Inhibitory Effect of Complex Formation with Oligodeoxyribonucleotide Ethyl Phosphotriesters on Transfer Ribonucleic Acid Aminoacylation†

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ABSTRACT: The oligodeoxyribonucleotide ethyl phosphotriesters d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A, which are complementary to the 3′-CpCpA terminus and -UpGpApA- anticodon region, respectively, of $tRNA^{Phe}_{coli}$, have been used as *in vitro* probes of the structure and function of tRNA. The effect of these triesters on the aminoacyl-tRNA synthetase catalyzed aminoacylation of tRNA was examined. At 0° both triesters inhibit the formation of phenylalanyl-$tRNA^{Phe}$ by approximately 50–60%. The inhibition decreases with increasing temperature. A Lineweaver-Burk analysis at 0° shows that the inhibition by both triesters is competitive in nature. The results suggest that the inhibition is a consequence of the formation of complexes between the triesters and the tRNA as described in the preceding paper (Miller, P. S., Barrett, J. C., and Ts'o, P. O. P. (1974), *Biochemistry 13,* 4887). As expected, d-Tp(Et)Gp(Et)G inhibits the aminoacylation of all five tRNAs examined (phenylalanine, lysine, tyrosine, proline, and leucine) by 55–80%, while the inhibition by d-Tp(Et)Tp(Et)Cp(Et)A is more specific and is greatest for $tRNA^{Phe}$. The effect of the triesters on various related reactions catalyzed by the aminoacyl-tRNA synthetases was investigated. d-Tp(Et)Gp(Et)G inhibits the enzymic deacylation of Phe-$tRNA^{Phe}$ at 0° by 15%, but has no effect on the synthetase-catalyzed ATP–pyrophosphate exchange reaction. d-Tp(Et)Tp(Et)Cp(Et)A does inhibit pyrophosphate exchange; however, the inhibition is not specific for any amino acid. The transfer of an activated amino acid from a preformed aminoacyl adenylate–synthetase complex to tRNA at 0° is inhibited by d-Tp(Et)Gp(Et)G for three amino acids (phenylalanine, tyrosine, and leucine), while d-Tp(Et)Tp(Et)Cp(Et)A only inhibits the transfer of phenylalanine. The effect of d-TpGpG, d-GpGpT, d-TpTpCpA, UpGpG, GpGpU, and UpUpCpA on aminoacylation of $tRNA^{Phe}$ was examined. At 0°, the inhibitory activities of these oligonucleotides directly parallel the magnitudes of their association constants with $tRNA^{Phe}$ as determined by equilibrium dialysis. These findings demonstrate that the triesters can inhibit tRNA aminoacylation by specifically masking complementary regions of the tRNA through complex formation. The results are discussed in terms of the current mechanism of aminoacylation of tRNA and of the role of the anticodon in the tRNA–synthetase interaction.

An understanding of the structure and the function of nucleic acids is of fundamental importance in molecular biology. In pursuit of this goal, we have developed oligonucleotide analogs which could be used to probe the structure and function relationship of nucleic acid in both biochemical and cellular systems. Previous publications from this laboratory (Miller *et al.*, 1971; Kan *et al.*, 1973; DeBoer *et al.*, 1973) have demonstrated that oligodeoxyribonucleotide ethyl phosphotriesters are useful tools in studying the structure, conformational dynamics, and hydrogen-bonded complexes of oligonucleotides. In addition, oligodeoxyribonucleotide phosphotriesters can form hydrogen-bonded complexes with complementary homopolynucleotides (Miller *et al.*, 1971) and, as described in the preceding paper, with complementary single-stranded regions of transfer RNA (Miller *et al.*, 1974). The complexes formed with the triesters[1] have greater stability than those formed with the parent oligodeoxyribonucleotides. These properties of the triesters suggested their use as probes of the structure and function relationship of nucleic acids by virtue of their ability to mask single-stranded, exposed regions of polynucleotides through complex formation. Furthermore, the stability of the triesters toward enzymic hydrolysis (Miller *et al.*, 1971, 1974) and their ability to enter into living cells and remain undegraded (Barrett, 1974) make the oligonucleotide triesters potential probes of the structure and function of nucleic acids in cellular systems.

We have studied the effect of triesters on the *in vitro* aminoacylation[2] of tRNA catalyzed by the aminoacyl-tRNA synthetase. The oligodeoxyribonucleotide ethyl phosphotriesters d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G form specific, hydrogen-bonded complexes with the -UpGpApA- anticodon sequence and the 3′-CpCpA amino acid accepting terminus, respectively, of $tRNA^{Phe}$ from *Escherichia coli* (Miller *et al.*, 1974). A comprehensive study was made on the effects of the triesters on the synthetase-catalyzed formation of phenylalanyl-$tRNA^{Phe}$ and other

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[1] The system of abbreviations used is the same as that described in the preceding paper (Miller *et al.*, 1974). Oligodeoxyribonucleotide ethyl phosphotriesters will be referred to as triesters in the text.

[2] Aminoacylation and aminoacyl-tRNA refer to the esterification of tRNA with an amino acid.

aminoacyl-tRNAs. At 0°, both d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G inhibit aminoacylation of $tRNA^{Phe}$. We conclude that the inhibition is a consequence of the complex formation between the triester and its complementary region of the tRNA.

Experimental Section

Materials and Methods. The radioactivity which was retained by glass fiber filters (Whatman, GF/C) was determined by liquid scintillation in a mixture containing 4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene. The liquid scintillation counter used was a Beckman LS-200B with a 19% counting efficiency for the tritium isotope on the filters and 38% efficiency for the tritium isotope in aqueous solution using the same toluene mixture containing varying amounts of Biosolv 3 (Beckman Instruments, Inc.).

A mixture of aminoacyl-tRNA synthetases from *E. coli* was isolated by the method of Muench and Berg (1966). Protein concentrations were determined by the method of Lowry *et al.* (1951). The formation of ^3H-labeled aminoacyl-tRNA was detected using the filter assay of Mans and Novelli (1961) with Whatman GF/C glass fiber filters. The tRNA was a mixture of tRNAs from *E. coli* (Schwarz/Mann). The concentration was calculated using 5.3×10^5 as the molar extinction of $tRNA^{Phe}$ at 260 nm (Blum, 1971). The tritium-labeled amino acids were all obtained from Schwarz/Mann and were used without further purification.

Preparation of Oligonucleotides. The oligonucleotides were synthesized as described in the preceding paper (Miller *et al.*, 1974). After isolation, they were purified by paper chromatography and passage through a Sephadex G-10 column. Autoclaved water was used in preparing all oligonucleotide solutions. The guanine-containing oligonucleotide triester solutions contained less than 1% dimethyl sulfoxide to facilitate solubility. This solvent had no effect on the aminoacylation reaction at the concentration employed. The concentrations of the oligonucleotides were determined spectroscopically from the extinction coefficients of the oligomers (Miller *et al.*, 1974).

Kinetics of Aminoacylation of $tRNA^{Phe}$ and Inhibition Studies. The inhibition of aminoacylation of tRNA by oligonucleotides was measured by comparing the rates of reaction in the presence and absence of the oligonucleotides. The mixture consisted of solution A (100 mM Tris-HCl (pH 7.5)–40 mM $MgCl_2$–5 mM KCl–4 mM ATP), 0.04–0.07 mg/ml of a mixture of aminoacyl-tRNA synthetases, 0.4–1.0 μM tRNA, 4–40 μM L-[^3H]phenylalanine (6 Ci/mmol), and the specified amount of oligonucleotide. The ratio of amino acid to $tRNA^{Phe}$ was approximately 200. Increasing the ratio had no effect on the results. The tRNA and the oligonucleotide were preincubated at 55° for 20 min to disperse any aggregates. This procedure itself had no effect on the aminoacylation of the tRNA, and all control reactions were treated in an identical manner. The reaction mixture (100–300 μl) was then preincubated at the specified temperature of the experiment for 20 min in the absence of enzyme to allow for temperature equilibration. The reaction was initiated by addition of enzyme. Kinetics of the reaction were determined by removal of aliquots (10–50 μl) at various times and phenylalanyl-$tRNA^{Phe}$ formation was assayed by the filter technique. The protocol was altered when desired by omitting the ATP and including the enzyme in the temperature equilibration step. The reaction was then initiated by addition of ATP.

Deacylation of Phe-$tRNA^{Phe}$. ^3H-Labeled phenylalanyl-$tRNA^{Phe}$ was prepared by incubating 1 ml of tRNA (2 mg/ml), 100 mM Tris-HCl (pH 7.5), 40 mM $MgCl_2$, 4 mM ATP, 5 mM KCl, 16.7 μM L-[^3H]phenylalanine (6 Ci/mmol), and 0.17 mg/ml of a mixture of *E. coli* aminoacyl-tRNA synthetases at 36° for 10 min. The reaction mixture was cooled to 0° and 0.2 ml of 2 M KOAc (pH 5.0) was added. The tRNA was precipitated by addition of three volumes of 95% ethanol, collected by centrifugation, and washed with 95% ethanol. The kinetics of nonenzymic deacylation of [^3H]Phe-$tRNA^{Phe}$ were determined by incubating [^3H]Phe-$tRNA^{Phe}$ (6 μM total tRNA) in solution A with 20 μM L-phenylalanine. Enzyme-catalyzed hydrolysis of [^3H]Phe-$tRNA^{Phe}$ was determined under the same conditions by including 80 μg/ml of aminoacyl-tRNA synthetase. Aliquots of the reaction mixture were withdrawn at various times and the amount of [^3H]Phe-$tRNA^{Phe}$ remaining was determined by the filter assay.

Specificity of Inhibition by Oligonucleotides. The aminoacylation of a number of tRNAs was determined under the same conditions used for $tRNA^{Phe}$ aminoacylation by substituting the amino acid under study for [^3H]phenylalanine. The amino acids used were L-[^3H]lysine (60 Ci/mmol), L-[^3H]leucine (16 Ci/mmol), L-[^3H]tyrosine (52 Ci/mmol), and L-[^3H]proline (30 Ci/mmol). The tRNA concentration in these experiments was 0.4 μM and the amino acid concentrations were 2–10 μM.

Pyrophosphate Exchange Assay. Pyrophosphate–ATP exchange was measured in solution A containing 10 μM L-amino acid, 2 mM sodium pyrophosphate (^{32}P, 1.6 mCi/mmol, obtained from New England Nuclear and diluted to the desired specific activity) and 0.08 μg/ml of the mixture of aminoacyl-tRNA synthetases. The formation of [^{32}P]ATP was assayed by the method of Berry and Grunberg-Manago (1970). No reaction occurred in the absence of added amino acid, and blanks, determined by the omission of the enzyme, were subtracted from all measurement.

Isolation of Enzyme–Aminoacyl Adenylate Complex and Transfer of the Aminoacyl Moiety to tRNA. Enzyme–aminoacyl adenylate complexes were formed by incubation of 250 μl of 40 mM $MgCl_2$, 4 mM ATP, 20 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol, 2 μM [^3H]-labeled L-amino acid, and 1.3 mg/ml of a mixture of aminoacyl-tRNA synthetases for 5 min at 22°. The amino acids used were L-[^3H]phenylalanine (59 Ci/mmol), L-[^3H]tyrosine (52 Ci/mmol), and L-[^3H]leucine (16 Ci/mmol). The complexes were isolated by chromatography of the reaction mixture on a Sephadex G-50 (coarse) column (1 cm × 59 cm) with a flow rate of 3.8 ml/min. The column buffer was 0.01 M sodium cacodylate (pH 6.0), 0.5 mM EDTA, and 0.05 M KCl. Two-milliliter fractions were collected and assayed for radioactivity. The complex was eluted in the void volume and was separated from the free amino acid. The isolated complex was stable for 3–4 hr after isolation as determined by rechromatography. The transfer of the amino acid from the aminoacyl adenylate–enzyme complex to tRNA was measured by incubation of 3–7 pmol/ml of complex at 0° with 1 μM tRNA, 50 mM KCl, 0.01 M sodium cacodylate (pH 6.0), and either 1 mM EDTA or 40 mM $MgCl_2$. The transfer was measured by assaying aliquots of the reaction mixture for aminoacyl-tRNA by the filter assay.

Lineweaver–Burk Analysis of the Inhibition. A Line-

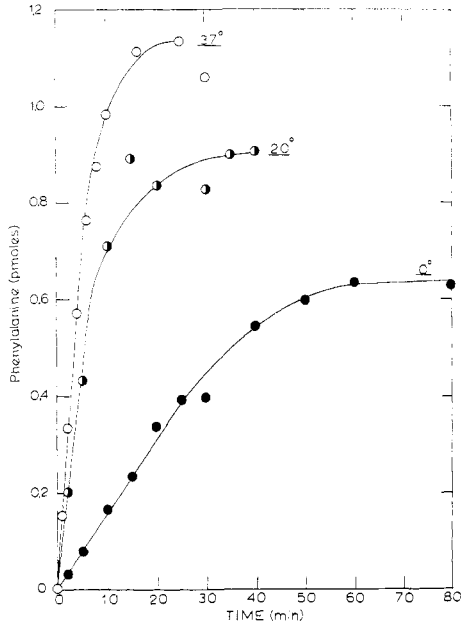

FIGURE 1: Effect of temperature on $tRNA^{Phe}$ aminoacylation. Solution A containing 0.04 mg/ml of aminoacyl-tRNA synthetase, 0.4 μM tRNA and 10 μM L-[^{3}H]phenylalanine (6 Ci/mmol) was incubated at the specified temperature. Aliquots (50 μl) were withdrawn at various times and assayed for [^{3}H]Phe-$tRNA^{Phe}$ formation by the filter assay.

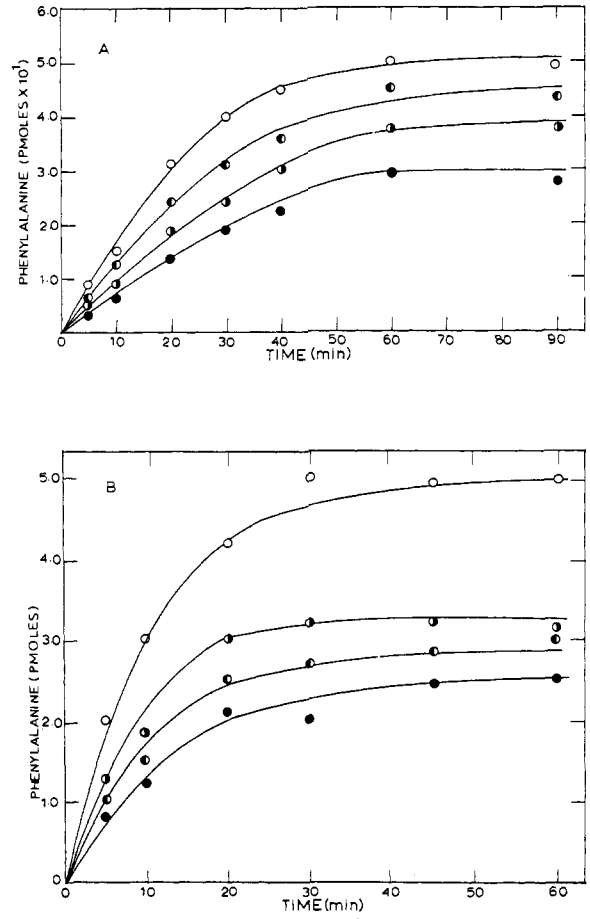

FIGURE 2: Inhibition by d-Tp(Et)Tp(Et)Cp(Et)A (A) and by d-Tp(Et)Gp(Et)G (B) of $tRNA^{Phe}$ aminoacylation at 0°: (A) solution A containing 0.4 μM tRNA, 40 μM L-[^{3}H]phenylalanine, 0.04 mg/ml of aminoacyl-tRNA synthetase, and d-Tp(Et)Tp(Et)Cp(Et)A at 0 μM (○), 139 μM (◐), 277 μM (◑), or 416 μM (●) was incubated at 0°; (B) solution A containing 4 μM tRNA, 40 μM L-[^{3}H]phenylalanine, 0.06 mg/ml of aminoacyl-tRNA synthetase, and d-Tp(Et)Gp(Et)G at 0 μM (○), 154 μM (◑), 307 μM (◐), and 460 μM (●) was incubated at 0°. Each point represents a 50-μl aliquot of the reaction mixture.

weaver-Burk analysis of the inhibition by d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G was performed using the unfractionated tRNA (*coli*). The reaction mixture was the same as that used for the kinetic studies except the tRNA concentration was varied from 1.2 to 12.0 μM. The initial velocity of the reaction was determined in the absence and presence of the triesters. The kinetics of the reaction were determined first to ensure that the reaction rate was linear at the time chosen to measure the initial rate. The analysis was performed at 0 and 37°. The reciprocal of the initial rate was plotted as a function of the reciprocal of the total tRNA concentration. The data were fitted to a straight line by the least-squares method using a Nova computer (Data General Corporation).

Results

Inhibition by d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G of the Aminoacylation of $tRNA^{Phe}$ at 0°. Since the triester-tRNA complex is most stable at low temperature, the effect of temperature on the aminoacylation of tRNA was first investigated. Figure 1 shows the effect of temperature on the formation of phenylalanyl-$tRNA^{Phe}$ in solution A containing 0.4 μM tRNA and 0.04 mg/ml of a mixture of *E. coli* tRNA synthetases. At 0°, the reaction proceeds at a lower rate and to a reduced extent than at 37°. However, the extent of aminoacylation at 0° can be increased to the same level observed at 37° (50 pmol/A_{260} unit) by increasing the amount of the enzyme (data not shown).

The effect of d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G on the formation of phenylalanyl-tRNA at 0° is shown in Figure 2. Both the rate and the extent of the aminoacylation decrease with increasing concentration of either triester. At a very high concentration of triester (~400 μM), which is equivalent to a 115-fold excess of triester to tRNA, the reaction is inhibited between 50 and 60%.

The inhibition is not due to inactivation of the enzyme by the triesters. In the presence of d-Tp(Et)Gp(Et)G increasing the tRNA concentration from 0.4 to 0.8 μM at a time when the extent of Phe-$tRNA^{Phe}$ formation has reached a plateau results in increased $tRNA^{Phe}$ synthesis to a new plateau level (Figure 3). Thus, the synthetase enzyme is still active in the presence of the triester.

The effect of d-Tp(Et)Gp(Et)G on the enzymic and nonenzymic deacylations of [^{3}H]Phe-$tRNA^{Phe}$ is shown in Figure 4. At 0° the triester has no effect on the nonenzymic hydrolysis of [^{3}H]Phe-$tRNA^{Phe}$. However, the triester does inhibit the enzyme-catalyzed deacylation reaction by approximately 15%. This is considerably less than the inhibition of the aminoacylation reaction (50–60%) observed under the same conditions.

Specificity of Inhibition by d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G. d-Tp(Et)Tp(Et)Cp(Et)A, which is complementary to the anticodon sequence of $tRNA^{Phe}$, and d-Tp(Et)Gp(Et)G, which is complementary to the 3′-CCA terminus commonly found in all sequenced tRNAs, were examined for their ability to inhibit the aminoacylation of five different tRNAs at 0°. In Table I, the inhibition of the extent of the reaction is reported; identical results were observed for the inhibition of the initial rate of the reactions. d-Tp(Et)Gp(Et)G was found to inhibit the aminoacylation of all five tRNAs to about the same degree (55–80%) with

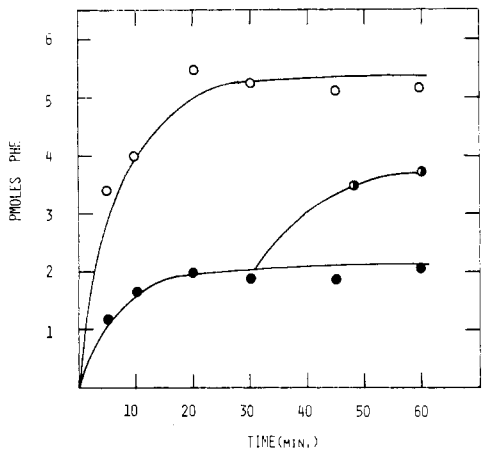

FIGURE 3: Effect of addition of tRNA to a tRNA^{Phe} aminoacylation reaction inhibited by d-Tp(Et)Gp(Et)G. Three reactions containing solution A, 0.4 μM tRNA, 0.04 mg/ml of tRNA-aminoacyl synthetase, and 0 μM (○) or 500 μM (◑; ●) d-Tp(Et)Gp(Et)G were incubated at 0°. After 30 min, tRNA in a negligible volume of solution was added to the reaction containing d-Tp(Et)Gp(Et)G (◑). This addition increased the tRNA concentration to 0.8 μM. Each point represents a 50-μl aliquot of the reaction mixture.

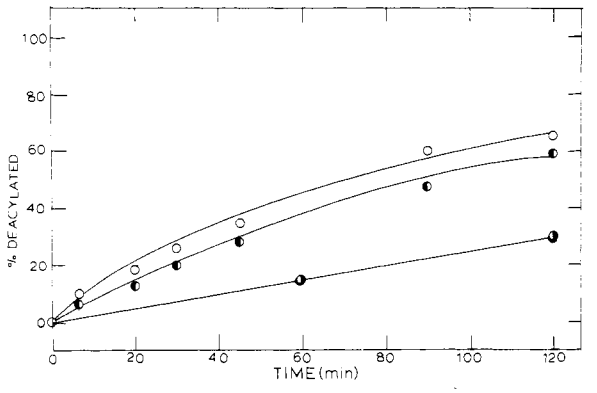

FIGURE 4: Effect of d-Tp(Et)Gp(Et)G on the enzymic and nonenzymic deacylation of [^{3}H]phenylalanine-tRNA.[^{3}H]Phe-tRNA^{Phe} (6 μM) in solution A containing 10 μM L-phenylalanine was incubated with 0 μM (○) and 500 μM (◐) d-Tp(Et)Gp(Et)G or with 0.08 mg/ml of aminoacyl-tRNA synthetase and 0 μM (◑) or 500 μM (●) d-Tp(Et)Gp(Et)G at 0°. Each point represents a 50-μl aliquot of the reaction mixture.

no apparent specificity. In contrast, d-Tp(Et)Tp(Et)Cp(Et)A inhibited the aminoacylation of the different tRNAs to varying degrees. Phenylalanyl-tRNA^{Phe} formation was inhibited to the greatest extent (45%), but not appreciably greater than leucyl-tRNA^{Leu} formation (35%). Lysyl-, tyrosyl-, and prolyl-tRNA syntheses were all inhibited, but to a significantly lesser degree (0.8–12%). The Pro-tRNA^{Pro} formation is inhibited only slightly by d-Tp(Et)Tp(Et)Cp(Et)A, but by a large amount by d-Tp(Et)Gp(Et)G. It should be noted that the conditions of the reaction were suboptimal for aminoacylation of tRNA^{Pro}.

Effect of the Triesters on the Pyrophosphate Exchange Reaction Catalyzed by Aminoacyl-tRNA Synthetase. The aminoacyl-tRNA synthetases catalyze the tRNA independent reaction of pyrophosphate exchange, which is the reverse reaction of the amino acid activation. To test whether the triesters inhibit tRNA aminoacylation by complex formation with the tRNA or by an interaction directly with the synthetase enzyme, their effect on the tRNA independent reaction was examined.

The pyrophosphate exchange reaction proceeds very rapidly in the absence of tRNA. The kinetics of L-phenylalanine-dependent [^{32}P]ATP formation from [^{32}P]pyrophosphate at 37° are linear for at least 15 min with 0.1 mg/ml of the mixture of synthetases and the normal tRNA aminoacylation conditions. No reaction occurs in the absence of added L-phenylalanine, indicating that no endogenous amino acids are present in the synthetase preparation. The reaction rate under these conditions is linearly proportional to the amount of enzyme present.

The effect of d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A on the phenylalanine-dependent pyrophosphate exchange reactions at various temperatures is shown in Table II. The pyrophosphate exchange was unaffected by d-Tp(Et)Gp(Et)G, while the reaction was inhibited by d-Tp(Et)Tp(Et)Cp(Et)A. The inhibition increased from 10% at 36° to 25% at 0°. Table III shows the effect of the triesters on the synthetase-catalyzed pyrophosphate exchange reaction dependent specifically for four different amino acids at 0°. As in the case of the phenylalanine-dependent reaction, d-Tp(Et)Gp(Et)G had no effect on any of the reactions. At a concentration of 270 μM, d-Tp(Et)Tp(Et)Cp(Et)A inhibited phenylalanine-, leucine-, tyrosine-, and proline-dependent pyrophosphate exchange from 2 to 10%. In contrast to the inhibition of the overall aminoacylation reaction, the inhibition by d-Tp(Et)Tp(Et)Cp(Et)A of the pyrophosphate exchange reaction demonstrated no specificity for the phenylalanine-dependent reaction.

At 20° the pyrophosphate exchange reaction is inhibited by the triester, d-Tp(Et)Tp(Et)Cp(Et)A, as well as by the diester, d-TpTpCpA (Table IV). Both the diester and triester contain the base sequence PyCA which is similar to the 3′-CCA terminal base sequence of tRNA. The triesters d-Tp(Et)Tp(Et)C and d-Tp(Et)Gp(Et)G show no effect on the pyrophosphate exchange reaction.

Effect of the Triesters on the Transfer of the Activated Amino Acid from the Aminoacyl Adenylate–Enzyme Complex to tRNA at 0°. In the absence of tRNA, many aminoacyl-tRNA synthetases form stable complexes with the aminoacyl adenylate, and these complexes can be isolated by Sephadex gel chromatography. The transfer of the activated amino acid from the complex to tRNA can be observed as a separate reaction (Eldred and Schimmel, 1972). The kinetics of this transfer are very rapid, but are measurable at 0°.

A phenylalanyl adenylate–synthetase complex was formed by incubation of L-[^{3}H]phenylalanine with the mixture of aminoacyl-tRNA synthetases in the absence of tRNA. The complex was isolated free from amino acid by

TABLE I: Inhibition by Triesters of the Overall Process of tRNA Aminoacylation at 0°.

tRNA[a]	% Inhibition	
	dTp(Et)Gp(Et)G[b]	dTp(Et)Tp(Et)Cp(Et)A[c]
Phenylalanine	60	45
Lysine	70	12
Tyrosine	65	12
Proline	80	0.7
Leucine	55	35

[a] 0.4 μM. [b] 270 μM. [c] 364 μM.

TABLE II: Effect of Triesters on Phenylalanine-Dependent ATP–Pyrophosphate Exchange.

	% Inhibition	
Temp (°C)	d-Tp(Et)Tp(Et)-Cp(Et)A[a]	d-Tp(Et)Gp(Et)G[b]
0	25	0
22	15	0
36	10	0

[a] 340 μM. [b] 380 μM.

TABLE III: Effect of Triesters on Amino Acid Dependent ATP–Pyrophosphate Exchange at 0°.

	% Inhibition	
	d-Tp(Et)Tp(Et)-Cp(Et)A[a]	d-Tp(Et)Gp-(Et)G[b]
Phenylalanine	10	0
Leucine	10	0
Tyrosine	12	0
Proline	2	0

[a] 270 μM. [b] 340 μM.

TABLE IV: Effect of Oligomers on Phenylalanine-Dependent ATP–Pyrophosphate Exchange at 20°.

	Concn (μM)	% Inhibition
d-Tp(Et)Tp(Et)Cp(Et)A	340	18
d-TpTpCpA	420	14
d-Tp(Et)Tp(Et)C	765	0
d-Tp(Et)Gp(Et)G	380	0

Sephadex gel chromatography (see Experimental Section). In a buffer containing tRNA, 20% of the phenylalanine was transferred from the complex to $tRNA^{Phe}$ at 0°. The lack of complete transfer was not due to degradation of the complex, which was shown to be 80% intact by rechromatography, nor was it due to insufficient tRNA. An increase in tRNA concentration had no effect on the reaction. The reaction did not proceed in the absence of Mg^{2+}.

Similar synthetase complexes with tyrosyl adenylate and leucyl adenylate were formed and isolated. Each enzyme complex has a different magnesium ion requirement for the transfer of the amino acid to tRNA. Leucyl-tRNA formation in the transfer occurs with the same rate and to the same extent (57%) in the presence and absence of magnesium ions, while tyrosyl-tRNA formation occurs with a maximum rate and extent (65%) in the presence of magnesium ions, but with a slower rate in the absence of magnesium ions.

The effects of various concentrations of d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G on the transfer of phenylalanine from the complex to tRNA at 0° are shown in Figure 5. Both triesters inhibit the rate and the extent of the reaction. d-Tp(Et)Gp(Et)G inhibited the transfer of

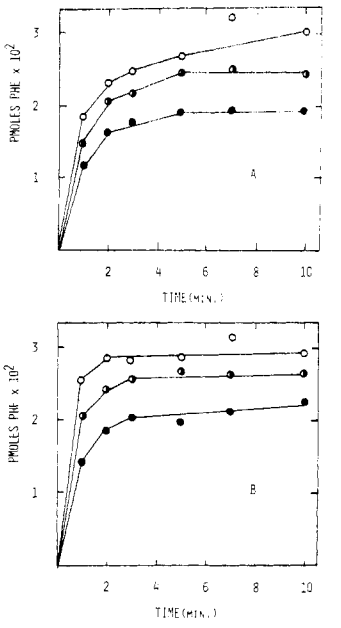

FIGURE 5: Inhibition by d-Tp(Et)Tp(Et)Cp(Et)A (A) and by d-Tp(Et)Gp(Et)G (B) of the transfer of phenylalanine from a synthetase–AMP–Phe complex to $tRNA^{Phe}$. The isolated complex (4 nM) was incubated with 0.01 M sodium cacodylate (pH 6.0), 50 mM KCl, 40 mM $MgCl_2$, and 1 μM tRNA at 0° with 0 μM (○), 210 μM (◑), 316 μM (●) d-Tp(Et)Tp(Et)Cp(Et)A in (A); 0 μM (○), 230 μM (◑), or 460 μM (●) d-Tp(Et)Gp(Et)G in (B). Each point represents a 50-μl aliquot of the reaction mixture.

TABLE V: Effects of Triesters on Transfer of Amino Acids from the Synthetase–Aminoacyl Adenylate Complex to tRNAs at 0°.

	% Inhibition	
Amino Acid	dTp(Et)Tp-(Et)Cp(Et)A[a]	dTp(Et)Gp-(Et)G[b]
Phenylalanine	33	25
Tyrosine	0	43
Leucine	0	75

[a] 316 μM. [b] 450 μM.

phenylalanine, tryosine, and leucine to their respective tRNAs (Table V). d-Tp(Et)Tp(Et)Cp(Et)A was absolutely specific for the inhibition of the transfer of phenylalanine and had no effect on the transfer of tyrosine and leucine.

Effect of Temperature on the Inhibition by the Triesters of Phe-$tRNA^{Phe}$ Formation. The kinetics of Phe-$tRNA^{Phe}$ formation in the presence of d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A was also examined at 20 and 37°. The experimental conditions were the same as those for the reaction at 0° except that the tRNA concentration was 0.4 μM and the synthetase concentration was 0.04 mg/ml. In the presence of 460 μM d-Tp(Et)Gp(Et)G, the percent inhibition of the extent of the aminoacylation decreased from 52% at 0° to 40% at 20° and to 31% at 37°. However, a greater percent inhibition of the initial rate of aminoacylation reaction is observed with increasing temperature, *e.g.*, 62% at 0°, 74% at 20°, and 86% at 37°. In the presence of 416 μM d-Tp(Et)Tp(Et)Cp(Et)A, the percent inhibition of both the initial rate and the extent of the reaction decreased

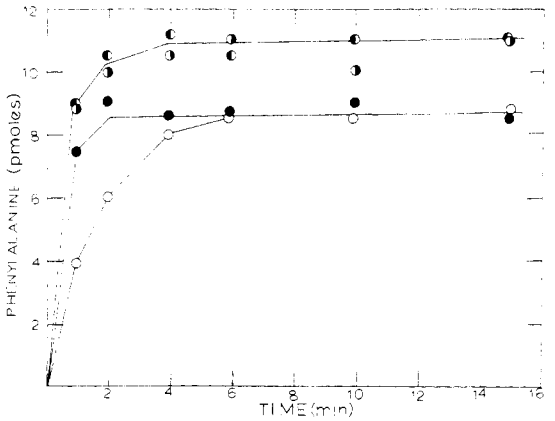

FIGURE 6: Effect of enzyme preincubation on the inhibition by d-Tp(Et)Gp(Et)G of $tRNA^{Phe}$ aminoacylation. The reaction mixture containing 100 mM Tris-HCl (pH 7.5), 40 mM $MgCl_2$, 5 mM KCl, 1 μM tRNA, and 10 μM L-[^{3}H]phenylalanine was preincubated at 20° for 20 min with 4 mM ATP (◐), 0.06 mg/ml of aminoacyl-tRNA synthetase (◑), 0.06 mg/ml of aminoacyl-tRNA synthetase, and 460 μM d-Tp(Et)Gp(Et)G (●), or 4 mM ATP and 460 μM d-Tp(Et)Gp(Et)G (○). The reactions were inititated by addition of ATP or synthetase. Each point represents a 50-μl aliquot of the reaction mixture.

with increasing temperature. For the initial rate the decrease was from 50% at 0° to 32% at 20°, and to 20% at 37°, and for the extent of the reaction the decrease was from 55% at 0° to 22% at 20° and to 18% at 37°.

The temperature effect on the inhibition by d-Tp(Et)Gp(Et)G of the initial rate was found to be dependent upon the experimental protocol. Normally, the aminoacylation reaction was performed by preincubation of the triester and the tRNA in the absence of the synthetase at the temperature of the experiment for 20 min. The reaction was then initiated by the addition of the enzyme (enzyme initiation). If the reaction mixture is preincubated in the presence of the synthetase, but without added ATP, the reaction does not proceed. The reaction commences only after the addition of ATP (ATP initiation). In the absence of triester, the kinetics and extent of the ATP initiated reaction are identical with those observed for the enzyme-initiated reaction.

However, the preincubation with the enzyme had a marked influence on the inhibition of aminoacylation by d-Tp(Et)Gp(Et)G at 20 and 37° (Figure 6). The inhibition of the initial rate was reduced from 56% for the enzyme-initiated reaction to 17% for the ATP-initiated reaction. Using the protocol of preincubation of the tRNA, enzyme, and triester, the inhibition of the initial rate was approximately equal to the inhibition of the extent of the reaction. Inhibition of the extent of reaction was the same using either protocol. At 0°, inhibition of the initial rate and the extent of the reaction by d-Tp(Et)Gp(Et)G was unaffected by the protocol employed, in contrast to the reactions at higher temperatures. Also, the inhibition by d-Tp(Et)Tp(Et)Cp(Et)A is unaffected by the protocol used at any temperature.

Lineweaver–Burk Analysis of the Inhibition. The inhibition of tRNA aminoacylation by d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A was studied at various concentrations of tRNA. At 37° a Lineweaver–Burk plot (Figure 7A) of the data indicates that the inhibition by d-Tp(Et)Gp(Et)G is a mixture of competitive and noncompetitive (hyperbolic noncompetitive). However, at 0° (Figure 7B) the inhibition is competitive with a K_i of 5×10^{-4} M. Competitive inhibition was also observed with d-Tp(Et)Tp(Et)Cp(Et)A at 0° with a K_i of 4×10^{-4} M (Figure 7C). It was not possible to distinguish the type of inhibition with d-Tp(Et)Tp(Et)Cp(Et)A at 37° due to the low level of inhibition.

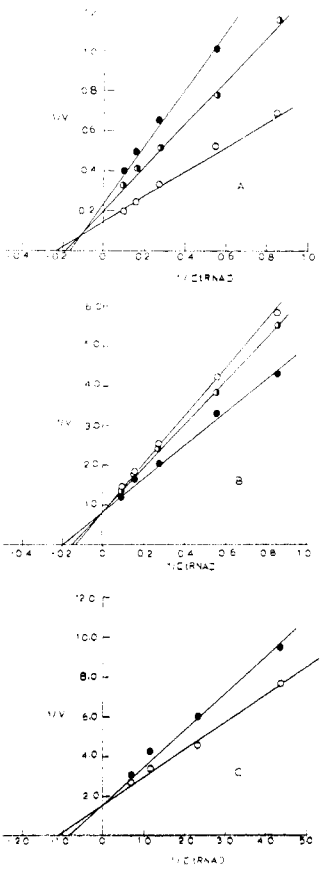

FIGURE 7: Lineweaver–Burk analysis of the inhibition by d-Tp(Et)Gp(Et)G at 37° (A) and 0° (B) and by d-Tp(Et)Tp(Et)Cp(Et)A at 0° (C). The conditions of the experiment are detailed in the text. The concentrations of d-Tp(Et)Gp(Et)G used at 37° (A) and 0° (B) were 0 μM (○), 100 μM (◑), or 200 μM (●). The concentration of d-Tp(Et)Tp(Et)Cp(Et)A used at 0° (C) was 0 μM (○) or 200 μM (●).

Inhibition by Other Oligonucleotides of the Aminoacylation of $tRNA^{Phe}$. Other oligodeoxyribonucleotide ethyl phosphotriesters were tested for their effect on the aminoacylation of $tRNA^{Phe}$ using the enzyme-initiated protocol. Two dimers, d-Tp(Et)G and d-Gp(Et)G, were tested at 0° and found not to be inhibitory at a concentration where d-Tp(Et)Gp(Et)G was effective. However, at a very high concentration (700 μM), d-Gp(Et)G did inhibit the extent of the reaction by 30%. The trimer d-Tp(Et)Tp(Et)C inhibited the extent of Phe-$tRNA^{Phe}$ formation at 0° by 21% at a concentration of 850 μM.

The oligodeoxyribonucleotides d-TpGpG and d-TpTpCpA, which have significantly lower association constants with $tRNA^{Phe}$ than their corresponding triester derivatives (Miller *et al.*, 1974), were observed to have considerably less inhibitory activity than the triesters (Table VI). The effect of the oligoribonucleotides UpGpG, GpGpU, and UpUpCpA on the aminoacylation of $tRNA^{Phe}$ was also examined at 0°. The association constants for complex formation between these oligoribonucleotides and $tRNA^{Phe}$ are higher than the association constants for the comparable oligodeoxyribonucleotides (Miller *et al.*, 1974). As shown in Table VI, the oligoribonucleotides are significantly better inhibitors of the aminoacylation reaction than are the oligodeoxyribonucleotides.

Discussion

The oligodeoxyribonucleotide ethyl phosphotriesters d-Tp(Et)Tp(Et)Cp(Et)A and d-Tp(Et)Gp(Et)G are complementary to the anticodon region and 3′-CCA terminus of $tRNA^{Phe}_{coli}$, respectively. In the preceding paper these triesters were shown by equilibrium dialysis experiments to form specific complexes with their complementary sequences of $tRNA^{Phe}_{coli}$ at 0° under moderate and low salt conditions (Miller *et al.*, 1974). The investigation on the inhibitory effect of the complex formation with the triesters on the aminoacylation of tRNA is described in this paper. Since complex formation occurs to the greatest extent at low temperature, most of the studies on the effect of the triesters on tRNA aminoacylation were conducted at 0°. This *in vitro* procedure appears to be biologically meaningful, since protein synthesis can occur normally in *E. coli* at 0° (Goldstein *et al.*, 1964).

As shown in Figure 1, the aminoacylation of $tRNA^{Phe}$ proceeds at a slower rate and to a lesser extent at low temperatures. However, the reaction is still significant at 0°. In the presence of a higher concentration of synthetase, the reaction proceeds to the same extent as observed at 37°.

At 0° the aminoacylation of tRNA does not proceed to completion, but instead reaches a plateau level. Incomplete reactions in tRNA aminoacylation, even at 37°, have been observed by others (Bonnet and Ebel, 1972, and references therein). In the case of the formation of valyl-$tRNA^{Val}_{yeast}$, the incomplete reaction has been interpreted by Bonnet and Ebel (1972) to be a consequence of two deacylation reactions which occur in addition to the synthetase-catalyzed acylation reaction. These additional reactions are the spontaneous hydrolysis and a synthetase-catalyzed deacylation of Val-$tRNA^{Val}$. These reactions are independent of the aminoacylation reaction. As a result, the aminoacylation of tRNA is not a unidirectional reaction but instead is a dynamic system in which the extent of aminoacylation is a reflection of the steady-state equilibrium between the acylation and deacylation reactions. This hypothesis also explains the temperature effect observed in Figure 1. At 0° the initial rate of the reaction, which reflects primarily the rate of the forward reaction of acylation, is reduced relative to the initial rate at 37°. The rate of the reverse reaction, deacylation, appears to be reduced to a relatively lesser degree. Therefore, the extent of the Phe-$tRNA^{Phe}$ formation is reduced at 0° relative to that at 37°. An increase in the enzyme concentration at 0° compensates for the slower forward rate. Thus, by adjusting the concentration of the enzyme, the same extent of aminoacylation can be reached at different temperatures.

At 0°, d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A inhibit both the initial rate and the extent of phenylalanyl-tRNA formation catalyzed by the aminoacyl-tRNA synthetase. Since triester-tRNA complex formation is reversible, one might expect the triesters to inhibit the rate of aminoacylation, but not the extent. If sufficient time is allowed, all the tRNA must dissociate from the triester-tRNA complex and become available for aminoacylation. In fact, the triesters, and other oligomers, inhibit the extent of the reaction as well as its rate. Under conditions where the plateau was reached in 30 min in the presence of the triesters, there was no further change in the level of aminoacylation for an additional 2 hr. This is not due to inactivation of the synthetase enzyme by the triester, since upon addition of tRNA aminoacylation resumes (Figure 3).

TABLE VI: Effect of Oligonucleotides on the Aminoacylation of $tRNA^{Phe}$ at 0°.

Oligomer	Assn Constant[a] (M^{-1})	Concn (μM)	% Inhibition
d-Tp(Et)Gp(Et)G	2,000	400	45
d-TpGpG	500	400	5
d-GpGpT	12,000	400	23
UpGpG	4,900	200	47
GpGpU	204,000	50	50
d-Tp(Et)Tp(Et)Cp(Et)A	2,400	300	50
d-TpTpCpA	1,300	400	20
UpUpCpA	45,000	50	54

[a] Association constant with $tRNA^{Phe}_{coli}$ in 0.1 M NaCl–10 mM-Mg^{2+} (Miller *et al.*, 1974. [b] Inhibition of the extent of formation of Phe-$tRNA^{Phe}$.

The observation that the triesters inhibit the extent as well as the rate of tRNA aminoacylation is also consistent with the hypothesis of Bonnet and Ebel (1972). The results suggest that the extent of aminoacylation is determined by the steady-state equilibrium of the acylation and deacylation reactions. Triesters have been shown to inhibit the forward aminoacylation reaction. Enzymic deacylation is inhibited by d-Tp(Et)Gp(Et)G but to a lesser degree than is aminoacylation. The nonenzymic hydrolysis of aminoacyl-tRNA is not affected by d-Tp(Et)Gp(Et)G. Thus the diminution of the extent of the aminoacylation reaction by the formation of tRNA-triester complexes is due to a greater inhibition of the forward aminoacylation reaction compared to inhibition of the reverse, deacylation reaction.

The adoption of the oligodeoxyribonucleotide triesters as probes of the structure and the function of nucleic acids is predicated on the ability of the triesters to inhibit nucleic acid function by a specific *complex formation* with the target nucleic acid. It has already been shown that the triesters can form specific complexes with $tRNA^{Phe}_{coli}$ (Miller *et al.*, 1974). The present study shows that the triesters are inhibitors of the aminoacylation of tRNA catalyzed by the aminoacyl-tRNA synthetase. However, there are two possible mechanisms for the observed inhibition. The first is due to an interaction of the triesters with the tRNA, and the second is due to an interaction of the triesters directly with the synthetase.

Several lines of evidence support the first mechanism. The specificity of the inhibition by the triesters of the aminoacylation of tRNA was first examined. All sequenced tRNAs have a 3′-CCA terminus and can thus potentially form a Watson–Crick base-paired complex with d-Tp(Et)Gp(Et)G. However, of the five tRNAs examined, only $tRNA^{Phe}$ has a -UpGpApA- sequence (in the anticodon region) which is complementary to d-Tp(Et)Tp(Et)Cp(Et)A. Therefore, d-Tp(Et)Gp(Et)G should inhibit the aminoacylation of all tRNAs, but d-Tp(Et)Tp(Et)Cp(Et)A should specifically inhibit aminoacylation of $tRNA^{Phe}$.

When five different tRNAs were examined for their ability to be aminoacylated in the presence of the two triesters, d-Tp(Et)Gp(Et)G was found to be an inhibitor of the aminoacylation of all the tRNAs; d-Tp(Et)Tp(Et)Cp(Et)A was found to inhibit Phe-tRNA synthesis to the greatest extent but without absolute specificity. This observation suggests

the presence of an additional mechanism of inhibition by the triesters.

This additional, nonspecific mechanism appears to be the inhibition by d-Tp(Et)Tp(Et)Cp(Et)A of the pyrophosphate exchange and presumably aminoacyl adenylate formation catalyzed by the aminoacyl-tRNA synthetases. This observation indicated that d-Tp(Et)Tp(Et)Cp(Et)A could interact directly with the synthetase enzyme. It has been suggested that the 3′-terminal adenosine of tRNA can compete with ATP for the ATP binding site on the aminoacyl-tRNA synthetase (Bruton and Hartley, 1970). Santi *et al.* (1971) have observed inhibtion by $tRNA^{Phe}$ of the ATP-PP_i exchange reaction catalyzed by the *E. coli* phenylalanyl-tRNA synethetase. Thus, the enzyme may "recognize" the -PyCA sequence of d-Tp(Et)Tp(Et)Cp(Et)A as the -CCA sequence of tRNA. In support of this hypothesis is the observation that d-TpTpCpA also inhibits pyrophosphate exchange to the same extent as d-Tp(Et)Tp(Et)Cp(Et)A, while the trimer, d-Tp(Et)Tp(Et)C, which lacks the terminal A residue, has no inhibitory activity (Table IV).

It should be noted that the pyrophosphate exchange reaction occurs approximately 100 times faster than the complete aminoacylation reaction but is inhibited less than 25%. It is difficult to predict the magnitude which this inhibitory effect would have on the overall aminoacylation reaction. Similarly, the sensitivity of different aminoacyl-tRNA synthetases to this type of inhibition, *i.e.*, the specificity of the inhibition, is also unpredictable.

It is possible that phenylalanyl-tRNA synthetase is particularly sensitive, relative to the other enzymes, to inhibition of amino acid activation. Then the "specificity" of inhibition of tRNA aminoacylation by d-Tp(Et)Tp(Et)Cp(Et)A could be a reflection of the relative sensitivity to inhibition of the first step of the aminoacylation reaction and not be the result of complex formation with $tRNA^{Phe}$. To examine this possibility, the aminoacyl adenylate–enzyme complex was obtained. By preactivating the amino acid, the aminoacylation of tRNA can be examined as a simple transfer of the activated amino acid to the tRNA. Any inhibitory effects on aminoacyl adenylate formation are thus circumvented. At 0°, d-Tp(Et)Gp(Et)G inhibits phenylalanyl-, leucyl-, and tyrosyl-tRNA formation with the enzyme–aminoacyl adenylate complex. In contrast, d-Tp(Et)Tp(Et)Cp(Et)A inhibits only Phe-$tRNA^{Phe}$ formation and has no effect on the formation of the other aminoacyl-tRNAs in this transfer reaction. This result indicates a high degree of specificity for inhibition by the triesters of the transfer reaction and thereby supports the conclusion that specific complex formation with the tRNA is the mechanism of inhibition.

The alternative mechanism for the triester inhibition of tRNA aminoacylation is an interaction of the triester directly with the synthetase enzyme. This mechanism of inhibition is discounted by the following experimental evidence. (1) The enzyme is not inactivated by the triesters. (2) Triesters of shorter length than d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A do not significantly inhibit. The amount of inhibition by other oligonucleotides is directly related to their ability to bind to tRNA (Table VI). (3) Preincubation of the synthetase enzyme with the tRNA and the triester results in a *decrease* in the inhibition of the initial rate suggesting that the enzyme competes with the oligomer for the tRNA during this incubation. If the triester interacts with the enzyme, this preincubation should lead to greater, not less inhibition. (4) d-Tp(Et)Gp(Et)G has no effect on the tRNA-independent, pyrophosphate exchange reaction, suggesting that this triester does not interact with the enzyme. However, d-Tp(Et)Tp(Et)Cp(Et)A does inhibit this reaction suggesting that it interacts with the enzyme. The results with d-TpTpCpA and d-Tp(Et)Tp(Et)C indicate that the effect of d-Tp(Et)Tp(Et)Cp(Et)A on the pyrophosphate exchange reaction is a special case. This may be due to the similarity of the -PyCA sequence of the oligomers to the 3′-CCA sequence of tRNA. Therefore, d-Tp(Et)Tp(Et)Cp(Et)A probably inhibits by two mechanisms. One is the nonspecific inhibition of amino acid activation, and the second is the specific inhibition of $tRNA^{Phe}$ by complex formation. (5) The specificity of inhibition of a tRNA species by a triester parallels the sequence complementarity between the triester and tRNA species. The specificity is very striking when the step of amino acid activation is circumvented by preactivation of the amino acid and the transfer of the aminoacyl moiety to tRNA is examined directly. This is due to the removal of the nonspecific interaction of d-Tp(Et)Tp(Et)Cp(Et)A with the enzyme as described in 4.

It is of interest to note that aminoacylation of $tRNA^{Leu}$ is inhibited by d-Tp(Et)Tp(Et)Cp(Et)A more than any other tRNA except $tRNA^{Phe}$. $tRNA^{Leu}$ has the base sequence -UpUpCpA- in its anticodon region. d-Tp(Et)Tp(Et)Cp(Et)A has the same base sequence. This suggests that the inhibition of aminoacylation of $tRNA^{Leu}$ may be due in part to competition by d-Tp(Et)Tp(Et)Cp(Et)A with the anticodon of $tRNA^{Leu}$ for the enzyme.

Complex formation between oligonucleotides and polynucleotides decreases with increasing temperature. Thus, the inhibition of tRNA aminoacylation by the triesters should have a similar temperature dependence. Inhibition of the initial rate and the extent of Phe-$tRNA^{Phe}$ formation by d-Tp(Et)Tp(Et)Cp(Et)A follows this prediction. For d-Tp(Et)Gp(Et)G, the same is true for the inhibition of the extent of the reaction, but the opposite effect on the inhibition of the initial rate is observed. Increased inhibition of the initial rate was observed with increasing temperature.

The extent of the aminoacylation reaction reflects the steady-state equilibrium attained between the acylation and deacylation reactions. In the presence of the triester, this state is a consequence of the kinetics of these interactions. The temperature effect on the inhibition of the initial rate is dependent upon the relative effects of temperature on the kinetics of the multiple interactions among the triester, tRNA, and synthetase. Using the enzyme-initiation protocol, the experiments were performed by preincubating the triester with the tRNA at the desired temperature. In the presence of the triester, the initial rate of the reaction is dependent upon the amount of free or uncomplexed tRNA available immediately after addition of the enzyme. The initial rate of aminoacylation increases at higher temperature. Therefore, the per cent inhibition caused by the triester will increase at higher temperature unless the amount of free tRNA also increases. The amount of free tRNA depends upon the rate of dissociation of the triester from the triester–tRNA complex. Thus, in the presence of d-Tp(Et)Gp(Et)G, an increased inhibition of the initial rate with higher temperature would result if the thermal enhancement of the aminoacylation rate in the absence of the triester is greater than the thermal enhancement of the rate of dissociation of the d-Tp(Et)Gp(Et)G–tRNA complex.

Podder (1971) has shown that guanine-containing oligo-

ribonucleotide complexes dissociate slowly. This observation suggests that the thermal enhancement of the forward aminoacylation reaction from 0 to 37° is larger than the thermal enhancement of the dissociation rate of the d-Tp(Et)Gp(Et)G–tRNA complex, but smaller than that of the d-Tp(Et)Tp(Et)Cp(Et)A–tRNA complex. This suggestion is strongly supported by the observation that the inhibition of the initial rate of the aminoacylation in the presence of d-Tp(Et)Gp(Et)G is greatly reduced if the triester, tRNA, and synthetase are preincubated at 37° using the ATP-initiation protocol. In fact, the inhibition of the extent and of the initial rate is very similar in this protocol. This result suggests that the interactions of the triester, tRNA, and synthetase have reached a thermodynamic equilibrium after preincubation.

An understanding of this problem may also help explain the results of the Lineweaver–Burk analyses of the kinetics using the enzyme-intiated protocol. Competitive inhibition was observed for both d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A at 0°. At 37° the inhibition by d-Tp(Et)Gp(Et)G was a mixture of competitive and noncompetitive inhibition. This may be due to the differential effect of thermal enhancement on the chemical reaction rate *vs.* the dissociation rate of the d-Tp(Et)Gp(Et)G–tRNA complex. At 37° the inhibition of the initial rate was too small to be measured by the ATP-initiation protocol for d-Tp(Et)Gp(Et)G or by the enzyme-initiation protocol for d-Tp(Et)Tp(Et)Cp(Et)A. Another possible explanation for the hyperbolic noncompetitive inhibition is that the triester–tRNA complex can bind to the enzyme, but the tRNA cannot be aminoacylated.

Both ribosyl- and deoxyribosyloligonucleotide phosphodiesters were also found to inhibit aminoacylation of $tRNA^{Phe}$ at 0°. The direct parallel between the magnitude of the association constant and the inhibitory activity of the oligonucleotide (Table VI) further supports the conclusion that complex formation between the oligomer and tRNA can inhibit aminoacylation of the tRNA.

The inhibition of aminoacyl-tRNA synthetases by oligo- and polynucleotides has been reported previously (Deutscher, 1965; Hayashi and Miura, 1966; Letendre *et al.*, 1966, 1969; Berry and Grunberg-Manago, 1970). However, this inhibition appeared to be a result of the competition of the oligo- and polynucleotides with tRNA for the enzyme binding site (Letendre *et al.*, 1969; Berry and Grunberg-Manago, 1970). No evidence was presented in these reports to suggest complex formation between tRNA and these oligo- or polynucleotides as the mechanism of inhibition.

Perhaps it is not surprising that d-Tp(Et)Gp(Et)G inhibits tRNA aminoacylation since it masks a portion of the tRNA which must participate in the acceptance of the aminoacyl group. However, d-Tp(Et)Tp(Et)Cp(Et)A complexes with the anticodon region of the tRNA, which is distant from the aminoacylation site. There are two possible mechanisms by which this may occur. (1) The binding of the oligonucleotide may cause a conformation change in the tRNA affecting the conformation of the 3′ terminus. This has interesting implications for conformation changes of tRNA upon binding mRNA. (2) The anticodon region of the tRNA may be important in the interaction with the aminoacyl-tRNA synthetase; and the masking of the anticodon region prevents this interaction. No attempt in this work has been made to distinguish between the two possibilities.

There have been numerous studies attempting to elucidate the role of the anticodon in tRNA–synthetase interactions (Thiebe and Zachau, 1968; Barnett and Jacobson, 1964; Gefter and Russel, 1969; Hörz and Zachau, 1973; Chambers *et al.*, 1973; Dube, 1973; Schimmel *et al.*, 1972; Schulman and Goddard, 1973, and references therein). These studies have not ruled out the possibility that the entire anticodon region is an interaction site, but not necessarily a recognition site, for the synthetase enzyme. Some of these studies have suggested an obiligatory requirement of particular residues for activity of certain tRNAs (Chambers *et al.*, 1973; Schulman and Goddard, 1973). The work of Schulman and coworkers (Schulman, 1970; Schulman and Goddard, 1973) suggests that the entire anticodon region should not be disgarded as a synthetase interaction site based on the effect of modification of a single base residue in the anticodon region.

The advantage of our approach is that the entire sequence of the anticodon, and not a single nucleotidyl unit, is involved in complex formation with the triester. There is no chemical modification of the nucleotidyl units. Our results clearly indicate that the anticodon region of at least $tRNA^{Phe}_{coli}$ plays a role in the interaction of the tRNA with the synthetase. This conclusion is made with the realization that the entire secondary and tertiary structures of tRNA may be involved in the recognition process (Campbell and Ts'o, 1971; Loftfield, 1972).

The preceding paper and the present study demonstrate the utility of oligodeoxyribonucleotide ethyl phosphotriesters as probes of the structure and function of nucleic acids. In the *in vitro* tRNA-synthetase system as a model, triesters complementary to the anticodon and the 3′-terminal regions of $tRNA^{Phe}$ inhibit aminoacylation of the tRNA. The inhibition results from masking regions of the nucleic acid by complex formation with the triesters. Preliminary experiments (Barrett, 1974) have demonstrated that triesters are able to penetrate the membranes of mammalian cells and remain undegraded inside the cells. Thus oligonucleotide phosphotriesters are also potential probes for cellular studies.

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Lipid Interactions in Membranes of Extremely Halophilic Bacteria. I. Electron Spin Resonance and Dilatometric Studies of Bilayer Structure†

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ABSTRACT: Dispersions of *Halobacterium cutirubrum* polar lipids in buffer and in salt solutions have been investigated over a wide temperature range (but above the phase transition) by volumetric dilatometry and electron spin resonance (esr) spin-labeling with nitroxide-labeled stearic acids and with vanadyl sulfate. The polar lipid fraction is essentially a binary mixture of the diphytanyl ether analogs of phosphatidyl glycerophosphate and of a glycolipid sulfate, in an approximate mole ratio of 2:1. Stearic acid spin-labels were used to study each pure major component as well. The unusual chemical features of these lipids include uniform, highly branched phytanyl hydrocarbon chains, large anionic head groups, and ether linkages to the phytanyl chains. The thermal coefficient of expansion of the lipids in the presence of added salts (4 M NaCl and/or 0.1 M $MgCl_2$) was found to decrease with increasing temperature and appeared to be discontinuous at −11, 11, 23, and 39–45°. There is evidence from spin-labels for a transition occurring in the polar head group of the major component at 23°. In the absence of added salts the lipid fluidity is increased and the head-group transition is shifted to 8°. Some of the stearic acid spin-labels appear to show transverse displacement in the bilayer, reporting on the head-group region at low temperatures and on the hydrocarbon region of the bilayer at high temperatures. The unusual dilatometric properties and the behavior of the labels can be explained by a model that postulates trans-gauche cooperative kinking of densely packed lipid hydrocarbon chains, occurring preferentially at the methyl-branched chain carbons. The head-group behavior is assumed to be a consequence of changes in the packing of the hydrocarbon phase.

The cell envelope of extremely halophilic bacteria, such as *Halobacterium cutirubrum,* contains two classes of lipids: (a) polar lipids (Kates, 1972; Hancock and Kates, 1973) consisting primarily of the diphytanyl ether analogs of phosphatidyl glycerophosphate[1] (55% of the total lipids by weight) and of a glycolipid sulfate (1-*O*-[β-D-galactopyranosyl-3′-sulfate-(1′→6′)-*O*-α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (32% of the total lipids by weight), and (b) nonionic, or neutral lipids (Tornabene

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[1] Abbreviations used are: PGP, diphytanyl ether analog of phosphatidyl glycerophosphate; GLS, diphytanyl ether analog of 1-*O*-[β-D-galactopyranosyl-3′-sulfate-(1′→6′)-*O*-α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DTBN, di-*tert*-butyl nitroxide.