaporated, and 24 μL (0.1 mmol) of tri-*n*-butylamine was added. Water was removed by repeated evaporation with DMF. The tributylammonium salt was dissolved in 1 mL of DMF, and the formation of the imidazolidate was accomplished by the addition of 80 mg (0.5 mmol) of 1,1′-carbonyldiimidazole dissolved in 2 mL of DMF and stirring 5 h at room temperature. The reaction was followed by TLC [cellulose, solution E; $R_f$ (imidazolidate) = 0.7]. Excess 1,1′-carbonyldiimidazole was destroyed with 30 μL of methanol. Tri-*n*-butylammonium phosphate (0.5 mmol) in 5 mL of DMF was added after 30 min, and the reaction mixture was kept for 1 day at room temperature. After removal of the solvent in high vacuo the residue was dissolved in water and chromatographed on a 25 × 2.5 cm ion-exchange column (Dowex 1-X2, $HCO_3^-$ form). Triethylammonium tubercidin 5′-diphosphate was eluted with a linear gradient of 2000 mL of solution of A/2000 mL of water between 0.7 and 0.8 M. Evaporation of the solvent yielded 730 $A_{270}$ units (60%, with $\epsilon$ = 12200 at 270 nm) of amorphous diphosphate: TLE (silica gel, solution C) $R_e$ = 1.8 (+) relative to tubercidin [1.0 (−)]; TLC (PEI-cellulose, solution D) $R_f$ = 0.25; $^{31}P$ NMR ($D_2O$) δ 7.80 (d, $J$ = 20.2 Hz), 11.04 ppm (d, $J$ = 20.2 Hz).

*Polymerization of ADP–TuDP Mixtures at Various Molar Rations (Analytical Scale).* Reaction mixtures contained 75 mM Tris-HCl, pH 8.3, 7.5 mM $MgCl_2$, 8 units/mL polynucleotides phosphorylase (Godefroy-Colburn & Grunberg-Manago, 1972), and the following concentrations (mM) of $Na_3ADP$ and the triethylammonium salt of TuDP: (a) 15.4:19.2; (b) 16.0:16.0; (c) 20.0:10.0; (d) 16.0:4.0; (e) 20.0:4.0; (f) 15.4:2.5; (g) 17.5:2.5. The mixtures were incubated at 37 °C for 18 h. The total incubation mixture was applied to a cellulose thin-layer plate and developed with solvent E. The material at the starting point was scratched off, extracted with water, and centrifuged. The water layer was removed and the extraction of the cellulose repeated with 500 μL of water. The yield of the copolymers was found to be as follows: (a) 19%; (b) 17%; (c) 18%; (d) 16%; (e) 12%; (f) 13%; (g) 11%.

*Determination of Adenosine and Tubercidin Residues from the Polynucleotides a–g.* A total of 300 μL 0.03 M Tris-HCl (pH 8.5) containing ~0.6 $A_{260}$ units of polymers (a–g) was treated with 2 μL of phosphodiesterase (1 mg/mL) suspension. The solution was incubated at 37 °C for 4 h and then applied to a silica gel thin-layer plate. The material was developed electrophoretically (C), and AMP [$R_e$ : 1.0 (+)] and TuMP [$R_e$ = 0.7 (+)] were isolated by scratching off the nonmigrating material and eluting from the silica gel with 3 × 500 μL of water. The absorbance was separated by centrifugation and the water brought to a final volume of 1 mL. The absorbance (AMP at 260 nm, TuMP at 270 nm) was read in a 1-cm cuvette and the molar ratio of AMP/TuMP determined as (a) 0.4, (b) 0.5, (c) 0.7, (d) 1.9, (e) 3.5, (f) 4.7, and (g) 5.7.

*Synthesis of Polynucleotides (Preparative Scale).* (*1*) *Poly(Tu).* The incubation mixture contained 366 μL (366 $A_{270}$ units, 30 μmol) of 83 mM TuDP, 70 μL of 1 M Tris-HCl (pH 8.3), 70 μL of 0.1 M $MgCl_2$, and 0.8 mg (4 units) of PNPase in 200 μL of water. The mixture was incubated for 18 h at 37 °C, diluted with 1 mL of water, cooled to 0 °C, and extracted twice with $CHCl_3$/isoamyl alcohol (5:3) and once with ether. After removal of the ether with a stream of nitrogen the aqueous layer was diluted with 5 mL of 0.04 M $NH_4HCO_3$. The solution was then applied to a 35 × 2.5 cm Ultrogel AcA 34 column. The polymer was eluted with solution F in the void volume. After lyophilization 2 mg (18%) of colorless amorphous material was obtained. See Table I for UV data and Figure 4 for CD data.

(*2*) *Poly(A,Tu$_2$).* Enzymatic synthesis was performed in a manner similar to that used for poly(Tu) by use of 244 μL (244 $A_{270}$ units, 20 μmol) of 83 mM TuDP, 300 μL (300 $A_{260}$ units, 20 μmol) of 67 mM ADP, 100 μL of 0.1 M $MgCl_2$, 100 μL of 1 M Tris-HCl (pH 8.3), and 1.2 mg (6 units) of PNPase in 300 μL of water. A total of 4.3 mg (30%) of colorless amorphous material was obtained. See Table I for UV data and Figure 4 for CD data.

(*3*) *Poly(A$_2$,Tu).* The preparation was the same as described for poly(Tu) and poly(A,Tu$_2$) but 61 μL (61 $A_{270}$ units, 5 μmol) of 83 mM TuDP, 300 μL (300 $A_{260}$, units, 20 μmol) of 67 M ADP, 60 μL of 0.1 M $MgCl_2$, 60 μL of 1 M Tris-HCl (pH 8.3) and 0.8 mg (4 units) of PNPase in 200 μL of water was used. The yield was 3.5 mg (35%) of colorless material. See Table I for UV data and Figure 4 for CD data.

*Protein Biosynthesis Reactions. E. coli* A19 30S and 50S ribosomal subunits (Cronenberger & Erdmann, 1975) and the necessary enzyme fraction (Traub et al., 1971) were isolated as described. The polylysine synthesizing system contained in 150 μL of TMAI buffer [10 mM Tris-HCl (pH 7.6 at 25 °C), 30 mM $NH_4Cl$, 10 mM $MgCl_2$, 6 and mM β-mercaptoethanol]. Furthermore, it contained all components described for the poly(U) system (Traub et al., 1971) with the following exceptions: poly(U) was replaced by poly(A), poly(A$_2$,Tu), poly(A,Tu$_2$), or poly(Tu), and phenylalanine was replaced by $^{14}C$-labeled lysine (27 μM, 100000 cpm/assay). The length and temperature of the incubations are indicated in the legends of Figures 5–7. The polymerization was stopped by chilling the samples to 0 °C and addition of 1 drop of 1% bovine serum albumin and 2 mL of trichloroacetic acid (containing 0.25% tungstinic acid; Gottesman, 1971). The samples were then heated at 90 °C for 10 min and filtered through glass fiber filters. Subsequently, the filters were dried, placed in toluene liquid scintillation fluid, and counted. In the poly(A)-dependent polylysine-synthesizing system usually 100% activity correspond to 15–20 mol of lysine polymerized/mol of 70S ribosome.

## Results and Discussion

Tubercidin 5′-diphosphate is a key intermediate in the synthesis of tubercidin-containing polynucleotides using polynucleotide phosphorylase. The monophosphate (Freist &

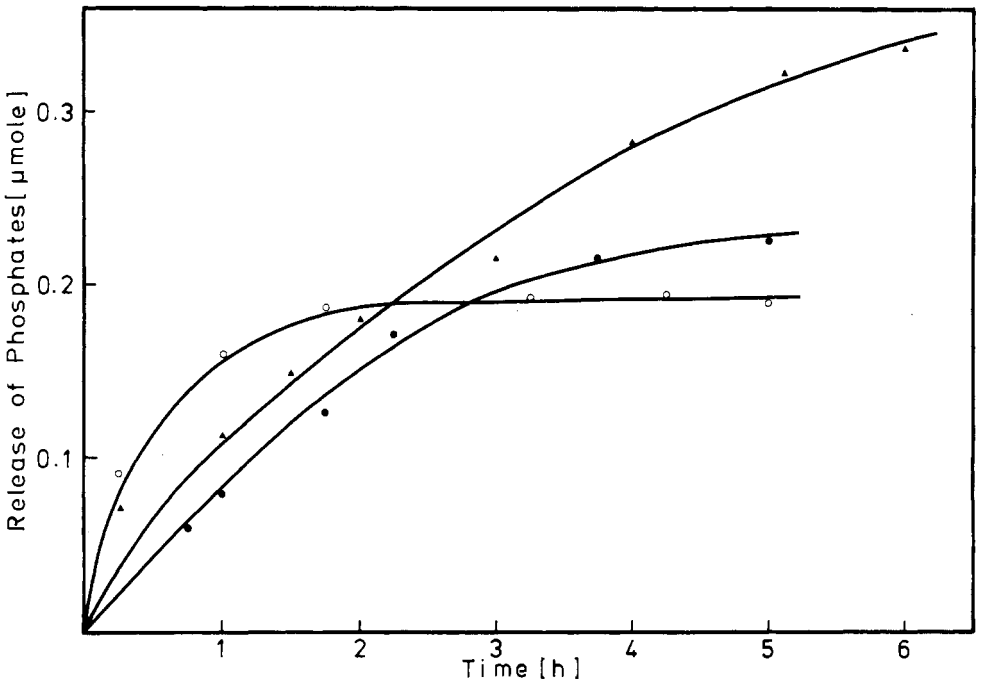


FIGURE 1: Time dependence of phosphate release during polymerization of 10 μmol of ADP (○), 10 μmol of ADP/TuDP 1:1 (▲), and 10 μmol of TuDP (●) by polynucleotide phosphorylase at 37 °C. The reaction mixture (0.5 mL) was 0.01 M in $MgCl_2$, and 0.1 in Tris-HCl (pH 8.3) and contained 0.8 mg (4 units) of PNPase. The reaction was started by adding the enzyme. The phosphate content was determined from 50-μL samples by addition of 0.95 mL of phosphate reagent and 4 mL of water. The probes were incubated for 30 min at 37 °C, and the absorbance was read at 820 nm. 1 μmol of phosphate corresponds to 24 $A_{820}$ units.

Cramer, 1978; Smith et al., 1973) was synthesized according to a method of Yoshikawa (Yoshikawa et al., 1967) by phosphorylation of the nucleoside with phosphorus oxychloride in trimethyl phosphate. After chromatography on an anion-exchange resin the 5′-monophosphate was obtained as the analytically pure triethylammonium salt as characterized by TLC and $^{31}P$ NMR spectroscopy. The conversion of the 5′-monophosphate to the diphosphate was accomplished by a method of Hoard (Hoard & Ott, 1965). Activation of the tri-*n*-butylammonium of TuMP with 1,1′-carbonyldiimidazole gave the imidazolidate, and condensation with tri-*n*-butylammonium phosphate yielded TuDP. Its $^{31}P$ NMR spectrum showed doublets at 7.80 and 11.04 ppm and coupling constants of 20 Hz, typical for a diphosphate.

*Incorporation of Tubercidin in Adenosine–Tubercidin-Containing Polynucleotides.* The velocity of polymerization can be determined by measurement of the phosphate release with the molybdenum blue method (Chen et al., 1956). As shown in Figure 1, the initial rate of polymerization was lower for TuDP as compared with ADP. If mixtures of ADP and TuDP are used during enzymatic polymerization, the initial velocity is between these limits (Figure 1). In contrast to the homopolynucleotides, the polymerization of an ADP–TuDP mixture shows higher plateaus for the phosphate release suggesting a higher degree of polymerization.

Reaction mixtures containing varying amounts of ADP and TuDP (1:1–7:1) were then polymerized with polynucleotide phosphorylase. In these analytical-scale experiments the resulting copolymers were separated from nonpolymerized mononucleotides by TLC, extracted, and quantified. The yields of the polymers were estimated by their UV absorbance.

For determination of the amounts of incorporated adenosine and tubercidin, each of the polymers was digested with snake venom phosphodiesterase, and the resulting 5′-monophosphates were separated by thin-layer electrophoresis. AMP and TuMP were recovered quantitatively by extraction from silica gel, and the absorbances ($A_{260}$ units for AMP and $A_{270}$ units for TuMP) were measured. The ratio of the molar amounts of incorporated residues was determined on the basis of the molar absorbancies at the corresponding wavelength ($\epsilon_{AMP}$ = 15 000, $\epsilon_{TuMP}$ = 12 200).

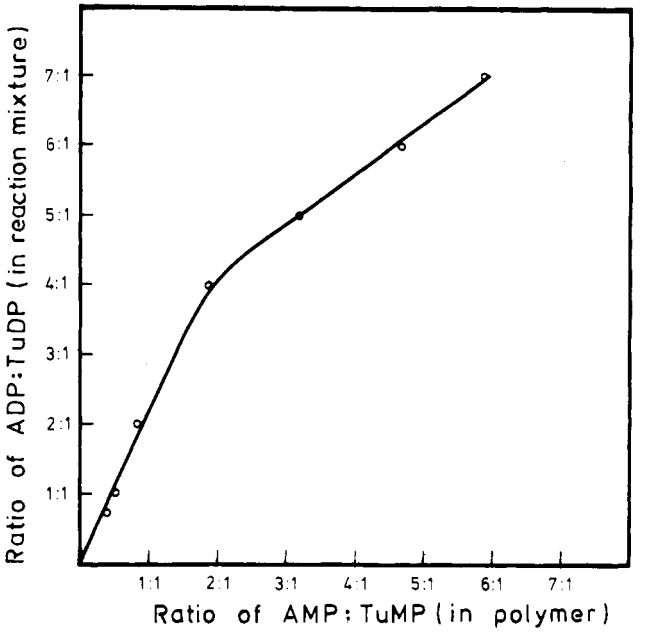


FIGURE 2: Incorporation of adenosine and tubercidin residues into copolymers by polynucleotide phosphorylase. The ratio of ADP/TuDP in the reaction mixture was varied between 0.8:1 and 7:1.

As shown in Figure 2, TuDP is apparently more easily incorporated than ADP. As can be seen from the figure, an optimum of tubercidin incorporation exists when $A,Tu_2$ copolymers are formed. The preferential incorporation of TuDP can be due to a stronger binding of the antibiotic to the active center of PNPase by either an increased $k_{on}$ or a decreased $k_{off}$ rate. The slower polymer formation (Figure 1) implies that a slower $k_{off}$ rate may determine the process. The more flexible conformation of tubercidin, which was shown by $^1H$ NMR (Evans & Sarma, 1975) and CD measurements, may

Table I: UV Data and Hypochromicities of Poly(A), Poly(Tu), and Copolymers a-g[a]

| polymer | UV max (nm) | UV min (nm) | hypochromicity (%) |
|---|---|---|---|
| poly(A) | 258 | 229 | 37 |
| poly(Tu) | 269 | 242 | 23 |
| a | 264 | 238 | 24 |
| b | 264 | 238 | 28 |
| c | 261 | 238 | 26 |
| d | 260 | 236 | 30 |
| e | 260 | 236 | 31 |
| f | 260 | 236 | 34 |
| g | 260 | 236 | 30 |

[a] Hypochromicities were determined in 0.01 M Tris-HCl (pH 8.5) containing 0.1-0.4 absorption units of the polymers; the latter were measured before and after digestion with 1 μL of phosphodiesterase (1 mg/mL) for 4 h at 37 °C.

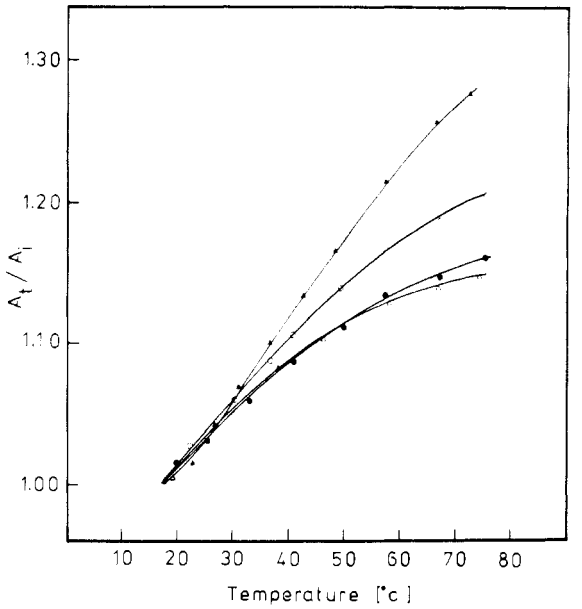


FIGURE 3: Melting profiles of poly(A) (▲), poly($A_2$,Tu) (○), poly(A,$Tu_2$) (●), and poly(Tu) (△) in 0.01 M sodium cacodylate (pH 7.0) and 0.1 M NaCl. $A_t/A_i$ is the ratio of absorbance at a given temperature ($t$) to that of the initial temperature ($i$). The melting curves were measured in a 1-cm cuvette; the temperature was increased stepwise and controlled inside the cell with a Pt resistor.

induce a better fitting of the antibiotic to the active center and would explain its slower processing and favored incorporation into polymers.

*UV–CD Spectra and Melting Profiles of Tubercidin-Containing Polynucleotides.* The UV spectrum of poly(Tu) shows an 11-nm bathochromic shift for the maximum and a 13-nm shift of the minimum compared to that of poly(A) (Table I). The absorption maxima and minima of the copolymers show values between these limits. This shift allows a rough calculation of the amount of copolymerized antibiotic. For the copolymers a–g the hypochromicity was determined by measurement of the absorbance before and after digestion with phosphodiesterase. As Table I shows, the hypochromicity increases with an increasing amount of adenosine in the polymers. Hypochromicities, calculated after digestion of the polymers, were found to be almost twice as high as those determined from the melting profiles between 17 and 80 °C: poly(A) = 37% (cleavage) and 22% (melting); poly(Tu) = 23% (cleavage). For other data, see Table I and Figure 3. Since the hypochromicity of a polynucleotide is a measure of the stacking interaction of bases in the polynucleotide chain, it can be concluded that tubercidin weakens the stacking interactions and disorders the polynucleotide structure.

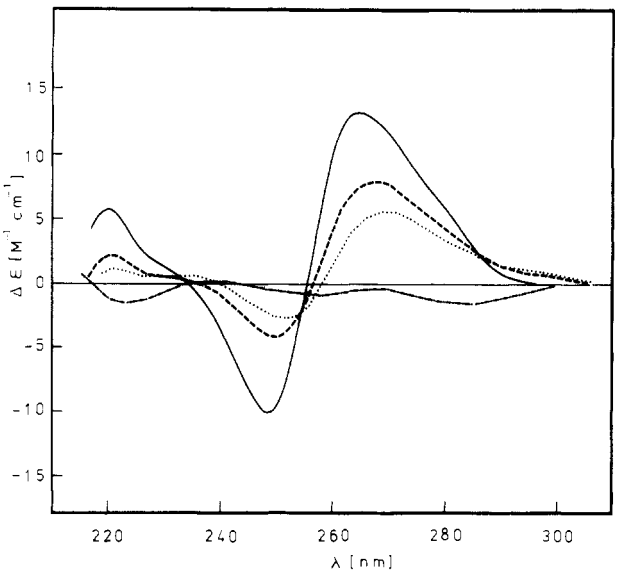


FIGURE 4: CD spectra of poly(A) (—), poly($A_2$Tu) (---), poly(A,$Tu_2$) (···), and poly(Tu) (-·-) in 0.01 M Tris-HCl (pH 7.4) and 0.1 M NaCl.

Poly(Tu), like poly(A), does not show cooperative melting at 0.1 M NaCl (Ikehara & Fukui, 1968). For the copolymers containing increasing amounts of Tu similar results were obtained (Figure 3). As shown in the figure a significant difference is observed in the melting profiles of the four polymers. Since a relative increase of Tu to A in the polymers leads to an earlier melting, a less stacked structure can also be seen from these experiments. This weakening of the stacking interaction may be due to either the change of the transition moments or/and the less hindered rotation of the nucleobase around the N-glycosylic bond. If the salt concentration for poly(A,$Tu_2$) was increased to 0.2 or 0.5 M NaCl, relatively sharp transitions with a $T_m$ of 48 °C were observed.

The less ordered structure of polymers containing increasing amounts of tubercidin can also clearly be demonstrated by comparison of the CD spectra (Figure 4). According to current theories (Bloomfield et al., 1974), the strong CD bands in polynucleotides are primarily due to interactions among the bases and arise also from the influence of the asymmetry of the ribose on the absorption band of the base. This asymmetry is also influenced by the freedom of rotation around the N-glycosylic bond. As can be seen from Figure 4 the CD bands of the polynucleotides are significantly reduced if the amount of tubercidin is increased.

*Efficiency of Polynucleotides in Ribosome-Dependent Polylysine Synthesis.* Previously, Hagenberg et al. (1973) had demonstrated that poly(Tu) is able to mediate nonenzymatic, codon-specific Lys-$tRNA^{Lys}$ binding to ribosomes. It was, therefore, of interest to compare the efficiency in polylysine synthesis of poly($A_2$,Tu), poly(A,$Tu_2$), and poly(Tu) with poly(A).

The extent of polylysine synthesis was first analyzed by varying the concentration of the four different polymers. The results are summarized in Figure 5 and show that poly(A), poly($A_2$,Tu), and poly(A,$Tu_2$) reach optimal biological activities at similar concentrations (0.36 μg/assay). Poly(Tu) differs from the other three polymers in that twice as much material is required to reach plateau values in polylysine synthesis and in that the optimal activity approaches only 60% of that with the other polymer.

Next, the time dependence of polylysine synthesis was analyzed at saturating amounts of the synthetic mRNAs (0.72 μg/assay for all four polynucleotides). From these kinetics (Figure 6) it is clear that in all cases maximal synthetic ac-

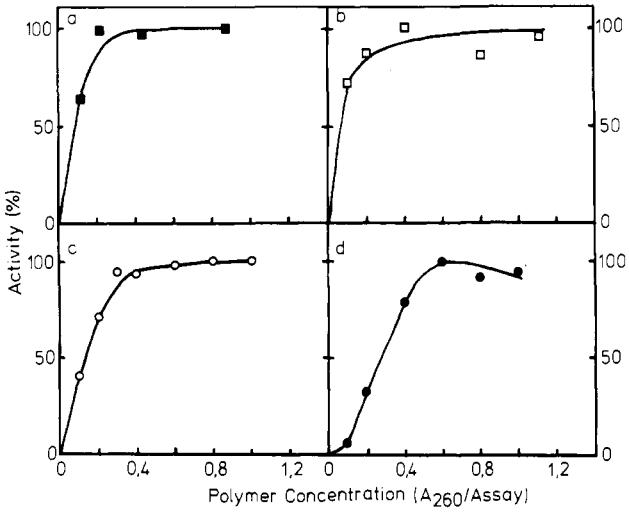


FIGURE 5: Polylysine synthesis in response to (a) poly(A) (■), (b) poly($A_2$,Tu) (□), (c) poly(A,$Tu_2$) (○), and (d) poly(Tu) (●) concentration. All samples were incubated at 37 °C for 90 min. 100% activity corresponds in samples a–c to 12 lysines and in sample d to 7 lysines polymerized per 70S ribosome (background in all experiments was <0.7 lysines/70S ribosome in absence of mRNA). For further details see Materials and Methods.

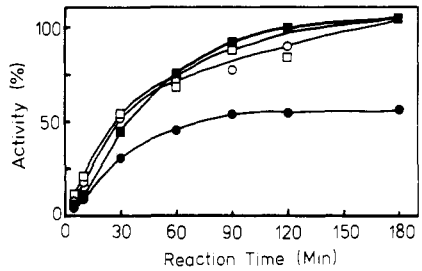


FIGURE 6: Time dependence of (■) poly(A)-, (□) poly($A_2$,Tu)-, (○) poly(A,$Tu_2$)-, and (●) poly(Tu)-directed polylysine synthesis. Temperature of incubation was 37 °C. 100% activity is defined to be the 120-min value of poly(A) and corresponds to 13 lysines polymerized/70S ribosome. Other details are given under Materials and Methods.

tivities are obtained after 90 min and that these values do not change if the incubation period is extended to 180 min. As with the concentration-dependence studies (Figure 5), poly($A_2$,Tu) and poly(A,$Tu_2$) exhibit activities comparable to poly(A), while poly(Tu) activity is considerable lower.

Since the thermal melting experiments with poly(A), poly($A_2$,Tu), poly(A,$Tu_2$), and poly(Tu) have shown (Figure 3) that Tu destabilizes the polymers, a temperature-dependent polylysine synthesis was performed (Figure 7). From the temperature dependence it is apparent that the three different Tu-containing polymers are more active than poly(A) at 20 and 30 °C. A comparison of the relative activities at 20 °C shows that poly(A,$Tu_2$) is 7 times and poly($A_2$,Tu) 2.5 times more active in polylysine synthesis than poly(A). At 30 °C, poly($A_2$,Tu) and poly(A,$Tu_2$) are 1.4 times as active as poly(A).

Since there is an apparent correlation between thermal unfolding of the polymers and their biological activities, it is suggested that partial destacking of the mRNA favors its binding to the ribosome and/or its ability to enhance codon–anticondon-specific synthesis of polylysine. On the basis of previous results (Hagenberg et al., 1973; Pezzuto & Hecht, 1980; Hecht et al., 1976) and those reported here, it seems promising to use the poly(A), poly($A_2$,Tu), poly(A,$Tu_2$), and poly(Tu) system in detailed misreading studies to broaden our knowledge of the mechanism ensuring fidelity in protein biosynthesis.

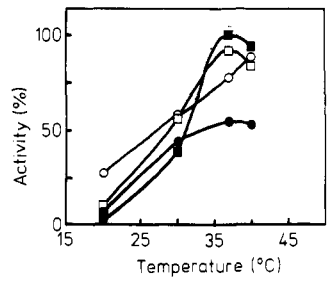


FIGURE 7: Temperature dependence of (■) poly(A)-, (□) poly($A_2$,Tu)-, (○) poly(A,$Tu_2$)-, and (●) poly(Tu)-directed polylysine synthesis. 100% activity corresponds to the poly(A)-directed synthesis at 37 °C which amounted to 15 lysines polymerized/70S ribosome.

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# Inactivation of L-Lactate Monooxygenase with 2,3-Butanedione and Phenylglyoxal†

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ABSTRACT: L-Lactate monooxygenase from *Mycobacterium phlei* is inactivated by reaction either with 2,3-butanedione in borate or in 2,6-lutidine buffer or with phenylglyoxal in 2,6-lutidine buffer. The inactivation with 2,3-butanedione in borate buffer is irreversible in the presence of excess borate, but essentially complete recovery of activity occurs on exchange of phosphate for boate buffer. In 50 mM borate, inactivation with 2,3-butanedione exhibits saturation kinetics with respect to increasing concentrations of 2,3-butanedione, whereas second-order kinetics for inactivation are seen in 200 mM borate. In 2,6-lutidine buffer, the inactivation is rapid, irreversible on change of buffer, and second order overall. Complete inactivation of the enzyme by phenylglyoxal in 2,6-lutidine buffer occurs on incorporation of 2 equiv of phenylglyoxal per subunit, but only one arginyl residue per subunit is modified. The inactivation is irreversible and second order in phenylglyoxal. There is substantial protection from inactivation in the presence of D-lactate, a competitive inhibitor of the enzyme. It is suggested that an arginyl residue in the active site in L-lactate monooxygenase is involved in the binding of the carboxyl group of substrates to the enzyme. An explanation for the unusual kinetics of inactivation with 2,3-butanedione in borate and with phenylglyoxal in 2,6-lutidine is offered.

Flavin-dependent enzymes which catalyze the oxidation of alcohol or amine groups in biological molecules are especially important because they couple the two-electron oxidation chemistry of such substrates to the one-electron chemistry of molecular oxygen or of metalloproteins in the electron-transport chain (Walsh, 1980). There has been considerable mechanistic study of these enzymes, focused mainly on the early steps in catalysis in which the substrate is oxidized and the flavin coenzyme is reduced (Bright & Porter, 1975; Bruice, 1975; Walsh, 1978). At least for the oxidation of alcohol and amine substrates having an adjacent carboxyl group considerable evidence has been accumulated that a carbanion intermediate or transition state is generated by removal of a proton from the carbon being oxidized (Walsh et al., 1971; Porter et al., 1973; Bruice, 1975; Ghisla & Massey, 1977). Studies on the enzyme L-lactate monooxygenase have been important in the development of this proposal, and a considerable body of research on the kinetics, spectral and fluorescence properties, and mechanism of this enzyme is available. Although the enzyme is an internal monooxygenase which catalyzes the four-electron oxidation of L-lactate to acetate and carbon dioxide with the reduction of $O_2$ to water, the mechanism is well established to proceed by oxidation of lactate to pyruvate, which is subsequently oxidatively decarboxylated (Lockridge et al., 1972). Recently, evidence of a covalent adduct of the substrate glycollate and N(5) of the flavin mononucleotide (FMN) coenzyme as a catalytically competent intermediate has been reported (Massey et al., 1980; Ghisla & Massey, 1980).

In view of the extensive mechanistic study of L-lactate monooxygenase, it is surprising that so little information on active-site amino acid residues necessary for catalysis is available. Modification by diethyl pyrocarbonate was used by Choong et al. (1977) to show that lactate monoxygenase from *Mycobacterium smegmatis* has a single histidine residue in the active site essential for normal catalysis. The binding of substrates and inhibitors and the chemical reactivity of the coenzyme were not altered by reaction of the enzyme with diethyl pyrocarbonate, and the suggestion was made that the essential histidine residue may function as the base which generates the substrate carbanion during catalysis. In addition to the carboxylate group of substrates, lactate monooxygenase has been found to bind a wide variety of anions at its active site, indicating the presence of one or more cationic residues (Massey et al., 1969; Lockridge et al., 1972; Ghisla & Massey, 1977). Choong et al. (1978) have reported that lactate monooxygenase from *M. smegmatis* is inactivated by treatment with 2,4-dinitrofluorobenzene and that a histidine and a lysine residue in the active site are modified. Since this modified enzyme was unable to bind anions, they suggested the lysine residue might be involved in the binding of anions to the enzyme. However, about 50% of the FMN coenzyme in the modified enzyme was slowly reduced by lactate and formed a complex with bisulfite. The lactate monooxygenase from *Mycobacterium phlei* (Takemori & Katagiri, 1975) appears to be similar to the enzyme from *M. smegmatis* in most respects except subunit structure (six for the *M. phlei* and eight for the *M. smegmatis* enzyme), but it has been less well studied. In connection with a study of some unusual hydrolytic activities of the *M. phlei* enzyme, an investigation of the residue(s) involved in the binding of anions to the active site of this enzyme was begun.

Through the use of the arginine-selective reagents 2,3-butanedione and phenylglyoxal, arginine residues have been implicated in the binding of anions, especially derivatives of phosphate, to a variety of enzymes (Riordan et al., 1977; Schneider, 1978). The flavoenzymes D-aspartate oxidase and

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