The Function of Pseudouridylic Acid in Transfer Ribonucleic Acid

SUMMARY

Inhibition of the nonenzymatic binding of phenylalanyl transfer RNA to polyuridylic acid-coded ribosomes at 20 mM magnesium was used as an assay to determine whether the tetranucleotide ribothymidyl-pseudouridylyl-cytidylyl-guanosine 3'-phosphate possessed any specific ability to bind to ribosomes. Inhibition of binding at the peptidyl site was studied by adding tetracycline to block aminoacyl site binding activity, and aminoacyl site activity was examined in the presence of excess deacylated tRNA to suppress peptidyl site binding. Deacylated tRNA was bound competitively with aminoacyl-tRNA at the peptidyl site, but was without effect at the aminoacyl site.

At both of these sites, T-$-C-Gp was more inhibitory than any other oligonucleotide tested. At the peptidyl site, T-$-C-Gp was 10 times more active than A-A-A-Gp, and 3 times more active than (A,U,C)Gp. At the aminoacyl site, T-$-C-Gp was again 3 times more active than (A,U,C)Gp and 20 times more active than A-A-Gp or (A,C)Gp. Alkaline hydrolysis destroyed the inhibitory activity and cyanoethylation of the pseudouridine residue with acrylonitrile decreased the activity to 50% although a control treated with propionitrile was fully active. Inhibitory activity was resistant to boiling.

Inhibition by T-$-C-Gp was not competitive with free phenylalanyl-tRNA nor affected by ribosome concentration. However, T-$-C-Gp does act at the ribosomal level since ammonium chloride-washed particles were 4 times more sensitive to T-$-C-Gp inhibition, and aging of these particles further increased their sensitivity. Variation of the polyuridylic acid or Mg$^{2+}$ concentration was without effect.

Since T-$-C-Gp was able to block aminoacyl-tRNA binding sites on ribosomes more effectively than other similar nucleotides this sequence probably represents part of the normal ribosome binding site in tRNA. The failure to compete with unbound aminoacyl-tRNA and the effect of ammonium chloride washing on the ribosomes suggests that the actual reaction mechanism is complex.

In the process of protein synthesis, specific aminoacyl transfer RNA molecules become bound to ribosomes in response to the directive influence of the messenger RNA code word. Current concepts of this process envision the existence of a common site on the ribosome for binding all aminoacyl-tRNA molecules but that stabilization of this association requires the additional interaction of the anticodon with the code word triplet (1) and an intact acceptor end (2-4). It has been generally recognized that the existence of a common site on the ribosome implies the existence of a complementary common site in all tRNA molecules, but there has been so far no experimental evidence available to help in identifying this site.

The discovery by Zamir, Holley, and Marquisee (5) of a common pentanucleotide sequence, GpTpGpCpGp, in most tRNAs of Escherichia coli, yeast, and rat liver indicated that this sequence might be involved in the ribosome binding site although the authors were careful to point out that their data was suggestive only of an involvement in a function which did not discriminate among different tRNA molecules. The importance of this pentanucleotide has subsequently been emphasized by the determination of the complete primary structure of 17 tRNA molecules (6-16) encompassing bacterial, plant, and animal sources which show that this sequence occurs in every tRNA at residues 20 to 24, counting from the amino acid acceptor end. In the only two minor exceptions to this rule, tRNA$^{\text{Met}}_{E. coli}$ (10)$^1$ and tRNA$^{\text{Ser}}_{\text{yeast}}$ (17), the 3'-terminal guanosine is replaced by an adenosine. In the cloverleaf two-dimensional representation of tRNA this sequence has been placed in a loop region (6), although it is clear from several lines of evidence that, in actuality, this region is shielded when the tRNA is in a native conformation (18-23).

1 The abbreviations used are: tRNA$^{\text{Met}}_{E. coli}$, E. coli tRNA species specific for methionine which can be formylated; tRNA$^{\text{Ser}}_{\text{yeast}}$, one of the two brewers' yeast tRNA species specific for serine; poly U, polyuridylic acid; tRNA$^{\text{Phe}}$, tRNA species specific for phenylalanine.
It seems reasonable to suppose that this unique sequence containing two unusual nucleotides is not so specifically localized for fortuitous reasons. Nevertheless, there has been no evidence up to the present to show whether this region of the molecule is in fact involved in the ribosome binding site or in some other common functional feature shared by all tRNAs.

In this report we show that the tetrancotide T-$-C-Gp is able to inhibit the poly U-directed binding of phenylalanyl-tRNA to ribosomes at both the peptidyl-tRNA binding site and at the aminoacyl-tRNA site. Other similar control oligonucleotides possess this inhibitory effect to a smaller extent and hydrolysis to mononucleotides abolishes the effect. Inhibition by T-$-C-Gp was not competitive with free phenylalanyl-tRNA but was affected by treatment of the ribosomes with ammonium chloride and aging at 4°.

These findings have been interpreted to mean that T-$-C-Gp is specifically interfering with some aspect of the aminoacyl-tRNA-ribosome binding mechanism, and therefore that the T-$-C-Gp sequence does in fact make up part of the normal ribosome binding site in tRNA. A preliminary account of this work has appeared (24).

EXPERIMENTAL PROCEDURE

Materials

Yeast and E. coli tRNA were obtained from Schwarz BioResearch and General Biochemicals, T1 RNase (Sankyo) from Calbiochem, venom phosphodiesterase and E. coli alkaline phosphatase from Worthington, 14C-phenylalanine from Amersham/Searle, Des Plains, Illinois, DEAE-Sephadex A-25 and G-25 beads from Pharmacia, Bio-Gel P2 (100 to 200 mesh) from Bio-Rad Laboratories, Richmond, California, and poly U from Miles Laboratories, Inc., Elkhart, Indiana. Tetrahydrofuran (Tetra-cyn) was obtained from Charles Pfizer Company, New York, through Dr. E. Jawetz of the University of California. It was dissolved in water (final pH about 2.7) and stored frozen. Its concentration was determined from absorbance at 355 nm.

14C-Phenylalanyl-tRNA

With or without the other 13C amino acids 14C-phenylalanyl-tRNA was prepared and assayed as previously described (25) using 6 µM 14C-phenylalanine (specific activity 785 cpm per µmole) and 12 µm each of the other 19 13C-amino acids when added. Aminoacyl-tRNA was isolated by extraction with phenol saturated with 90 mM triethylammonium formate buffer, pH 4.6, 5 mM mercaptoethanol. After ethanol precipitation, residual salts and phenol were removed by gel filtration. The phenylalanyl-tRNA preparation (1.5 to 1.6% phenylalanyl-tRNA) was stored at -15°. Based on charging assays of uncharged, treated tRNA, these preparations were 90 to 100% charged with phenylalanine.

Uncharged, Treated tRNA

This was prepared by phenol extraction and Sephadex G-25 chromatography of E. coli tRNA as described above. It was then stripped by incubation in 1.8 M Tris, pH 8.0 (26), for 30 min at 37°, and the Tris removed by Sephadex U-25 filtration.

Ribosomes

They were prepared from a frozen cell paste of E. coli B by the pressure cell method of Nirenberg (27) modified as follows. Stationary phase cells grown on a rich medium (28) were used, ammonium chloride replaced potassium chloride. DNase treatment and prior incubation were omitted, the first ribosomal pellet was extracted with 50 mM Tris, pH 8.0, 0.04% sodium deoxycholate for 10 min at 0°, and the ribosomes were subsequently washed by three cycles of differential centrifugation. Ribosomes were stored at -70° and used only once. These ribosomes are largely free of endogenous mRNA but are highly active when supplemented with viral mRNA (29) and preliminary experiments showed them to be also somewhat more active in nonenzymatic poly U-dependent binding of phenylalanylintRNA than those made by the procedures of Kurland (30) or Seeds, Retema, and Conway (31). However, the extent of contamination with T factor, G factor, initiation factors, or GTP is not known.

Oligonucleotides

Isolation—All of the oligomers used in this study were obtained by fractionation of a complete T1 RNase digest of yeast tRNA on DEAE-Sephadex-urea columns (32). A typical preparation is described. Baker's yeast tRNA (2 g, 6870 pmoles of nucleotide) (Schwarz BioResearch) in 12 ml was desalted on a column (2.5 x 80 cm) of Sephadex G-25 equilibrated in 10 mM EDTA, pH 8.0, in order to remove potentially inhibitory heavy metals (33). Digestion was carried out for 36 hours at 37° in 120 ml of 0.05 M Tris, pH 7.6, containing 10 mmoles of RNA nucleotide from the Sephadex column, 40,000 units of T1 RNase (Sankyo), and a few milliliters of chloroform. The reaction was terminated by addition of 60 g of urea, loaded on a column (4 x 80 cm) of DEAE-Sephadex A-25 equilibrated with 7 M urea, 0.18 M NaCl, and 0.02 M Tris, pH 7.6, at 23°, and eluted at 80 ml per hour by a 0.0264 M per liter linear NaCl gradient containing Tris and urea.

Operation of DEAE-urea columns in this manner gives partial fractionation of isoplicths in the di- to tetranucleotide range and consequently many peaks. However, because the T-$-C-Gp tetrancotide is common to all tRNAs and other tri- and tetranucleotides bounded by guanylate residues are not so frequently found, the T-$-C-Gp oligomer stands out as a major peak. Its location was confirmed by testing for the bathochromic shift in alkali characteristic of pseudouridine residues. The ratio of A260 (40 mM NaOH):A280 (pH 7) served as a reliable indicator for confirming the location of the T-$-C-Gp peak after making appropriate blank corrections. Usually ratios of 1.5 to 1.7 were found while other oligonucleotides in that region gave ratios around 1.0.

The peak was desalted by the method of Uziel and Cohn (34) on Bio-Gel P2 (100 to 200 mesh) equilibrated with 10-4 M Tris, pH 8.0, at 23°. Salt was monitored by conductivity and urea by refractive index. The salt-free oligoucleotide solution was lyophilized.

Separation of T-$-C-Gp from the other oligonucleotides in this fraction was done by chromatography on DEAE-Sephadex in 7 M urea, pH 2.7 (32). Three thousand optical density units (an optical density unit is that amount of material per ml of solution which produces an absorbance of 1 in a 1-cm light path cell at 260 nm), of oligonucleotide from the previous column were
made 7 M in urea, pH 2.7, with HCl, and the measured salt concentration was adjusted to 0.03 M. The sample (20 ml) was loaded on a column (2 x 50 cm) of DEAE-Sephadex A-25 equilibrated at 23° with 7 M urea, adjusted to pH 2.7 with HCl, 0.03 M NaCl, and eluted at 80 ml per hour with a 0.06 M linear salt gradient in the same buffer. Usually four symmetrical peaks were observed well separated from each other and from minor contaminants. The first peak came out virtually at the void volume because it consisted only of adenosine, cytidine, and guanosine residues. In several earlier experiments, with the use of bakers' yeast tRNA obtained from General Biochemicals, this peak was rather pure A-A-A-Gp (see "Identification"). However, in a later run, using bakers' yeast tRNA from Schwarz BioResearch, a mixture of two trimonucleotides A-A-Gp and (A,C)Gp was found in this peak. It is not known whether this difference was caused by variation in the source of the tRNA or to slightly different chromatography, or selection, or both of the T-Ψ-C-Gp peak from the pH 7.6 column.

The second peak was an oligonucleotide or oligonucleotides containing 1-methyl adenylate. The third peak, eluting just before T-Ψ-C-Gp, was pGp presumably derived from the 5'-end of some tRNA chains. T-Ψ-C-Gp was the fourth and largest peak, eluting at about 0.09 M salt. The bathochromic shift in alkali was used to confirm the presence of pseudouridine in this peak, taking care first to neutralize the solution before measuring absorbance.

After neutralization with NH₄OH, each peak was desalted and concentrated as described above. Where necessary, the concentrates were desalted again on small gel columns. Molar concentration was determined from absorbance measurement at 260 nm, pH 7, on alkali-hydrolyzed samples by the use of the base composition information of Table I and extinction coefficients from the literature (35, 36). Yields of T-Ψ-C-Gp averaged about 300 optical density units per pmole of tRNA nucleotide, or 70% of the theoretical yield if all the ultraviolet-absorbing material in the commercial preparation was tRNA.

As long as only fresh solutions and fresh DEAE-Sephadex columns were used, no problems with RNase were encountered. However, it is especially important to keep the Bio-Gel columns in the presence of 0.02% NaN₃ when not actually in use in order to prevent bacterial growth and consequent release of RNase.

For the preparation of (A, U, C)Gp, the tetrarnucleotide fraction from several small pH 7.6 column runs was pooled and recrchromatographed on DEAE-Sephadex, 7 M urea, pH 2.7, as described above. Several major peaks appeared. The one eluting at 0.054 M NaCl was desalted and concentrated as described above. It was subsequently identified as a tetranucleotide containing the four common nucleotides (see below).

Identification—Several T-Ψ-C-Gp preparations were made. They were characterized by base composition (Table I) and by chain length as determined by phosphorus analysis (Table II). The preparations designated "a" and "b" were not analyzed quantitatively for nucleotide composition. However, qualitative inspection of the chromatogram after digestion to nucleosides showed results very similar to the other preparations, namely strong spots of ribothymidine, pseudouridine, cytidine, and guanosine, and faint spots of adenosine and uridine. A chain length determination (Table II) was done on "b" but no further analysis of "a" was performed.

The other oligonucleotides were identified similarly, bearing in mind that they all must terminate in a 3'-guanylate residue since they were derived from a T₁ RNase digest. The data of Tables I and II suggested that A-A-Gp/(A, C)Gp was a mixture of two trimonucleotides and this was confirmed by separation into two peaks on a DEAE-Sephadex-urea column at pH 4.0. At this pH, (A, C)Gp is expected to elute before A-A-Gp. On this assumption, the relative molar amounts of the two peaks were calculated to be 59 and 41%, respectively, in good agreement with the proportions calculated from the data of Table I. The identity of pGp was confirmed by showing that treatment with alkaline phosphatase alone was sufficient to convert all of the material to guanosine.

Methods

Binding Assays

These assays were performed as described in the legends to the various figures and were designed to allow the measurement of maximal phenylalanyl-tRNA binding to either the aminoacyl or peptidyl-tRNA site under conditions of limiting ribosomes. Maximal binding at the peptidyl site (i.e. in the presence of

<table>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td><strong>Base composition of oligonucleotide preparations</strong></td>
</tr>
<tr>
<td><strong>Values are reported relative to ribothymidine for T-Ψ-C-Gp and relative to guanosine for the other oligonucleotides. The method of analysis is as described under ‘Methods.’</strong></td>
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<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Guanosine</th>
<th>Adenosine</th>
<th>Uridine</th>
<th>Cytidine</th>
<th>Ribothymidine</th>
<th>Pseudo-uridine</th>
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<tr>
<td>T-Ψ-C-Gp-1</td>
<td>0.68</td>
<td>0.02</td>
<td>0.06</td>
<td>1.09</td>
<td>1.00</td>
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<td>T-Ψ-C-Gp-2</td>
<td>1.39</td>
<td>0.14</td>
<td>0.39</td>
<td>1.37</td>
<td>1.00</td>
<td>1.17</td>
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<td>T-Ψ-C-Gp-3</td>
<td>1.00</td>
<td>1.00</td>
<td>0.82</td>
<td>1.08</td>
<td></td>
<td></td>
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<tr>
<td>(A, U, C)Gp</td>
<td>1.00</td>
<td>1.39</td>
<td>0.67</td>
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<tr>
<td>A-A-Gp/(A, C)Gp</td>
<td>1.00</td>
<td>3.05</td>
<td>0.21</td>
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<td></td>
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<tr>
<td>A-A-Gp</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pGp</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* This preparation consists of approximately 60% (A, C)Gp and 40% A-A-Gp.

**Phosphorus content of oligonucleotide preparations**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Concentration</th>
<th>Porganic</th>
<th>Pterminal</th>
<th>Fe:Phosphorus</th>
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<tbody>
<tr>
<td>T-Ψ-C-Gp-1</td>
<td>4.08</td>
<td>20.0</td>
<td>4.35</td>
<td>4.6</td>
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<tr>
<td>T-Ψ-C-Gp-2</td>
<td>5.85</td>
<td>25.8</td>
<td>5.52</td>
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<td>T-Ψ-C-Gp-3</td>
<td>8.68</td>
<td>32.1</td>
<td>9.30</td>
<td>3.5</td>
</tr>
<tr>
<td>(A, U, C)Gp</td>
<td>12.2</td>
<td>55.1</td>
<td>12.5</td>
<td>4.4</td>
</tr>
<tr>
<td>A-A-Gp/(A, C)Gp</td>
<td>1.94</td>
<td>6.00</td>
<td>1.91</td>
<td>3.1</td>
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<tr>
<td>A-A-Gp</td>
<td>1.80</td>
<td>8.90</td>
<td>2.30</td>
<td>3.9</td>
</tr>
<tr>
<td>pGp</td>
<td>12.4</td>
<td>23.3</td>
<td>24.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Computed from absorption at 260 nm, pH 7, after alkaline hydrolysis, 0.3 M NaOH, 18 hours, 37°, with the use of the ε (pH 7) given in the literature (35, 36) for nucleotides. For T-Ψ-C-Gp, the value of ε = 30,400.

**Porganic** was determined as total phosphorus minus inorganic phosphorus.

**Pterminal** was determined as the ΔP₁ produced after alkaline phosphatase treatment. Of the oligonucleotide 30 to 40 monomers was incubated with 24 units of enzyme in 0.1 M Tris, pH 9.0, at 37° for 30, 60, and 90 min. A stable plateau value was obtained in every case.

* A mixture of 60% (A, C)Gp and 40% A-A-Gp (see Table I).
tetracycline) under the conditions described in Fig. 2 was reached in 20 min in the presence or absence of T-Ψ-C-Gp (50% inhibition), was stable to 60 min, and decreased 23% by 70 min. Variation in pH from 5.4 to 7.0 had no effect although Tris buffer was 1.3 times better than cacodylate. Poly U was saturating at less than half the standard concentration even with 50% inhibition by T-Ψ-C-Gp, 90% saturation being reached at one-fifth the standard level. Phenylalanyl-tRNA was present in a 1.5- to 4-fold excess as shown by the saturation data of Fig. 2. The presence or absence of the other αC-aminoacyl-tRNAs made no difference since the phenylalanyl-tRNA saturation curves were identical, inhibition by uncharged tRNA (Figs. 1 and 2) was the same, and inhibition by T-Ψ-C-Gp seemed about the same. As expected under these conditions, the amount of binding was directly proportional to the amount of ribosomes added. Since 95% of the binding was poly U-dependent, no corrections were made for polymer-independent binding in this work.

Maximal aminoacyl site binding (with excess uncharged tRNA as in Fig. 1) required 60 min of incubation and 0.15 μM phenylalanyl-tRNA in the standard assay. Phenylalanyl-tRNA, 0.07 μM, gave 83% saturation, and 0.3 μM gave no more than 0.15 μM. Saturation with poly U at this site was not directly tested but seems likely since the standard assay used 4 times the amount giving 80% saturation at the peptidyl site. As with peptidyl site binding, use of phenylalanyl-tRNA with or without αC-aminoacyl-tRNA made little difference either in the amount of phenylalanyl-tRNA bound or in the degree of inhibition observed by T-Ψ-C-Gp (compare Figs. 6 and 7). Addition of GTP (0.4 mM) to binding reactions blocked at the peptidyl site by 4-fold excess of uncharged tRNA had no effect on the amount bound at 60 or 80 min.

It should be noted that hydrolysis of phenylalanyl-tRNA is continually taking place during these assays. Under the conditions used here, the half-life of phenylalanyl-tRNA is 47 min so that after 30 min of incubation only 65% of the original phenylalanyl-tRNA is still present. Although the uncharged tRNAph so generated would be expected to interfere with peptidyl site binding, a number of factors make it difficult to quantitate the effect. For example, phenylalanyl-tRNA bound to ribosomes is less susceptible to hydrolysis than free phenylalanyl-tRNA (37), the rate of release of phenylalanyl-tRNA and of tRNAph from ribosomes appears slower than the rate of binding (38), and the ratio of deacylated to acylated tRNA, while constantly increasing, is doing so in the face of increased total binding of phenylalanyl-tRNA with time. Empirically, a plateau in the amount of binding with time can be reached, and while it may only represent some steady state value, all inhibition assays have been done under these constant conditions. Moreover, as shown below, the presence of a reasonable amount of deacylated tRNAph in the assay does not seriously affect the calculation of relative binding constants for an added modified tRNA.

Similarly, aminoacyl site assays which are incubated for 70
min should result in only 40% of the added phenylalanyl-tRNA remaining at the end of the reaction. This may account for the abnormally higher concentration of added phenylalanyl-tRNA needed to saturate the same amount of ribosomes at this site as compared to the peptidyl site. However, since deacylated tRNA does not inhibit at this site (Fig. 1), the presence of extra uncharged tRNA should have no effect other than slightly increasing the normal 4-fold excess of uncharged tRNA that is added in order to suppress peptidyl site binding.

Analysis of Single Site Binding—For the case of limiting binding sites to ribosomes in the presence of excess aminoacyl-tRNA and competitor, the relationship at equilibrium can be readily shown to be

\[ \frac{(B_o/B) - 1}{K_d/K_a} = \left( \frac{\text{tRNA}}{\text{aminoacyl-tRNA}} \right) \]

where \( B_o \) is the maximum amount of aminoacyl-tRNA bound in the absence of competitor tRNA, \( B \) is the amount of aminoacyl-tRNA bound in the presence of a given amount of tRNA, \( K_d \) is the association constant for tRNA and ribosomes, and \( K_a \) is the association constant for aminoacyl-tRNA and ribosomes. Thus a plot of \( (B_o/B) - 1 \) versus \( \frac{\text{tRNA}}{\text{aminoacyl-tRNA}} \) will be a straight line through the origin whose slope is a ratio of \( K_a/K_d \). The unbound concentrations are needed in this equation and are obtained as follows. The free phenylalanyl-tRNA is simply that added minus the amount bound to ribosomes which can be directly calculated from the counts per min bound. This correction is usually small. The free tRNA is the amount added minus the tRNA bound to ribosomes. Since the tRNA is unlabeled this quantity must be obtained indirectly. It is assumed that with excess phenylalanyl-tRNA all ribosome sites are filled so that \( B_o - B \) in moles is the amount of deacylated tRNA bound. When only small amounts of tRNA are added, this correction becomes important.

The effect of partial deacylation of the added aminoacyl-tRNA such as occurs during the assay is not very great. When enough excess aminoacyl-tRNA is present so that the amount unbound is approximately equal to that initially added, \( K_a/K_a \) becomes \( K_a/(1 - f)K_d \) where \( f \) is the fractional acylation of aminoacyl-tRNA and the concentration of tRNA is calculated as described above. If \( R_o \) is defined as the observed slope of the line equal to \( K_a/(1 - f)K_d \) and \( R_T \) as the true association constant ratio, \( K_a/K_d \), then

\[ R_T = \frac{fR_o}{1 - (1 - f)R_o} \]

and this equation can be used to calculate a true \( R_T \) from \( R_o \) and various assumed degrees of acylation.

In general, if \( R_o > 1 \), increasing deacylation leads to increased values for \( R_T \) compared to \( R_o \). If \( R_o \) is 1, \( R_T = 1 \) independent of the degree of deacylation, and if \( R_o < 1 \), increased deacylation decreases \( R_T \) relative to \( R_o \). However, the effects are not too marked. For example, if \( R_o \) is 1.2 (as in Fig. 2), 30% deacylation such as occurs by the end of a 35-min incubation will increase \( R_T \) only to 1.3.

Analysis of Nucleotide Composition

Oligonucleotides were digested by the combined action of venom phosphodiesterase and alkaline phosphatase and subjected to two-dimensional thin layer cellulose chromatography. The solvents and general procedures have been described previously (39). For the first dimension, Solvent E was used followed after drying by Solvent C in the same dimension. Solvent D was used for the second dimension. Standards were applied in each dimension and identification was done by comparison of \( R_T \) values on the same plate. In one case, A-A-A-Gp, Solvent A was used two times in the first dimension since a preliminary run had shown the presence of only adenosine, guanosine, and possibly uridine.

For quantitation the spots were eluted with 0.1 N HCl and the absorbance at 260 nm determined. Appropriate extinction coefficients were calculated from handbook data (35) and available spectra, and blank corrections were made as needed.

Other Methods

Total and inorganic phosphate was determined by the method of Ames and Dubin (40). Absorbance of tRNA and ribosome solutions was measured at 260 nm in 10 mM magnesium acetate, 3 mM Tris, pH 7.6. Under these conditions, tRNA of E. coli has an e/phosphorus) of 6800.

RESULTS

Site Specificity of Binding Assays—In order to study the effect of T-Ψ-C-Gp on the binding of aminoacyl-tRNA to ribosomes it was first necessary to devise an assay which would measure binding at either the aminoacyl or peptidyl site but not both. This was accomplished by the use of tetracycline and deacylated tRNA since it was known from the work of Seeds et al. (31) that these two compounds inhibit at different ribosomal sites.

![Fig. 2. Effect of the ratio of deacylated to acylated tRNA on peptidyl site binding. Reaction mixtures (0.15 ml) consisted of assay buffer, pH 7.8, 0.62 optical density unit of ribosomes, 0.4 mm tetracycline HCl, 0.68 mm poly U nucleotide, 14C phenylalanyl-tRNA (181 μmoles of phenylalanine, 142,000 cpm per μmole of RNA nucleotide) without other 14C-amino acids, and uncharged, treated tRNA where indicated. Incubation was at 30° for 30 min. Reactions were stopped and analyzed as described in Fig. 1. In all cases the amounts of phenylalanyl-tRNA added were saturating since addition of 4.8, 7.2, 14.4, or 21.6 μmoles of phenylalanyl-tRNA yielded 1380, 1340, 1420, and 1380 cpm bound, respectively, in the absence of uncharged tRNA. Phenylalanyl-tRNA (micromoles) added: 7.2, ○; 14.4, Δ; 21.6, ●. (B_o/B) - 1 and deacylated/acylated ratios were calculated as described under "Experimental Procedure." For comparison the percentage inhibition observed is plotted on the right-hand ordinate.](http://www.jbc.org/Downloadedfrom)
The results shown in Fig. 1 illustrate the effects observed. In the absence of tRNA, tetracycline blocked approximately 50% of the binding, and the remainder could be blocked by the addition of excess deacylated tRNA. However, the same amount of tRNA in the absence of tetracycline only inhibited about 50%. Clearly the effect of the two compounds was additive, suggesting that they act at different sites. Since the decrease was one-half in each case, it is reasonable to assume that the two sites correspond to the ribosomal aminoacyl and peptidyl sites. The same conclusions were reached by Seeds et al. (31) with the use of ribosomes prepared in a different way.

Designation of the tetracycline-sensitive site as the aminoacyl site was initially based on the finding that peptidyl-tRNA could be bound to ribosomes in the presence of tetracycline (42), and further substantiated by the work of Sarkar and Thach (43) who showed that phenylalanyl-tRNA bound in the presence of tetracycline could still react with puromycin, a property normally associated only with complexes bound at the peptidyl site. By difference it is assumed that the tRNA-sensitive site is the peptidyl site.

It should be pointed out that only a small amount of peptide bond formation takes place during these binding assays. When the amount of diphenylalanine produced was analyzed by the method of Pestka (44) and related to the total amount of ribosome-bound material, it was found that 23% was diphenylalanine when no inhibitor or excess tRNA was added, and only 6% was diphenylalanine when tetracycline was present. In addition, a low level, 4 to 7% of (phenylalanine)₂₃ was produced with or without inhibitors. Furthermore these values are maximal ones since they include any unbound oligophenylalanine formed which would not be measured by the Millipore binding assay.

The nature of the tRNA inhibition at the peptidyl site was analyzed, as described under "Methods," in terms of an equilibrium competition between deacylated tRNA and aminoacyl-tRNA. This method provides a way of linearizing the data and allows a direct quantitative comparison of various inhibitors relative to aminoacyl-tRNA. Fig. 1B shows that the results obtained in Fig. 1A do fall on a straight line when analyzed in this way, and Fig. 2 shows that a true competition exists between tRNA and aminoacyl-tRNA. Thus, as long as ribosome sites are limiting, variation in the amounts of the tRNA species added has an inhibitory effect only in terms of a changed ratio of the two species.

In this analysis, the slope of the line corresponds to the ratio of association constants for deacylated tRNA and aminoacyl-tRNA, respectively. The values obtained, 1.25 and 1.20, indicate essentially equivalent binding constants for both tRNA species at this site.

Since this assay does not depend on labeling the competitor...
tRNA, it can be used for assessing the ribosome-binding ability of modified tRNAs which cannot, by reason of the modification, be charged with amino acid or otherwise specifically labeled. For example, it has been used to show that tRNA-Phe is no longer able to bind to the peptidyl site after cyanoethylation of the pseudouridine residues with acrylonitrile. In addition, this assay is potentially useful for testing fragments of tRNA for their binding ability since not only is it unnecessary to label the material, but very low association constants can be detected simply by using high concentrations of inhibitor.

**Inhibition by T-Ψ-C-Gp and Other Oligonucleotides at Peptidyl Site**—The inhibition of binding of phenylalanyl-tRNA by T-Ψ-C-Gp and other oligonucleotides is illustrated in Fig. 3. A number of experiments using three different T-Ψ-C-Gp preparations are summarized in this figure. It is clear that T-Ψ-C-Gp can inhibit two-thirds or more of the binding capacity while another tetranucleotide, A-A-A-Gp, is far less active. The complete inactivity of pGp provides evidence that the effect is not one of simple Mg++ binding nor one caused by guanine nucleotides in general. In a separate experiment, the effect on the inhibition by T-Ψ-C-Gp of raising the Mg++ concentration from 20 to 40 mM was examined directly. Although the amount of binding in the absence of tetranucleotide increased from 1540 to 1930 cpm the percentage inhibition by 88 μmole of T-Ψ-C-Gp was unchanged, being 49% at 20 mM magnesium and 50% at 40 mM magnesium. Cyanoethylation of T-Ψ-C-Gp reduced the inhibitory activity by 50% but did not inactivate the oligonucleotide. This reduction was significant, however, since substitution of acrylonitrile by propionitrile in the incubation yielded a completely active tetranucleotide. Propionitrile is physically very similar to acrylonitrile but lacks the polarized double bond which makes nercylnitrile reactive (39). On the other hand, removal of the 3’-terminal phosphate from T-Ψ-C-Gp had no effect, neither enhancing nor diminishing its inhibitory power (results not shown).

In Fig. 3B, the above results were replotted as already described for the analysis of tRNA inhibition of peptidyl site binding. Although the data do appear to be linearized by this method, it is not at all clear that the theoretical aspect of the analysis is valid for the inhibition of aminoacyl-tRNA binding by these oligonucleotides. For example, the inhibition observed does not appear to be reversed by aminoacyl-tRNA (see Figs. 4 and 9). Nevertheless this type of plot does provide a convenient way of linearizing the data and thus of quantitating the inhibitory effect of the different oligonucleotides. It is used for this purpose here without any further assumptions regarding its theoretical significance.

An illustration of this failure of the inhibition to be affected by phenylalanyl-tRNA concentration is shown in Fig. 4. In this experiment, inhibition of binding by T-Ψ-C-Gp was examined at two phenylalanyl-tRNA concentrations both of which were in excess for the amount of ribosomes used. It is clear that there is no direct relation between the phenylalanyl-tRNA concentration and the T-Ψ-C-Gp concentration needed to reach a given level of inhibition. This figure also compared T-Ψ-C-Gp inhibition with another oligonucleotide, (A, U, C)Gp, which differs only in the replacement of a uridine by adenosine if one considers that ribothymidine and pseudouridine are formally equivalent to uridine. While neither was affected by variation in phenylalanyl-tRNA concentration, T-Ψ-C-Gp was 2.8 times more

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*J. Ofengand, unpublished results.

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**Effective Inhibition by T-Ψ-C-Gp**—In a similar way, the effect of T-Ψ-C-Gp on the phenylalanyl-tRNA binding at the peptidyl site was examined. The percentage inhibition is presented in Fig. 5A and the linearized data in Fig. 5B. There are several points to note. First, T-Ψ-C-Gp was the most potent inhibitor at this site, the tetranucleotide (A, U, C)Gp being only one-third as active. Second, tri- and hexanucleotides or pGp were virtually ineffective. Third, alkaline hydrolysis completely inactivated T-Ψ-C-Gp although the incubated control was fully active. Fourth, T-Ψ-C-Gp was 2.6 to 3.4 times more active at this site than at the peptidyl site (for example, compare Figs. 3 and 4 with Figs. 5 and 6).

Since the activity of (A, U, C)Gp was greater than originally anticipated, the effect of alkaline hydrolysis on this oligomer was tested in order to see if its inhibitory activity was also alkali-sensitive. Fig. 6 shows that, like T-Ψ-C-Gp, alkali abolished the activity although the incubated control was fully active. It appears that this oligomer has some intrinsic inhibitory power.
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FIG. 5. Oligonucleotide inhibition of ribosomal aminoacyl site binding. A, oligonucleotides were prepared and characterized as described under "Experimental Procedure" and Tables I and II. O, TΨCG-3; ●, TΨCG-3 heated for 20 min at 100°, pH 7; Δ, TΨCG-3 (OH); △, (A, U, C)G; ▽, AAG/(A, C)G; □, pGp. Alkaline hydrolysis of oligonucleotides (TΨCG(OH)) was done at 100° for 20 min in 0.13 M NaOH (45), 1 mM oligomer. Samples were tested after neutralization with HCl. Control oligonucleotides were similarly treated with 0.13 M NaCl, pH 7, in place of the alkali. Reaction mixtures, incubation conditions, and analyses were as described in Fig. 1A except that the 3C-phenylalanyl-tRNA preparation (198 pmoles of phenylalanine, 155,000 cpm per pmoles of RNA nucleotide) was not charged with 12C-amino acids, and 2.8 mM uncharged, treated tRNA (a 4-fold excess) was present in all tubes. 100% bound corresponds to 1.8 pmoles of phenylalanyl-tRNA. Binding at aminoacyl site was measured by the method described in Fig. 1A. Two sets of reactions were run, one with and one without tetracycline. The first set, without tetracycline, measured aminoacyl site binding plus residual peptidyl site binding not completely suppressed by the added tRNA. The second set with tetracycline measured only the residual peptidyl site binding. Subtraction of the second set of numbers from the first gave the desired values shown in the figure. This procedure was followed for each experimental point. B, analysis of aminoacyl site binding results. The data of A were replotted as described in Fig. 3B. TΨCG, TΨ-C-Gp; AUCG, (A, U, C)Gp; AAG, A-A-Gp; ACG, (A, C)Gp.

The ability of cyanoethylated TΨ-C-Gp to inhibit binding at this site was also examined (Fig. 7). As observed for inhibition at the peptidyl site, cyanoethylation yielded a product that was only 50% as active as the propionitrile-treated or untreated TΨ-C-Gp controls.

Competition between TΨ-C-Gp and phenylalanyl-tRNA for Ribosome Sites—The experimental agreement observed between the TΨ-C-Gp inhibition curves and the mathematical model proposed above for competition between tRNA (or fragments) and aminoacyl-tRNA for limiting ribosome binding sites suggested that TΨ-C-Gp was also competing with phenylalanyl-tRNA for ribosome binding sites, albeit with a very low relative association constant. This mechanism appeared particularly likely since the deacylated tRNA effect was competitive (Fig. 2). Nevertheless, a direct test for competition between added phenylalanyl-tRNA and TΨ-C-Gp was negative (Fig. 8). In this experiment, done at the aminoacyl site, the standard (excess) amount of phenylalanyl-tRNA and 3 times that amount were used in two sets of tubes to which were added variable amounts of TΨ-C-Gp. If TΨ-C-Gp was competing with phenylalanyl-tRNA according to the relation derived under "Methods," the slope of the inhibition line should have been decreased to one-third when the phenylalanyl-tRNA concentration was tripled. Clearly this did not occur.

It should be noted that the deacylated tRNA concentration (used to inhibit the peptidyl site) was not increased proportionately when the phenylalanyl-tRNA concentration was tripled because of the possibility of depleting the free magnesium concentration in the assay mixture. Consequently, more total binding (aminoacyl plus peptidyl site) was observed. However, as expected, more tetracycline-resistant binding was also observed, so that when the aminoacyl site-specific binding was calculated, no net decrease in the inhibition was found.

A similar failure of TΨ-C-Gp to compete with phenylalanyl-tRNA for binding at the peptidyl site was already noted (Fig. 4). A more complete experiment is illustrated in Fig. 9 and shows that inhibition by TΨ-C-Gp at this site could not be reversed by addition of more phenylalanyl-tRNA. Two concentrations of TΨ-C-Gp were tested, and failure to be reversed was clearly evident in both cases. The theoretical line was calculated from the relation derived under "Experimental Procedure" with the use of the percentage bound at the lowest concentration of phenylalanyl-tRNA to calculate a value of $K_d/K_a$. The remote possibility that TΨ-C-Gp was competing with poly U was also tested in this experiment. In the presence of 0.118 μmole of TΨ-C-Gp, 0.34 mM, 0.68 mM (the standard concentration), and 1.36 mM poly U nucleotide gave 46.5%, 45.8%, and 43.8% respectively, of the maximal binding without TΨ-C-Gp, indicating the absence of any poly U effect.
Effect of Ribosome Concentration and Ribosome Treatment on Inhibition by TψCG—In order to test the possibility that the true competitor with TψCG was a factor-phenylalanyl-tRNA complex and that the factor was being introduced with the ribosome preparation, the inhibitory effect of TψCG was measured at two different concentrations of ribosomes, both of which were limiting in the assay system (see inset, Fig. 10). Use of a 2-fold different concentration of ribosomes should have similarly altered the “factor” concentration, and thus an effect on the TψCG inhibition curve was expected. However, the results of this experiment (Fig. 10) show that when the percentage inhibition by TψCG at each ribosome level was determined and plotted in the appropriate way, no effect of ribosome concentration could be seen.

On the other hand, some component of the ribosome does appear to play an important role in the inhibitory phenomena described here. When ammonium chloride-washed ribosomes were employed in order to eliminate possible effects of the known ribosome-associated proteins, an increased sensitivity to TψCG was observed. Fig. 11 shows that NH₄Cl-washed ribosomes were almost 4 times more sensitive to TψCG than the standard preparation. This effect was in the direction expected if some necessary and limiting factor were removed or inactivated by the NH₄Cl wash. Moreover, the effect was not related to the Mg²⁺ concentration since changing the concentration from 20 to 30 mM had no effect on either the uninhibited binding of phenylalanyl-tRNA or on the degree of inhibition by TψCG (Table III). Nevertheless, a repeat of the experiment described in Fig. 10 with these NH₄Cl-washed ribosomes gave the same result, namely that the ribosome concentration had no effect on the degree of inhibition by TψCG (data not shown).

It appears that the effect of NH₄Cl washing on the sensitivity of ribosomes to TψCG is more complex. Storage at 4°C increased the sensitivity of the particles to TψCG inhibition although the uninhibited binding capacity remained essentially unchanged (Table IV). Several points emerge from the experiments summarized here. First, the effect of NH₄Cl was verified with two different ribosome preparations and shown to be a direct NH₄Cl effect (Experiments 2, 3, and 6). Second, the sensitivity to TψCG increased with storage at 4°C (compare Fig. 7. Inhibition of aminocayl site binding by TψCG and TψCeCG. Assay of the inhibition of binding and analysis of the data was done as described in Fig. 6 except that the ¹⁴C-phenylalanyl-tRNA preparation (192 μmole of phenylalanine, 150,000 cpm per μmole of RNA nucleotide) was charged with all the other ¹³C-amino acids, 2.9 mM uncharged, treated tRNA (a 2.9-fold excess) was present in all tubes, and reaction was initiated by addition of ribosomes to the chilled reaction mixtures; 100% bound equals 1.6 μmole of phenylalanyl-tRNA. O, TψCG; •, TψCG treated with propionitrile; X, TψCG treated with acrylonitrile. Reaction with either reagent was carried out in a solution of 6 M acrylonitrile (or propionitrile), 0.08 M sodium carbonate buffer, pH 9.44, at 30°C for 5½ hours in tightly closed vessels. After neutralization, the product was desalted on Bio-Gel P2 and lyophilized. Qualitative analysis of the nucleotide composition of the acrylonitrile-treated tetranucleotide by the procedures given under “Methods” showed that the pseudouridine spot had disappeared and that the only new spot to appear corresponded in Rₚ to a marker of N₁-cyanoethyl pseudouridine. A quantitative compositional analysis was not performed. TψCG, Tψ-C-Gp; TψCeCG, T-(N₁-cyanoethyl)-ψ-C-Gp.
FIG. 8. Failure of phenylalanyl-tRNA to reverse TyCG inhibition at aminoacyl site. Assay of the inhibition of binding and analysis of the results was done as described in Fig. 6 except that duplicate tubes were prepared at both the standard phenylalanyl-tRNA concentrations of 0.15 μM (○) and at 0.45 μM (●) phenylalanyl-tRNA; 100% bound corresponds to 1.3 and 1.8 μmoles of phenylalanyl-tRNA, respectively. The dashed line is the theoretical curve expected for 0.45 μM phenylalanyl-tRNA on the basis of competition between unbound TyCG and unbound phenylalanyl-tRNA for limiting aminoacyl binding sites on the ribosome using the equation derived under "Experimental Procedure." pheRNA, phenylalanyl-tRNA; TyCG, T-ψ-C-Gp.

FIG. 9. Nonreversal of peptidyl site inhibition by phenylalanyl-tRNA. Reaction mixtures, incubation conditions, and analyses were done as described in Fig. 2 except that uncharged tRNA was absent in this experiment. Variable amounts of phenylalanyl-tRNA (181 μmoles of phenylalanine, 142,000 cpm per μmole of RNA nucleotide) were added as indicated in the presence of 0 (▲), 0.113 (○), and 0.226 (●) μmoles of TyCG-2; 100% bound equals 1.9 μmoles of phenylalanyl-tRNA. The dashed line is the theoretical curve expected on the basis of competition between unbound TyCG and unbound phenylalanyl-tRNA for limiting peptidyl binding sites on the ribosome using the equation derived under "Experimental Procedure." pheRNA, phenylalanyl-tRNA; TyCG, T-ψ-C-Gp.

FIG. 10. Effect of varying ribosome concentration on TyCG inhibition. Assays were performed as described in Fig. 2 except that 21.3 μmoles of phenylalanyl-tRNA (167 μmoles of phenylalanine, 129,000 cpm per μmole of RNA nucleotide) was added and the amount of ribosomes was varied as indicated. 0.34 optical density units of ribosomes, ○; 0.68 optical density unit of ribosomes, ●. TyCG, T-ψ-C-Gp; ODU, optical density unit.

FIG. 11. Effect of NH₄Cl wash on ribosome response to inhibition by TyCG. Assays were performed as described in Fig. 2 except that 21.6 μmoles of phenylalanyl-tRNA (210 μmoles of phenylalanine, 149,000 cpm per μmole of RNA nucleotide) was added, incubation was at 30° for 25 min, and 0.68 optical density unit of standard ribosomes (○) or 0.70 optical density unit of NH₄Cl washed ribosomes (●) were used. Ammonium chloride washing was done by suspending 260 optical density units of standard ribosomes in 12 ml of 1 M NH₄Cl, 0.01 M Tris, pH 7.4, 0.01 M magnesium acetate, and 10⁻⁴ M dithiothreitol and centrifuging out the ribosomes as described by Erbe, Nau, and Leder (46). After three washes the ribosomes were suspended in the same buffer without NH₄Cl; 100% binding corresponded to 3.07 μmoles of phenylalanyl-tRNA bound per optical density unit for the standard ribosomes and 2.81 μmoles bound for the NH₄Cl-washed ribosomes. TyCG, T-ψ-C-Gp.
lished conditions which would measure binding at the aminoacyl-aminoacyl-tRNA to ribosomes, it was first necessary to establish the aminoacyl-tRNA binding ability, but that the exact nature of this interaction is unclear.

In order to study the effects of T-$C-Gp$ on the binding of aminoacyl-tRNA to ribosomes, it was first necessary to establish conditions which would measure binding at the aminoacyl and peptidyl sites independently. This was made possible by the analytical procedure developed here, which should make it possible to quantitatively examine the effects of various modifications of tRNA on its ability to bind to ribosomes. Although the fit of the data of Figs. 1 and 2 to the theoretical model was not attempted by any of these workers, a direct comparison with our findings is not possible. For example, Levin and Nirenberg (38) and Kuriki, Fukuma, and Kaji (2) both observed that at 20 mM Mg$^{2+}$ deacylated tRNA was less inhibitory than expected for equivalent levels of the ribosome preparations. For example, the results we obtained were quite similar to those reported by Seeds et al. (31) while the earlier report of Kurland (30) is more in agreement with Ravel's findings. Other workers have also described the effect of deacylated tRNA on the binding of aminoacyl-tRNA to ribosomes (2, 38, 49, 50). In those cases where comparable systems were examined, the results obtained did not disagree with our findings. For example, Levin and Nirenberg (38) and Kuriki, Fukuma, and Kaji (2) both observed that at 20 mM Mg$^{2+}$ deacylated tRNA was less inhibitory than expected for equivalent competition with aminoacyl-tRNA. In the latter case, even a 4-fold excess of tRNA blocked only 62% of the binding when 70 S ribosomes were used, although the theoretical inhibition was observed when binding to 30 S particles, thought to have only one available binding site (61), was measured. An discrimination between aminoacyl and peptidyl binding sites, for example by the use of tetracycline, was not attempted by any of these workers, a direct comparison with our findings is not possible.

Using this ribosomal system, the inhibitory effect of T-$C-Gp$ and several other oligonucleotides were compared at both the

### Table III

**Effect of Mg$^{2+}$ concentration on ammonium chloride washed ribosomes**

Assays were performed as described in Fig. 11 except that the magnesium concentration and amount of ribosomes was varied as described in the table, and a different preparation of NH$_4$Cl-washed ribosomes was used.

<table>
<thead>
<tr>
<th>T-$C-Gp$ concentration</th>
<th>0.5 optical density units ribosome</th>
<th>1.0 optical density units ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmolar</td>
<td>µmolar</td>
<td>µmolar loads of tRNA bound</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.38</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>1.45</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>1.39</td>
</tr>
<tr>
<td>44</td>
<td>23</td>
<td>0.88</td>
</tr>
<tr>
<td>44</td>
<td>30</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Experiments 2 through 6). Third, the NH$_4$Cl effect was observed both for T-$C-Gp$ and the control (A, U, C)Gp oligomers (Experiments 2 and 3). Fourth, the ratio of inhibitory activity of T-$C-Gp$ to (A, U, C)Gp varied with the ribosome preparation. For the standard M preparation it was only 1.5, rising to 1.8 to 1.9 for the control and NH$_4$Cl treated M ribosomes, while for the standard L ribosomes, the ratio was 3.0 in Experiment 5, a value virtually identical with that previously found for this ribosome preparation (Fig. 4). Lack of material prevented a further analysis of this effect in Experiment 6.

We conclude from these experiments that T-$C-Gp$ is interacting with the ribosome in such a way as to cause it to lose its aminoacyl-tRNA binding ability, but that the exact nature of this interaction is unclear.

### DISCUSSION

In order to study the effects of T-$C-Gp$ on the binding of aminoacyl-tRNA to ribosomes, it was first necessary to establish conditions which would measure binding at the aminoacyl and peptidyl sites independently. This was made possible by the report of Seeds et al. (31) that tetracycline and NH$_4$Cl treated M ribosomes, while for the standard L ribosomes, the ratio was 3.0 in Experiment 5, a value virtually identical with that previously found for this ribosome preparation (Fig. 4). Lack of material prevented a further analysis of this effect in Experiment 6.

We conclude from these experiments that T-$C-Gp$ is interacting with the ribosome in such a way as to cause it to lose its aminoacyl-tRNA binding ability, but that the exact nature of this interaction is unclear.

### Table IV

**Effect of T-$C-Gp$ and A-U-C-Gp on standard and ammonium chloride washed ribosomes**

Assays were performed as described in Fig. 11 using approximately 0.7 optical density unit of the various ribosome preparations. Preparations L and M were made in different batches made by the standard procedure and stored as described under "Experimental Procedure." Ammonium chloride washing was performed as described in the legend to Fig. 11. Control ribosomes were similarly washed but with 50 mM NH$_4$Cl and 5 mM glutathione in place of the dithiothreitol. Both control and NH$_4$Cl washed ribosomes were stored in this latter buffer at 4°C. Inhibitory slopes means the slope of the line obtained from the type of plot illustrated in Figs. 10, 11, and previous figures.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ribosome preparation</th>
<th>Treatment</th>
<th>Pre-tRNA bound per optical density unit of ribosomes</th>
<th>Inhibition slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L NH$_4$Cl</td>
<td>2.8</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M Standard</td>
<td>3.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M NH$_4$Cl</td>
<td>2.2</td>
<td>7.0</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>M Control</td>
<td>2.7</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>M NH$_4$Cl</td>
<td>2.8</td>
<td>7.3</td>
<td>4.1</td>
</tr>
<tr>
<td>6</td>
<td>M Control</td>
<td>2.8</td>
<td>2.5</td>
<td>1.3</td>
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<td></td>
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<td>M Control</td>
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<td>0.4</td>
</tr>
<tr>
<td></td>
<td>M Standard</td>
<td>2.7</td>
<td>2.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>
aminoacyl and peptidyl sites. As the essential results were the same at both sites, they are discussed together. The most important conclusion to be drawn from this work is that phenylalanyl-tRNA binding can be inhibited by oligomers as small as tetranucleotides and that T-Ψ-C-Gp is considerably more inhibitory than the other oligonucleotides tested. The trimucleotide mixture was virtually unreactive, being only 6% as inhibitory as an equivalent amount of T-Ψ-C-Gp while the two control tetranucleotides, A-A-A-Gp and (A, U, C)Gp were 10% and 33%, respectively, as active as T-Ψ-C-Gp. No doubt the length, base composition, and sequence of the oligomers all contribute to their inhibitory ability. Except as noted above, pentanucleotides or higher oligomers were not tested, nor were compounds with 3' termini other than guanylate. Removal of the 3'-phosphate from T-Ψ-C-Gp did not have any effect. It should be emphasized that the inhibitory effects observed here were not caused by depletion of magnesium ion or to competition with poly U, and were not the result of any heat-labile contaminant in our preparations. On the other hand, as expected for RNA oligonucleotides, alkaline hydrolysis destroyed the inhibitory activity.

It was interesting to note that cyanooethylolation of the pseudouridine residue in T-Ψ-C-Gp decreased its inhibitory power (presumably its affinity) for the ribosome system to one-half but did not abolish it. On the other hand, previous work in our laboratory has shown that cyanooethylolation of tRNA<sub>dry</sub> under conditions believed to modify the pseudouridine residue in the T-Ψ-C-Gp sequence but not the 4-thiouridine or other nucleotide residues inactivated almost all of its ability to bind to ribosomes as well as its ability to accept phenylalanine. One explanation of this apparent paradox would be that the T-Ψ-C-Gp sequence serves not only as a focal point for the common ribosome binding site but also acts to maintain the over all conformational integrity of the molecule.

The only evidence that T-Ψ-C-Gp inhibits by complexing to tRNA binding sites in the ribosome is the fact that this particular sequence seems to be necessary for optimal effect. Other similar oligonucleotides were less effective, and simply adding a cyanooethyl group to the N1 of the pseudouridine moiety was sufficient to reduce activity by half. On the other hand, direct competition with free phenylalanyl-tRNA does not occur, nor does there appear to be any competitive interaction with factors that might have been introduced with the ribosomes. However, the state of the ribosome does have a marked effect on the inhibition, as witnessed by the 4-fold increased sensitivity on washing with ammonium chloride and the further sensitization upon storage.

We suggest the following model as one way to rationalize our results. In native aminoacyl-tRNA, as in decylated tRNA, the T-Ψ-C-Gp region is shielded by interaction with other parts of the molecule. Only on complexing to the ribosome does aminoacyl-tRNA unfold, exposing the T-Ψ-C-Gp region which then can bind to another part of the ribosomal surface. This additional binding serves to stabilize the complex which otherwise is very unstable. In the presence of the T-Ψ-C-Gp tetranucleotide, conversion to the stable complex is inhibited because the additional binding site is blocked. Since in this model T-Ψ-C-Gp is competing not with free phenylalanyl-tRNA but with phenylalanyl-tRNA bound in an unstable complex with ribosomes, the free phenylalanyl-tRNA concentration is not expected to influence the inhibition when ribosomes limit the system.

To our knowledge, this report represents the first attempt to elucidate the ribosome binding site of tRNA by the use of specific tRNA fragments. Only two other studies on the binding of tRNA fragments to ribosomes have so far appeared. In the first (252), the anticodon arm of tRNA<sub>E.coli</sub>, a 19 nucleotide oligomer including both the loop and complete double-stranded arm, was shown to bind readily to ribosomes. However, binding could not be detected when a single-stranded 11 nucleotide oligomer was used even though it was derived from the 19 nucleotide unit and contained the entire anticodon loop. In the second report (53), binding of half-molecules of tRNA<sub>Val</sub> to ribosomes and ribosomal subunits was studied. It is interesting to note that some binding of the 3' half of the molecule, which contains an intact T-Ψ-C-Gp loop, was detected although the efficiency was much less than for the intact molecule. Clearly the binding of an oligomer as small as T-Ψ-C-Gp would be an even more difficult task, and no doubt explains in part why such a high molar concentration was needed to obtain the effects reported here.

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James Ofengand and Carole Henes


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