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Effect of Transfer Ribonucleic Acid Dimer Formation on Polyphenylalanine Biosynthesis†

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ABSTRACT: *Escherichia coli* $tRNA^{Phe}$ (anticodon GAA) as well as yeast $tRNA^{Phe}$ (anticodon GmAA) forms a strong complex with *E. coli* $tRNA^{Glu}$ (anticodon s^2UUC) through an interaction between their complementary anticodons. This interaction inhibits aminocylation of $tRNA^{Phe}$ but not the formation of a complex with elongation factor Tu. Moreover, at 0 °C, $tRNA^{Glu}$ strongly inhibits the binding of Phe-tRNA to poly(U)-programmed ribosomes via either the enzymic (EF-Tu-promoted) or nonenzymic pathway. At 15 °C, $tRNA^{Glu}$ effectively inhibits polyphenylanine synthesis in the *E. coli* system. The inhibition is reversed at 37 °C, where the Phe-tRNA·$tRNA^{Glu}$ dimer is dissociated. Calculations based upon the *E. coli* intracellular concentrations of tRNAs and the published rates of association and dissociation of the tRNA dimers suggest that this interaction may inhibit protein synthesis in vivo at temperatures below 15 °C.

The translation of mRNA[1] requires the correct codon–anticodon interaction to occur during the binding of AA-tRNA to ribosomes [for a review, see Pongs (1978)]. The discovery that tRNAs bearing complementary anticodons can form tight dimers with dissociation constants as low as 1×10^{-7} M (Eisinger, 1971; Grosjean et al., 1976, 1978; Grosjean & Chantrenne, 1980) raises the question of whether this interaction inhibits the rate of peptide chain elongation. Dimer formation might a priori affect the rate of aminoacylation of the tRNA, the interaction of AA–tRNA with EF-Tu·GTP, or the binding of this ternary complex[2] to ribosomes. Our previous results indicated that ternary complex formation was not inhibited by the interaction of the anticodons (GmAA yeast and GAA *Escherichia coli*) of the Phe-$tRNA^{Phe}$ with the complementary anticodon (s^2UUC) of Glu-$tRNA^{Glu}$ (Yamane et al., 1981). We then examined the effects of dimer formation upon the rates of aminoacylation of $tRNA^{Glu}$ and $tRNA^{Phe}$, upon the rate and extent of binding of Phe-$tRNA^{Phe}$ to ribosomes, and upon the rate of incorporation of Phe-$tRNA^{Phe}$ into polyphenylanine in an *E. coli* in vitro translation system. At 15 °C, the rates of both aminoacylation of $tRNA^{Phe}$ and polyphenylanine synthesis are substantially inhibited by $tRNA^{Glu}$,

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; tRNA, transfer ribonucleic acid; EF-Tu, elongation factor Tu; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; DTT, dithiothreitol; poly(U), poly(uridylic acid); poly(A), poly(adenylic acid).

[2] Throughout the text ternary complex denotes the species AA-tRNA-EF-Tu·GTP.

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which leads us to propose that at low temperatures tRNA dimerization may control the rate of protein synthesis.

Materials and Methods

E. coli $tRNA^{Phe}$ (anticodon GAA, specific activity 1100 pmol/A_{260} unit), yeast $tRNA^{Phe}$ (anticodon GmAA, specific activity 1020 pmol/A_{260} unit), *E. coli* $tRNA^{Glu}$ (anticodon s^2UUC, specific activity 1200 pmol/A_{260} unit), and *E. coli* $tRNA_1^{Val}$ (anticodon cm^5UAC, specific activity 1100 pmol/A_{260} unit) were purchased from Boehringer Mannheim. $tRNA^{Glu}$ was annealed at 65 °C for 3 min to ensure renaturation (Grosjean et al., 1976). *E. coli* Lys-tRNA was obtained from Dr. A. E. Johnson.

Elongation factor Tu·GTP was prepared as described before (Miller & Weissbach, 1970). Radioactive phenylalanine and glutamic acid were obtained from New England Nuclear Corp. A mixture of aminoacyl-tRNA synthetases was prepared from *E. coli* B by the method of Muench & Berg (1966). *E. coli* ribosomes and EF-G were provided by Dr. N. Brot. The aminoacylation assays were performed by using 200-μL reaction mixtures containing 100 μM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM KCl, 1 mM ATP, 1 mM DTT, 25 μg of bovine albumin, 0.91 μM *E. coli* $tRNA^{Phe}$ or 0.31 μM *E. coli* $tRNA^{Glu}$, and 95 μM [^{14}C]phenylalanine (114 cpm/pmol) or 35 μM [^{3}H]glutamic acid (537 cpm/pmol) plus 12 μg of a mixture of aminoacyl-tRNA synthetases from *E. coli* B. The mixtures were incubated at 15 °C. At timed intervals 40-μL aliquots were withdrawn and precipitated with 1 mL of 5% cold trichloroacetic acid containing 0.04 M phenylalanine or glutamic acid. The precipitates were collected on cellulose nitrate filters, and their radioactivity was measured. The conditions employed in the binding and polymerization assays are described in the figure legends. The binding of Phe-tRNA to ribosomes was determined by the method of Zamir et al. (1974). Polyphenylalanine synthesis was quantified by hot trichloroacetic acid precipitation followed by collection on Millipore filters.

Results and Discussion

Effect of $tRNA^{Glu}$ on Aminoacylation of $tRNA^{Phe}$. Increasing concentrations of $tRNA^{Glu}$ have a drastic effect on the rate of aminoacylation of *E. coli* $tRNA^{Phe}$ at 15 °C (Figure 1a). A 0.55-fold excess of $tRNA^{Glu}$ (0.28 μM), calculated to lower the concentration of free $tRNA^{Phe}$ by more than 40% (Grosjean et al., 1978), inhibited the initial rate by 35% at 15 °C but exerted no inhibitory effect at 35 °C (data not shown). A similar effect was observed on the rate of aminoacylation of $tRNA^{Glu}$ in the presence of $tRNA^{Phe}$ (Figure 1b). This inhibition might be due to either the synthetase recognition requiring the anticodon sequence or the anticodon–codon interaction ($tRNA^{Phe}$–$tRNA^{Glu}$ dimer formation in the present case) triggering exposure of the TψCG sequence (residues 54–57) (Schwarz et al., 1976; Moller et al., 1979) and consequently weakening the synthetase–tRNA interaction. Both ribo- and deoxyribonucleotides complementary to the anticodon region of *E. coli* $tRNA^{Phe}$, at concentrations >100 μM, have been shown to inhibit the synthesis of Phe-$tRNA^{Phe}$ at 0 °C (Barrett et al., 1974). Recently, it has been elegantly shown that the replacement of residues 34–37 in the anticodon loop of yeast $tRNA^{Phe}$ by various oligonucleotide sequences leads to different rates and extents of aminocylation by yeast phenylalanine synthetase. This result implies that the anticodon region is involved in the synthetase recognition (Bruce & Uhlenbeck, 1981). Since the uniqueness of a given tRNA resides in its anticodon, investigators have sought to attribute to it a critical role in the synthetase–tRNA recognition

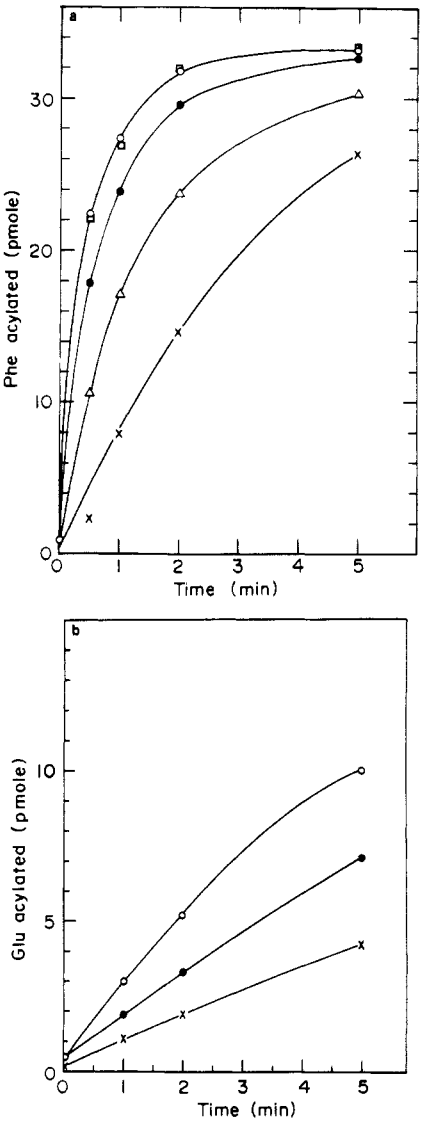

FIGURE 1: Effect of dimerization on the time course of aminoacylation of $tRNA^{Phe}$ and $tRNA^{Glu}$. (a) Effect of $tRNA^{Glu}$ or $tRNA^{Val}$ on $tRNA^{Phe}$ acylation: (○) 0.91μM $tRNA^{Phe}$; (●) 0.91 μM $tRNA^{Phe}$ plus 0.50 μM $tRNA^{Glu}$; (△) 0.91 μM $tRNA^{Phe}$ plus 2.1 μM $tRNA^{Glu}$; (X) 0.91 μM $tRNA^{Phe}$ plus 6.2 μM $tRNA^{Glu}$; (□) 0.91 μM $tRNA^{Phe}$ plus 4.3 μM $tRNA^{Val}$. (b) Effect of $tRNA^{Phe}$ on $tRNA^{Glu}$ acylation: (○) 0.31 μM $tRNA^{Glu}$; (●) 0.31 μM $tRNA^{Glu}$ plus 1.1 μM $tRNA^{Phe}$; (X) 0.31 μM $tRNA^{Glu}$ plus 3.3 μM $tRNA^{Phe}$. Reaction temperature 15 °C.

mechanism [for reviews, vide Ofengand (1977); Schimmel & Söll (1979), Schimmel (1979), Schulman (1979), and Ebel et al. (1979)]. However, it is still not clear how synthetases differentiate between different tRNA molecules. Although all tRNAs seem to have basically a similar structure, synthetases may not require the same recognition sites on tRNA.

Effect of $tRNA^{Glu}$ on Phe-$tRNA^{Phe}$ Binding to Ribosomes. Since the binding of AA-tRNA to ribosomes takes place primarily through the codon–anticodon interaction, one would expect, a priori, an inhibitory effect of $tRNA^{Glu}$ on Phe-tRNA binding. In fact, as shown in Figure 2, the rate of EF-Tu-promoted binding of Phe-tRNA to ribosomes programmed with poly(U) was inhibited by increasing concentrations of $tRNA^{Glu}$. The rate of EF-Tu-independent binding of Phe-tRNA was inhibited to about the same extent as the EF-Tu-dependent binding rate. It has been shown already that $tRNA^{Glu}$ does not disrupt Phe-tRNA·EF-Tu·GTP (Yamane et al., 1981). To confirm that by forming complexes $tRNA^{Glu}$

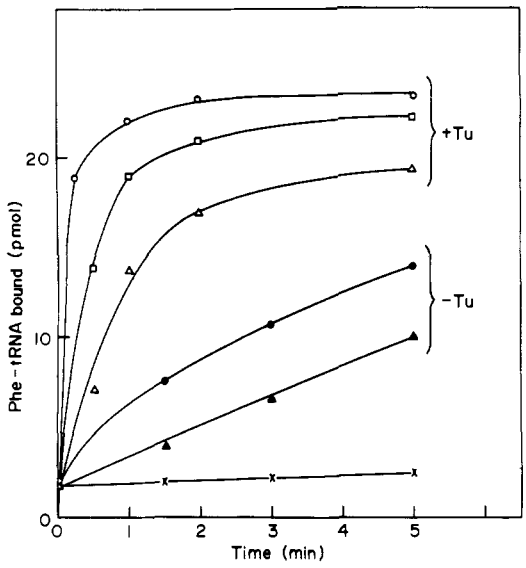

FIGURE 2: Effect of $tRNA^{Glu}$ and EF-Tu on the rate of binding of *E. coli* Phe-tRNA to the poly(U)–ribosome complex. The binding mixture contained, in a 50-μL total volume, 50 mM KCl, 100 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2.5 mM phosphoenolpyruvate, 0.1 μg of pyruvate kinase, 24 pmol of Phe-tRNA, 0.1 A_{260} unit of poly(U), 0.4 A_{260} unit of ribosomes, 38 pmol of EF-Tu·GTP, where indicated, and the following amounts of $tRNA^{Glu}$: (●, ○) 0.48 μM Phe-tRNA; (▲, △) 0.48 μM Phe-tRNA plus 1.2 μM $tRNA^{Glu}$; (□) 0.48 μM Phe-tRNA plus 1.2 μM $tRNA^{Glu}$ and 0.76 μM $tRNA^{Phe}$ (deacylated); (X) minus poly(U). The reactions were conducted at 0 °C and were intiated by adding ribosomes and poly(U).

inhibits Phe-tRNA binding to ribosomes, we performed an experiment in which unacylated $tRNA^{Phe}$ was added to a mixture of Phe-tRNA and $tRNA^{Glu}$ before conducting the ribosome binding reaction. The added $tRNA^{Phe}$ reversed the inhibition by $tRNA^{Glu}$ (figure 2) to the extent predicted by the contention that $tRNA^{Glu}$ inhibited by complexing with Phe-tRNA through the interaction of their complementary anticodons.

Effect of $tRNA^{Glu}$ on Polyphenylalanine Synthesis. Polyphenylalanine synthesis is also inhibited by $tRNA^{Glu}$ (Figure 3), as would be expected from the results of the ribosome-binding experiment. In the presence of saturating amounts of ribosomes and poly(U) at 15 °C, a 5-fold excess of $tRNA^{Glu}$ lowers the rate of phenylalanine incorporation by 80%. In contrast, at 37 °C $tRNA^{Glu}$ inhibits the incorporation rate by only 30%. The lower degree of inhibition at the higher temperature is readily explained by the decreased stability of the dimer at 37 °C. By extrapolation from the available data (Grosjean et al., 1976, 1978; Grosjean & Chantrenne, 1980), the association constant decreases from $\sim 20 \times 10^5$ M^{-1} at 15 °C to $\sim 1 \times 10^5$ M^{-1} at 37 °C. Thus, at the higher temperatures 50% of the Phe-tRNA would be dissociated from $tRNA^{Glu}$, which is enough to maintain the observed polymerization rate.

To be certain that $tRNA^{Glu}$ inhibits polyphenylalanine synthesis specifically, we repeated the experiment, substituting $tRNA^{Val}$ for $tRNA^{Glu}$ (Figure 4). Although the anticodon (cm^5U) of $tRNA^{Val}$ differs from the anticodon (s^2UUC) of $tRNA^{Glu}$ only in the second base, $tRNA^{Val}$ does not interact detectably with $tRNA^{Phe}$ (Grosjean et al., 1978). To demonstrate that $tRNA^{Glu}$ is not a general inhibitor of polymerization, we showed that it exerts no effect upon the rate of polylysine synthesis coded by poly(A) (results not shown).

If $tRNA^{Glu}$ inhibits polyphenylalanine formation by binding to Phe-tRNA, the inhibition should be reversed by adding deacylated $tRNA^{Phe}$. The interpretation of this experiment

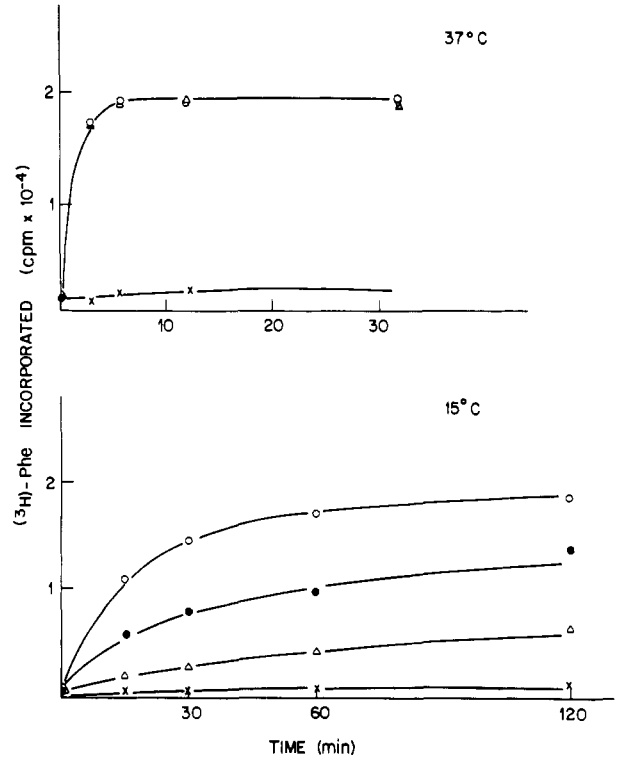

FIGURE 3: Effect of complementary $tRNA^{Glu}$ on the rate of incorporation of *E. coli* Phe-tRNA into polyphenylalanine at 37 and 15 °C. Each 50-μL complete reaction mixture contained the following: polymerization buffer (Jelenc & Kurland, 1979) (magnesium acetate, 10 mM; $CaCl_2$, 0.5 mM; potassium phosphate, 5 mM; KCl, 95 mM; putrescine, 8 mM; spermidine, 1 mM; NH_4Cl, 5 mM; DTT, 1 mM), phosphoenolpyruvate, 400 nmol; pyruvate kinase, 1 μg; ribosomes, 1.6 A_{260} units; poly(U), 0.4 A_{260} unit; Phe-tRNA, 120 pmol (298 cpm/pmol); EF-Tu·GTP, 200 pmol; GTP, 1 nmol; G factor, 4 μg; $tRNA^{Glu}$, 110 and 550 pmol. The reaction mixtures were incubated at 37 or 15 °C, samples were taken as indicated, 1 mL of 5% Cl_3C-COOH containing 0.5% Phe was added, and samples were heated at 100 °C for 15 min, filtered, and treated as described by Jelenc & Kurland (1979). The reaction mixtures contained the following amounts of $tRNA^{Glu}$: (○) none; (●) 110 pmol; (△) 550 pmol; (X) minus $tRNA^{Glu}$ and minus poly(U).

is complicated because $tRNA^{Phe}$ is itself a strong inhibitor of polyphenylalanine formation (Figure 4) (Kyner et al., 1973; Zasloff, 1973); however, it can be seen that the mixture of $tRNA^{Glu}$ and $tRNA^{Phe}$ inhibits polymerization less than would be expected from the combination of their separate inhibitory effects. This result can be explained qualitatively as follows: $tRNA^{Glu}$ binds to the same extent to either the ternary complex or $tRNA^{Phe}$, lowering the concentration of both inhibitor and substrate proportionally and maintaining approximately the same rate of synthesis. One should keep in mind that aminoacylated $tRNA^{Phe}$ was used for polyphenylalanine synthesis experiments. Since the inhibitory effect due to $tRNA^{Glu}$ is already observed at the aminoacylation level, one should expect a much higher effect if the polypeptide synthesis is carried out with nonacylated $tRNA^{Phe}$.

On the basis of these results, it seems possible that the formation of tRNA dimers might limit the rate of peptide chain elongation in vivo. To assess this possibility, we can estimate whether the availability of a free ternary complex can become rate determining. From the data of Maaløe & Kjeldgaard (1966), the concentration of total tRNA in *E. coli* cell is $\sim 5 \times 10^{-4}$ M. A specific AA-tRNA might be 2% of the total or 10 μM. Assuming that the intracellular concentrations of both Phe-tRNA and Glu-tRNA are ~ 10 μM and that the dissociation constant of the Phe-tRNA–Glu-tRNA dimer at 37 °C is 10 μM (Grosjean et al., 1976, 1978;

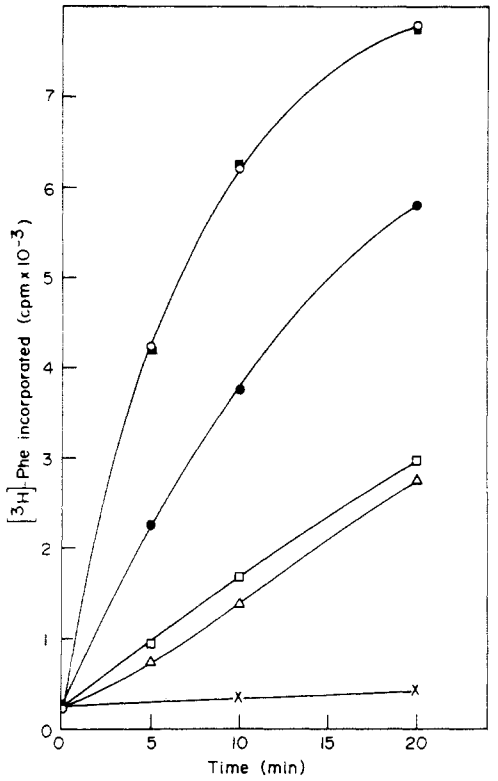

FIGURE 4: Effect of nonacylated $tRNA^{Phe}$ and $tRNA^{Glu}$ on polyphenylalanine synthesis. Conditions were the same as described in Figure 3, except the Phe-$tRNA^{Phe}$ concentration was 0.84 μM. (○) 0.84 μM Phe-$tRNA^{Phe}$; (●) 0.84 μM Phe-tRNA plus 1.1 μM $tRNA^{Glu}$, (□) plus 0.91 μM nonacylated $tRNA^{Phe}$, (△) plus 1.1 μM $tRNA^{Glu}$ plus 0.91 μM $tRNA^{Phe}$, (■) 0.84 μM Phe-tRNA plus 1.2 μM $tRNA^{Val}$, and (X) minus poly(U).

Grosjean & Chantrenne, 1980), it can be calculated that the concentration of "free" Phe-tRNA would be ~6 μM. The dimerization of tRNAs is not affected by the binding of EF-Tu·GTP (Yamane et al., 1981); therefore, the free Phe-tRNA would actually be bound to EF-Tu·GTP. This concentration of free ternary complex is about 1 order of magnitude higher than the Michaelis constant for the ternary complex binding to ribosomes (Miller & Weissbach, 1970).

The elongation rate might also be limited by the rate of dissociation of the dimers. The rate constant of dissociation of the $tRNA^{Phe}$–$tRNA^{Glu}$ dimer is ~51 s^{-1} at 37 °C [extrapolated from the data of Grosjean et al. (1976, 1978) and Grosjean & Chantrenne (1980)], which is 3 times as fast as the in vivo rate of peptide bond formation, applying no corrections for the frequency of appearance of specific codons. Thus the available data indicate that at 37 °C, dimer formation will not inhibit the rate of peptide chain elongation, i.e., the dimer dissociation rate is faster than the peptide chain elongation rate.

In constrast, at 15 °C the dimers are much more stable, and their rates of dissociation are much slower. Again from the data of Grosjean et al. (1976, 1978) and Grosjean & Chantrenne (1980), we calculation that the dissociation constant of the $tRNA^{Phe}$–$tRNA^{Glu}$ dimer drops to 5×10^{-7} M, and its dissociation rate constant declines to 2 s^{-1}. The concentration of free Phe-tRNA or free ternary complex would drop to ~2 μM, only about a factor of 2 above the Michaelis constant. Thus, it is possible that in vivo the elongation rate is controlled by dimer formation at temperatures below 15 °C.

The minimal growth temperature for a typical mesophile bacterium, *E. coli*, is 8 °C (Ingraham, 1958; Ron & Davis, 1971), i.e., cell multiplication does not occur below that temperature. In correlation with the cessation of cell division, a sudden increase in ribosomal subunit accumulation has been observed between 8 and 10 °C (Friedman et al., 1969). Because aminoacyl-tRNA synthetases were shown to be functioning normally even at 0 °C (Goldstein et al., 1964; Das & Goldstein, 1968; Barrett et al., 1974), the dimer formation between two complementary tRNAs, perhaps $tRNA^{His}$ and $tRNA^{fMet}$, is certainly a possible cause. Another possible factor implicated in the temperature dependence of protein synthesis is the crystallization of membrane lipids (Schecter et al., 1974; Towers et al., 1973). In addition, translocation of ribosomes along mRNA is greatly slowed at temperatures below 8 °C. This process also involves the breakage of codon–anticodon interactions. The effects of low temperatures on protein synthesis in mammalian cells in culture have been extensively investigated by Craig (1975, 1976, 1979) and Oleinick (1979), and they have concluded that the initiation step becomes rate limiting below 25 °C in mouse L and Chinese hamster ovary cells. This was not the result of membrane phase changes since the same effect could be reproduced in membrane-free subcellular systems (Craig & Fahrman, 1977).

In nature, there are organisms which undergo metabolic adaptation to changing temperature, e.g., a cold acclimation observed in toadfish, *Opsanus tau* (Plant et al., 1977; Nielsen et al., 1977). This organism, when transferred from 20 to 10 °C, initially undergoes a depression in its rates of metabolism and protein synthesis, but, after several days, an increase of overall protein synthesis is observed, higher than in fish maintained throughout at 20 °C. This increase in protein synthesis was correlated with increased EF-1 activity, but whether this is the main cause of the acclimatization is not known (Nielsen et al., 1977).

Because the degree of protein synthesis inhibition due to dimer formation depends upon competition between a complementary tRNA and ribosomes for a certain tRNA, one possibility for increasing the binding to ribosomes without affecting the anticodon region is by base modification. Although there is a growing interest in the regulatory roles of tRNAs in cells, no clear picture has yet emerged about the biological function of the partial modification of tRNAs in cell regulation or its effects upon the efficiency of codon reading. (Björk & Neidhardt, 1975; Marcu & Dudock, 1976; Roe & Tse, 1977).

In spite of potential inhibition of protein synthesis by tRNA dimer formation, many species thrive at low temperatures. It would be interesting to know whether these organisms have selected, through evolution, a complement of tRNAs that form weakly associated dimers. It is also possible the codons for weakly associating tRNAs are preferred. Further studies of low-temperature protein synthesis are in order.

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Synthesis of Nucleoside 3′-(*S*-Alkyl phosphorothioates) and Their Use as Substrates for Nucleases†

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ABSTRACT: The synthesis of cytidine, uridine, guanosine, and adenosine 3′-(*S*-methyl phosphorothioates) by treatment of the 2′,5′-di-*O*-(4-methoxytetrahydropyran-4-yl)ribonucleosides with 2-(methylthio)-4*H*-1,3,2-benzodioxaphosphorin 2-oxide is described. These nucleotide analogues are stable compounds both in the solid state and in neutral aqueous solution. All four of these compounds are degraded by RNase T_2 to the parent nucleotides and methanethiol. In addition, cytidine and uridine 3′-(*S*-methyl phosphorothioates) are substrates for bovine pancreatic ribonuclease and guanosine 3′-(*S*-methyl phosphorothioate) is a substrate for RNase T_1 and RNase U_1. When used in conjunction with a chromophore-producing reagent, nucleoside 3′-(*S*-methyl phosphorothioates) provide a means for direct kinetic measurement of ribonuclease activity over a wide pH range (pH 2–9). The reactivities of these substrates with ribonucleases are compared to the reactivities of other synthetic substrates as well as a number of natural substrates. The utility of ribonucleoside 3′-(*S*-methyl phosphorothioates) as substrates for the assay of ribonucleases is discussed.

Nucleoside phosphorothioates and their esters are substrates for a number of enzymes of nucleic acid metabolism (Goody & Eckstein, 1971; Eckstein & Gindl, 1970; Schlimme et al., 1970; Cook, 1970; Cook et al., 1969; Eckstein, 1970). Cook (1970) and Cook et al. (1969) prepared thymidine 5′-(*S*-ethyl phosphorothioate) by condensation of dilithium *S*-ethyl phosphorothioate and 3′-(*O*-acetyl)thymidine using dicyclohexylcarbodiimide. The dinucleoside phosphorothioate Tp(s)T was prepared by the same authors by the reaction of 5′-deoxy-5′-iodo-3′-(*O*-acetyl)thymidine with thymidine 3′-phosphorothioate. Eto et al. (1974) treated the 2′,3′-borate complex of the ribonucleoside 1 with 2-(methylthio)-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (MBTO)[1] (**2**) (Iio et al., 1973) in the presence of cyclohexylamine to form the ribonucleoside 5′-(*S*-methyl phosphorothioate) **3**.

Using the method of Eto et al. (1974), we have prepared the ribonucleoside 3′-(*S*-methyl phosphorothioate) analogues of the four major naturally occurring ribonucleotides in order

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[1] Abbreviations used: MBTO, 2-(methylthio)-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; TLC, thin-layer chromatography; DMF, dimethylformamide.

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