Reaction of Ribosome-bound Peptidyl Transfer Ribonucleic Acid with Aminoacyl Transfer Ribonucleic Acid or Puromycin*

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SUMMARY

Polylysyl transfer ribonucleic acid, free of lysyl-tRNA, has been isolated and its characteristics in an Escherichia coli polypeptide-synthesizing system in vitro have been studied. Ribosomes obtained from frozen E. coli cells were washed in 0.5 M NH₄Cl and assayed at MgCl₂ concentrations of at least 0.01 M.

Binding of this peptidyl-tRNA to ribosomes occurs in the presence of templates containing 3 or more adenylic acid residues. p-Chloromercuriphenylsulfonate or lysyl-tRNA prevents this attachment. Binding occurs most effectively when both 30 S and 50 S ribosomal subparticles are present. Unlike lysyl-tRNA, bound polylysyl-tRNA reacts with puromycin in the absence of added GTP and supernatant factors. The product of this reaction was shown to be polylysyl-puromycin.

Under analogous conditions, a limited incorporation of lysine into bound polylysyl-tRNA can be shown. In this limited addition reaction, a polyadenylate template longer than three nucleotides is required.

Chlortetracycline is a strong inhibitor of the limited addition of lysine to polylysyl-tRNA, but is without effect on the cleavage of polylysyl-tRNA by puromycin. Chloramphenicol, on the other hand, inhibits the reaction between polylysyl-tRNA and puromycin, but has little influence on the limited addition reaction. Gougerotin, a puromycin-like antibiotic, inhibits the puromycin reaction.

Phenylalanyl-tRNA reacts poorly with puromycin in the presence of ribosomes and oligouridylic acid, whereas N-acetylnaphthylalanyl-tRNA is cleaved extensively, forming N-acetylnaphthylalanylpurumycin. Phenylactyl-tRNA, although capable of binding to ribosomes, displays no detectable reactivity with puromycin. Chloramphenicol completely inhibits the cleavage of N-acetylnaphthylalanyl-tRNA by puromycin.

Recent studies on protein synthesis with systems in vitro have centered on the steps of initiation, elongation, and termination. Peptide chain initiation requires N-formylmethionyl-tRNA (1-3) and two ribosomal components (4, 5). Elongation has been studied with synthetic polynucleotide messengers at high magnesium ion concentrations. Under these conditions, the normal initiation requirements are circumvented. A requirement for GTP and several separable protein components, in addition to ribosomes, has been established for the elongation process (6).

The antibiotic puromycin functions as a codon independent analogue of aminoacyl-tRNA (7), and cleaves the growing peptide chain from the peptidyl-tRNA-messenger-ribosome complex with the formation of peptidyl-puromycin (8, 9). In order to simplify the requirements of the peptide bond-forming reaction, polylysyl-tRNA was isolated and bound to washed ribosomes in the presence of poly A. The requirements for further reaction, either with lysyl-tRNA or with puromycin, were then studied.

A similar system has already been reported by Rychlik (10).

A partial requirement for GTP and the supernatant fraction has been reported (11) for the reaction of puromycin with a poly U-polyphenylalanyl-tRNA-ribosome complex. The results obtained in the present experiments, however, indicate that at high Mg²⁺ concentrations only ribosomes and an appropriate codon are required for a single-step reaction between aminoacyl-tRNA or polylysyl-tRNA and puromycin. Peptide bond formation would be a uniquely ribosomal function, with GTP and supernatant fractions serving in frame-shifting and in reassortment of ribosomal binding sites (12). After completion of this work, it was reported that formylmethionyl-tRNA reacts with puromycin upon the addition of only ribosomes and template, although the presence of contaminating GTP was not excluded (13). Since formylmethionyl-tRNA is an analogue of peptidyl-tRNA, this observation supports the generality of the conclusions presented in this paper.

EXPERIMENTAL PROCEDURE

Materials

Polylysyl-tRNA—Polylysyl-tRNA was prepared by incubating radioactive lysine and poly A with previously incubated E. coli

1 The abbreviations used are: tRNA, transfer ribonucleic acid; TCA, trichloroacetic acid; CMS, p-chloromercuriphenylsulfonate.

* This investigation was supported in part by a grant from the National Science Foundation, and by a fellowship to the author from the Jane Coffin Childs Memorial Fund for Medical Research.

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The incubation mixture contained in a final volume of 1.0 ml: 0.04 M Tris-HCl, pH 7.8; 0.015 M MgAc2; 0.03 M KCl; 8.0 mM 2-mercaptoethanol; 6.0 mM phosphoenolpyruvate, potassium salt; 0.25 mM GTP; 1.5 mM ATP; 50 μg per ml of pyruvate kinase; 200 μg per ml of poly A; 5 mg per ml of stripped extract of 1.0 ml of water, and dialyzed overnight against 0.2 M KCl; 8.0 mM 2-mercaptoethanol; 6.0 mM Tris-HCl, pH 7.4-0.01 M MgCl2; 0.03 M MgAc2; 0.01 M Tris-HCl, pH 7.4-0.05 M NH4Cl, and five times with 0.01 M-polylysine, and an equivalent of 2.5 g of glass beads per g (wet weight) of cells. DNase I was then added, and the mixture was ground in the Omni-Mixer at half the maximum speed for 3 min in an ice water bath. Glass beads, cells, and cellular debris were removed by two centrifugations at 30,000 × g for 20 min each, and the ribosomes were precipitated by centrifugation for 4 hours at 100,000 × g. The ribosomes were washed once with 0.01 M MgCl2-0.01 M Tris-HCl, pH 7.4-0.50 M NH4Cl, and five times with 0.01 M MgCl2-0.01 M Tris-HCl, pH 7.4-0.50 M NH4Cl; these ribosomes were then stored at 0° in the final washing buffer at a concentration of 12 to 26 mg per ml.

The ability of the ribosomes to catalyze the puromycin reaction can be destroyed by prolonged storage at -15°, or by maintaining them at 0° at high dilution. Restoration of activity by the addition of supernatant factors and GTP was not successful. Washed ribosomes chromatographed on DEAE-cellulose according to the method of Salas et al. (20) retained the ability to carry out the puromycin reaction. In addition, washed ribosomes may be heated at 60° for 5 min without loss of activity, a result in agreement with that reported by Allen and Zamecnik (21) with reticulocyte ribosomes.

The 30 S and 50 S ribosomal subparticles were derived from a crude ribosomal pellet as follows. A suspension of unwashed E. coli B ribosomes (55 mg per ml) was dialyzed overnight against 0.01 M Tris-HCl, pH 7.4-10 mM MgCl2-5 × 10-4 M dithiothreitol, and the subparticles were separated on a 5 to 32% sucrose gradient containing the same reagents. The separated subparticles were stored at 0° and used within 3 days of their preparation.

**Templates**—Poly A and poly U were obtained from Miles Laboratories. The poly A was dialyzed against dilute EDTA and then against H2O. It was stored at -20° in dilute Tris-HCl, pH 7.4, at an O.D.260 of 45. Oligouridylicate was a gift from Dr. J. M. Clark, Jr. Oligoadenylate was prepared by the method of Wahba.2

**Antibiotics**—Chlorotetracycline and puromycin were obtained from Lederle, and chloramphenicol from Parke, Davis and Company. Gougerotin was a gift from Dr. J. M. Clark, Jr. Puromycin-5'-β-cyanoethyl phosphate was prepared as described by Smith et al. (9).

**Methods**

The standard incubation mixture (Mixture A) for the puromycin assay contained: 50 mM Tris-HCl buffer, pH 7.4, 12 mM 2-mercaptoethanol, and 100 mM NH4Cl. To this standard mixture were added 10 mM or 20 mM MgCl2 acylated tRNA, washed ribosomes, and template. In the polylysine system, 0.4 mM 14C-lysine was added, and incubation was usually carried out at 37° for the indicated time intervals. In the phenylalanine system, incubation was at 24° for 60 min. Samples of 0.05 to 0.10 ml were incubated and then treated as follows.

**Precipitation by TCA**—To the sample, 5 ml of ice-cold 5% TCA were added. After 10 min at 0°, the precipitate was gathered by filtration on a nitrocellulose membrane filter and washed three times with 5 ml of ice-cold TCA. The filter was then dried, 2.0 ml of tolune-0.4% 2,5-diphenyloxazole-0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid were added, and the radioactivity was determined in a Nuclear-Chicago scintillation counter. This assay (10) determines the extent to which the tRNA substrates have become deacylated during incubation; polylysine and polylysylpuromycin are soluble in 5% TCA.

**Precipitation of Polylysine by TCA-Tungstate**—To the sample (0.10 ml) was added 0.05 ml of 2.0 M NaOH. Following incubation for 10 min at 37°, the sample was cooled to 0° and 0.05 ml of 2.0 N acetic acid, 0.02 ml of 0.2% 14C-polylysine, and 3.0 ml of TCA-tungstate reagent were added. After 10 min at 0°, the precipitate was filtered on a nitrocellulose filter and washed three times with 5 ml of TCA-tungstate reagent, and the radioactivity was determined as above. This assay was used 2. A. J. Wahba, personal communication.
to detect the addition of radioactive lysine to unlabeled polylysyl-tRNA.

**Precipitation after Heating in TCA-Tungstate**—After incubation, polylysine carrier and 5 ml of TCA-tungstate reagent were added, and the mixture was heated for 10 min at 0°C. The sample was then maintained at 0°C for 10 min, filtered, and washed with TCA-tungstate reagent. Under these conditions, longer polylysine chains are precipitated more efficiently than shorter chains (23), and therefore the assay allows one to detect the addition of unlabeled puromycin or lysine to radioactive polylysine.

**Binding**—The assay was essentially that of Nirenberg and Leder (24). The sample was diluted with 5.0 ml of ice-cold mixture A, filtered on a nitrocellulose membrane filter, and washed rapidly with the same mixture. For binding to ribosomal subparticles, the procedure of Kaji, Suzuki, and Kaji (25) was modified as follows: to an incubation mixture (Mixture B) containing 0.0625 M Tris-HCl, pH 7.4, 0.025 M KCl, and 0.01 M MgCl₂, were added 300 μg per ml of poly A, 37,000 cpm per ml of 3H-polylysyl-tRNA (specific activity, 2.52 × 10⁶ cpm per μmole), and 1.9 O.D.₂₆₀ units per ml of 30 S ribosomal subparticles, or 4.0 O.D.₂₆₀ units per ml of 50 S ribosomal subparticles or both. Portions of 0.05 ml were incubated at 37°C, chilled, filtered on nitrocellulose membrane filters, and washed with ice-cold Mixture B. The filters were dried and their radioactivity was determined as indicated above.

Chromatography on carboxymethyl cellulose was performed as described by Smith and Stahmann (22). After incubation, the sample (5.0 ml) was mixed with 0.40 ml of 2.0 N NaOH and incubated at 37°C for 20 min. The precipitate was removed by centrifugation and 10 ml of 5% TCA were added to the supernatant fraction. After 10 min at 0°C, the mixture was centrifuged, and the supernatant fluid was extracted with ether. Mono-, di-, and trilysine carriers were added, and the mixture was diluted to 130 ml and adsorbed onto a column, 25 × 1 cm. The column was washed with water, and an exponential gradient of NaCl was applied (0.50 M NaCl into a mixing flask containing 500 ml of H₂O). To 10 ml of Bray's solution (26) were added 1.0 ml samples for radioactivity determinations; ninhydrin-reacting material was determined by the method of Troll and Cannan (27).

Chromatography on phosphocellulose was performed according to the procedure of Smith et al. (9). A shallower gradient (0 to 0.2 M NaCl) was found to give better separation of the polylysine and polylysylpuromycin oligomers. A 2.5-ml sample was chromatographed on a column, 20 × 1 cm, with a 1.0-liter linear gradient. Samples were monitored for radioactivity and ninhydrin reactivity as described above.

**RESULTS**

**Binding of Polylysyl-tRNA to Ribosomes**—Polylysyl-tRNA has been isolated, re-bound to ribosomes in the presence of an appropriate template, and allowed to form additional peptide bonds under specified conditions. The specificity of the rebinding reaction will be discussed first.

Fig. 1A shows that a template with a minimum of 3 adenylate acid residues is required for the binding of polylysyl-tRNA to ribosomes (24). ApA does not stimulate binding and is, in fact, inhibitory. Fig. 1B shows that poly A also stimulates this binding. The polylysyl-tRNA-polyadenylate complex (Fig. 1B) forms at lower Mg⁺⁺ concentration (0.01 M) and is more stable at higher temperatures (37°C) than the polylysyl-tRNA-oligoadenylate complex (Fig. 1A). CMS inhibits the binding observed with poly A (Fig. 1B). In other experiments, it was found that CMS causes unbinding of polylysyl-tRNA when it was added after the binding reaction was complete. The binding of polylysyl-tRNA was also suppressed by the simultaneous addition of lysyl-tRNA (Fig. 1B), suggesting competition for ribosomal binding sites. Pre-bound polylysyl-tRNA was displaced to only a small extent by the subsequent addition of lysyl-tRNA.

The 50 S and 30 S ribosomal subunits separated by sucrose gradient centrifugation in low magnesium were tested separately and together for their ability to bind polylysyl-tRNA. Fig. 2 shows that the 50 S subunit alone has negligible ability to bind polylysyl-tRNA. The small amount of binding observed with the 30 S subunit is stimulated (3.6-fold) by addition of the 50 S subunit. Lysyl-tRNA binding under these conditions was stimulated 2-fold. Thus, the binding of polylysyl-tRNA is qualitatively comparable to the binding of aminoacyl-tRNA.

**Reaction of Ribosome-bound Polylysyl-tRNA with Puromycin**—

![Fig. 1. A, template-dependent binding of 3H-polylysyl-tRNA to ribosomes. To mixture A plus 20 mM MgCl₂ were added: 0.4 mM 3H-lysine, 440 μg per ml of washed ribosomes, and 18,200 cpm per ml of 3H-polylysyl-tRNA (specific activity, 2.2 × 10⁶ cpm per μmole). Where indicated, 37 μg per ml of diadenosine monophosphate (A₁) or 46 μg per ml of triadenosine diphosphate (A₂) were added. Samples of 0.05 ml were incubated at 37°C for varying times and ribosome-bound 3H-polylysyl-tRNA was determined as described in "Methods." B, effect of lysyl-tRNA and CMS (pCMPS) on the binding of 3H-polylysyl-tRNA to ribosomes. The system was as described in A except that 10 mM MgCl₂ and 120 μg per ml of poly A were used. Where indicated, 140 μmole per ml of 3H-lysyl-tRNA or 1.0 mM CMS were added, 2-mercaptoethanol was omitted from vessels containing CMS. The 0.05-ml samples were incubated at 37°C for the indicated times and assayed for binding. The concentration of 3H-polylysyl-tRNA was 50 μmole per ml.](http://www.jbc.org/)

![Fig. 2. The binding of 3H-polylysyl-tRNA to ribosomal subparticles. The binding of 3H-polylysyl-tRNA to ribosomal subparticles was determined as indicated in "Methods."](http://www.jbc.org/)
The requirements of the reaction of polylysyl-tRNA with puromycin are shown in Tables I and II. Table I shows that triadenosinediphosphate, hexaadenosine pentaphosphate, or poly A served as templates for the puromycin reaction. Poly U did not substitute for poly A and inhibited the poly A-stimulated reaction. This inhibition by poly U is probably due to the formation of a double-stranded helix poly (A + U) which lacks template activity (14). As seen in Table II, there was no requirement for supernatant fraction of GTP. No inhibition was caused by the addition of GDP, a competitive inhibitor of GTP in the polypeptide-polymerizing reaction, or by the presence of a large excess of GTPase. Unlike polylysyl-tRNA, the reaction of lysyl-tRNA with puromycin in this system required that previous polymerization take place by the addition of both GTP and supernatant fraction; lysyl-tRNA alone does not react with puromycin. This observation eliminates the possibility that our preparations were grossly contaminated with GTP and supernatant. In addition, poly A is required for polymerization of lysyl-tRNA to polylysyl-tRNA; triadenosine diphosphate does not function in this reaction.

Bound polylysyl-tRNA is capable of reacting with puromycin in the presence of CMs. Although CMs causes an over-all decrease in the extent of cleavage by puromycin, this effect is due to a competing reaction which releases bound peptidyl tRNA rather than to inhibition of peptide bond synthesis per se.

The effect of the antibiotics chloramphenicol, chlortetracycline, and gougerotin on the reaction of puromycin with bound polylysyl-tRNA has been investigated. Chloramphenicol was slightly inhibitory, especially at early incubation times (Table III). Chlortetracycline had no significant inhibitory activity. Gougerotin, an analogue of puromycin (28), caused no release of polylysine from the ribosomes, and was itself an inhibitor of the puromycin reaction (29). Neither chloramphenicol nor chlortetracycline affected the binding of polylysyl-tRNA to ribosomes in the presence of poly A. Gougerotin stimulated the initial binding and increased the stability of the complex.

The product of the reaction of ribosome-bound polylysyl-tRNA with puromycin was analyzed to determine if peptide bond formation had occurred. This was accomplished by allowing 3H-puromycin-5′-β-cyanoethyl phosphate, which in this system behaved in a manner identical with that of puromycin, to react with bound 3H-labeled polylysyl-tRNA. The prod-

### Table I

**Effect of template on cleavage of polylysyl-tRNA by puromycin**

In Experiment I the following were added to Mixture A: 10 mM MgCl2; 0.4 mM 14C-lysine; 485 µg per ml of washed ribosomes; 15,000 cpm per ml of 1H-polylysyl-tRNA (specific activity, 5.8 × 106 cpm per µmole); 4.6 × 10-5 M puromycin; and, where indicated, 120 µg per ml of poly A, 46 µg per ml of triadenosine diphosphate, or 24 µg per ml of hexaadenosine pentaphosphate. The 0.05-ml samples were incubated for 10 min at 37°. Precipitation was by TCA. In Experiment II the following were added to Mixture A: 10 mM MgCl2; 940 µg per ml of washed ribosomes; 4,500 cpm per ml of 14C-polylysyl-tRNA (specific activity, 1.2 × 106 cpm per µmole); 4.6 × 10-5 M puromycin; and, where indicated, 50 µg per ml of poly A or 100 µg per ml of poly U. The 0.10-ml samples were incubated for 20 min at 37°, and then precipitated by TCA.

<table>
<thead>
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<th>Template</th>
<th>TCA-soluble</th>
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<tr>
<td>Triadenosine diphosphate</td>
<td>36</td>
</tr>
<tr>
<td>Hexaadenosine pentaphosphate</td>
<td>54</td>
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<tr>
<td>Poly A</td>
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<tr>
<td>Experiment II</td>
<td></td>
</tr>
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<td>None</td>
<td>3</td>
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<tr>
<td>Poly U</td>
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</tr>
<tr>
<td>Poly A</td>
<td>63</td>
</tr>
<tr>
<td>Poly U + poly A</td>
<td>8</td>
</tr>
</tbody>
</table>

### Table II

**Effect of GTP and supernatant on cleavage of 3H-polylysyl-tRNA by puromycin**

The incubation conditions were as described in Table I, Experiment I. Poly A was used as template. Puromycin was added after 10 min of preliminary incubation at 37°. Where indicated, 0.25 mM GDP, 0.25 mM GTP, G + T (4 µl per ml of the ammonium sulfate fraction), or purified G (200 µl per ml), were added after incubation. The samples (0.10 ml in Experiment I, or 0.05 ml in Experiment II) were then incubated for an additional 10 min at 37°. Precipitation was carried out in TCA as described under Methods. In the absence of puromycin, none of these factors caused significant cleavage of the 3H-polylysyl-tRNA.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TCA-soluble</th>
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<td>None</td>
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<tr>
<td>GDP</td>
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<tr>
<td>GTP</td>
<td>65</td>
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<tr>
<td>GTP + G + T</td>
<td>73</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>60</td>
</tr>
<tr>
<td>G</td>
<td>55</td>
</tr>
</tbody>
</table>

### Table III

**Effect of chloramphenicol and chlortetracycline on puromycin reaction**

To Mixture A were added 10 mM MgCl2; 0.4 mM 14C-lysine; 120 µg per ml of poly A; 1.04 mg per ml of washed ribosomes; and 16,000 cpm per ml of 14C-polylysyl-tRNA (specific activity, 3.2 × 106 cpm per µmole) in Experiment I or 18,000 cpm per ml of 3H-polylysyl-tRNA (specific activity, 2.0 × 106 cpm per µmole) in Experiment II. After 10 min of preliminary incubation at 37°, 1.0 × 10-4 M chloramphenicol, 1.0 × 10-4 M chlortetracycline, and 4.6 × 10-4 M puromycin were added, and the vessels were incubated for an additional 2 min (I) or 10 min (II) at 37°. In Experiment I, 0.05-ml samples were assayed for binding, and in Experiment II, 0.10-ml samples were precipitated with cold TCA.

<table>
<thead>
<tr>
<th>Additions</th>
<th>14C-Polylysyl-tRNA bound</th>
<th>3H-Polylysyl-tRNA cleaved by puromycin</th>
<th>Release</th>
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<td>Chloramphenicol</td>
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<td>55</td>
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<tr>
<td>Chlortetracycline</td>
<td>988</td>
<td>55</td>
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Downloaded from http://www.jbc.org/ by guest on October 14, 2017
mycin-5'-'cyanoethyl phosphate were eluted with urea, and the min at 37°, 800,000 cpm per ml of 3²P-puromycin-5'-&cyanoethyl
fig per ml of poly A, snd 80,000 cpm per ml of 'H-polylysyl-tRNA
limited alkaline hydrolysis. Incubation mixture. Precipitation was by TCA-tungstate after
per pmole) was then added and, where indicated, 1.0 x 10⁻³
GTP; and G and T DEAE-Sephadex eluate, 0.16 ml per ml of
lysyl-tRNA (200,000 cpm per ml, specific activity, 1.4 x 10⁶ cpm
ml of W-polylysyl-tRNA was incubated for 5 min at 37°. 3H-
lysine occurs, and the requirement for preexisting poly-
sines. This then is consistent with the formation of puromycin oligolysines. Free ²H-oligolysine is seen as well. The
source of the free ²H-oligolysine is not known; enzymes capable of
degrading peptidylpuromycin may be present in the system.
Peptide bond formation could be shown with either poly A or
triadenosine diphosphate, although more oligolysylpuromycin
relative to oligolysine was seen with the latter template.
The synthesis of oligolysylpuromycin can be detected more
simply by heating reaction products in TCA-tungstate (see
"Methods"). The two assays were consistent and indicated
that the reaction of puromycin with ribosome-bound polylysyl-
tRNA involved the formation of a peptide bond.

Reactions of Ribosome-bound Polylysyl-tRNA with Lysyl-tRNA—
The ability of the ribosome alone to form puromycin oligolysines suggested the possibility of an analogous reaction between
polylysyl and lysyl-tRNA. This was verified (Fig. 4) by the
demonstration of a transfer of radioactivity from lysyl-tRNA to polylysyl, dependent only on the addition of unlabeled
polylysyl-tRNA, ribosomes, and poly A. The kinetics of this
transfer reaction are consistent with the rapid utilization of a
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supernatant and GTP are required for further elongation.
The same reaction could be followed as an increase in the
acid insolubility of labeled polylysyl-tRNA on addition of
unlabeled lysyl-tRNA, as shown in Table IV. It can also be
seen in this table that although hexaadenosine pentaphosphate
promotes this addition reaction, triadenosine diphosphate does
not. This result contrasts with the corresponding experiment

FIG. 3. The formation of ³H-polylysyl-²P puromycin-5'-&cyanoethyl phosphate. To Mixture A plus 10 mm MgCl₂ were
added: 0.4 mm ³C-lysine, 440 µg per ml of washed ribosomes, 120
µg per ml of poly A, and 80,000 cpm per ml of ²P-polylysyl-tRNA
(specific activity, 6.3 x 10⁸ cpm per amole of lysine). After 10
min at 37°, 800,000 cpm per ml of ²P-puromycin-5'-&cyanoethyl
phosphate (specific activity, 2.7 x 10⁶ cpm per µmole) was added,
and the mixture (2.5 ml) was incubated for an additional 20 min
at 37°. After incubation, the mixture was adsorbed onto a col-
umn, 20 x 1 cm, the unreacted ³H-polylysyl-tRNA and azP-puro-
lysinetRNA were added, and the samples (0.10 ml) were incu-
bated for an additional 10 min at 37°. After heating for 10 min
phosphate were added. After 5 min
triadenosine diphosphate or 24 µg per ml of hexaadenosine penta-
Insoluble (see "Methods") (specific activity, 5.8
cpm per ml, TCA-insoluble, 3,000 cpm per ml of hot tungstate-
originating from puromycin chromatographed in multiple, dou-
ble-labeled peaks shifted with respect to the marker oligo-
sines. This then is consistent with the formation of puromycin oligolysines. Free ²H-oligolysine is seen as well. The
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FIG. 4. The incorporation of ³H-lysyl-tRNA into ³C-polylysyl-
tRNA. Mixture A plus 10 mm MgCl₂, 0.4 µm ³C-lysine; 485
µg per ml of washed ribosomes, and where indicated, 25 µmole per
ml of ³C-polylysyl-tRNA was incubated for 5 min at 37°. ³H-
lysyl-tRNA (200,000 cpm per ml, specific activity, 1.4 x 10⁷ cpm
per µmole) was then added and, where indicated, 1.0 x 10⁻² M
GTP; and G and T DEAE-Sephadex eluate, 0.16 ml per ml of
incubation mixture. Precipitation was by TCA-tungstate after
limited alkaline hydrolysis.

TABLE IV
Elongation of ³H-polylysyl-tRNA by ³C-lysyl-tRNA on
various templates

To Mixture A were added 20 mm MgCl₂; 0.40 µm ³C-lysine; 485
µg per ml of washed ribosomes; and ³H-polylysyl-tRNA, 37,500
cpm per ml, TCA-insoluble, 3,000 cpm per ml of hot tungstate-
insoluble (see "Methods") (specific activity, 5.8 x 10⁷ cpm per
µmole). Where indicated, 120 µg per ml of poly A, 46 µg per ml of
triadenosine diphosphate or 24 µg per ml of hexaadenosine penta-
phosphate were added. After 5 min at 37°, 163 µmole per ml of
³C-lysyl-tRNA were added, and the samples (0.10 ml) were incu-
bated for an additional 10 min at 37°. After heating for 10 min
at 90°, the samples were precipitated in TCA-tungstate. Blank
values were obtained by omitting ³C-lysyl-tRNA from the incu-
bation mixtures.

| Template                | Increase in hot TCA-
tungstate precipitable counts (less 350 cpm blank) |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>69</td>
</tr>
<tr>
<td>Triadenosine diphosphate</td>
<td>72</td>
</tr>
<tr>
<td>Hexaadenosine pentaphosphate</td>
<td>210</td>
</tr>
<tr>
<td>Poly A</td>
<td>190</td>
</tr>
</tbody>
</table>
for the puromycin reaction (see Table I), in which triadenosine
diphosphate functioned as template. It would seem that two
codons are required to allow polylysyl-tRNA to react with
lysyl-tRNA but only one is required for the reaction with puro-
mycin. This is consistent with the idea that the puromycin
reaction is codon-independent. With poly A as template, no
addition of phenylalanyl-tRNA to polylysyl-tRNA could be
detected. In common with the puromycin reaction, the lysyl-
tRNA reaction was not inhibited by GDP. The two reactions

Table V

<table>
<thead>
<tr>
<th>Effect of chloramphenicol and chlortetracycline on lysyl-tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Addition</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Chlortetracycline</td>
</tr>
</tbody>
</table>

![Graph](image)

Fig. 5. Chromatography of the limited addition product 1H-
polylysyl-tRNA + 14C-lysyl-tRNA. To Mixture A plus 10 mM
MgCl₂, were added 0.4 mM 14C-lysine, 485 μg per ml of washed
ribosomes, 120 μg per ml of poly A, and 60,000 cpm per ml
of H-polylysyl-tRNA (specific activity, 7.3 x 10⁶ cpm per μmole
of lysine). This mixture was incubated for 10 min at 37°. Then,
122,000 cpm per ml of 14C-lysyl-tRNA (specific activity, 1.47 x
10⁶ cpm per μmole) were added and the incubation mixture,
total volume 5.0 ml, was held for 20 min at 37° and then cooled to
0°. Carrier 1C-dilysyl and trilysyl were added, and the marked
polypeptides were detached from tRNA by limited alkaline
hydrolysis (cf. "Methods"). The peptides were chromatographed
on carboxymethyl cellulose as indicated in "Methods." The
elongation of a 1H-polylysine molecule with a 14C-lysine residue
moves it one peak ahead in the elution profile. Thus, the 1C-
labeled peptides are derived from preceding 1H-polylysine peaks.

Table VI

<table>
<thead>
<tr>
<th>Relative stability to attack by puromycin of 1C-phenylalanyl-tRNA, 14C-phenylactyl-tRNA, and N-acetyl-14C-phenylalanyl-tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate</strong></td>
</tr>
<tr>
<td>1C-Phenylalanyl-tRNA</td>
</tr>
<tr>
<td>14C-Phenylactyl-tRNA</td>
</tr>
<tr>
<td>N-Acetyl-14C-phenylalanyl-tRNA</td>
</tr>
</tbody>
</table>

The distribution of 14C-label among the various oligolysine
peaks suggests that each species of H-polylysyl-tRNA is equally
reactive with 1C-lysyl-tRNA, and that only one 1C-lysine is
added per reacting H-polylysyl-tRNA molecule. This evidence
for single addition is indirect, however, and the possibility
that in some cases more than one residue is added to a H-
polylysyl-tRNA has not been excluded. About 10% of the
added polylysyl-tRNA will react with lysyl-tRNA, compared
with about 70% with puromycin.

Reaction of Puromycin with Possible Peptidyl-tRNA Analogues
Lyyl-tRNA does not react at a measurable rate with
puromycin whereas polylysyl-tRNA is cleaved rapidly and
extensively. The reactivity of polylysyl-tRNA compared with
lysyl-tRNA might be ascribed to the absence of a charged α-
amino group or, alternatively, to the presence of a peptide bond
on the carboxyl-terminal amino acid of the peptidyl-tRNA. To
test these possibilities, the puromycin reactivity of 1C-phenyl-
lactyl-tRNA and N-acetyl-14C-phenylalanyl-tRNA were com-
pared. Although binding assays show that all three compounds
bind well to ribosomes in the presence of hexauridine pentaphos-
phate, N-acetylphenylalanyl-tRNA was cleaved by puromycin
at approximately 3 times the background rate obtained with
phenylalanyl-tRNA (Table VI). The product of this cleavage
was identified by electrophoresis as N-acetylphenylalanylpuro-
mecin. Phenylactyl-tRNA, on the hand, was even less reactive
than phenylalanyl-tRNA. This result indicates that the pre-
sumed ribosomal peptide synthetase distinguishes aminocetyl-
tRNA from peptidyl-tRNA by the peptide bond present in the
latter.
the conclusions of Suarez and Nathans (30) and Hierowski (31) is not inhibited by chlortetracycline but the limited addition inhibition by chlortetracycline. Thus, the puromycin reaction TCA precipitation were used.

were added at zero time where indicated. The binding assay or TCA precipitation were used.

As with the reaction between polylsyl-tRNA and puromycin, the release of N-acetyl-14C-phenylalanyl-tRNA by puromycin is inhibited by chloramphenicol but not by chlortetracycline; neither antibiotic inhibits binding. With the phenylalanine system, the inhibition by chloramphenicol is complete (Table VII). On the basis of these results, it is felt that ribosomes under the conditions used here can utilize a variety of peptidyl-tRNA analogues in the limited formation of peptide bonds without added GTP or supernatant.

DISCUSSION

Polylysyl-tRNA, isolated free of supernatant enzymes and ribosomes, is capable of reattaching to extensively washed ribosomes in the presence of a suitable template. The requirements for this reaction are similar to those observed for aminoacyl-tRNA, i.e., a sulfhydryl compound and Mg++. The template must be at least three nucleotides in length. With respect to binding to ribosomal subunits, a large stimulation in the binding of polylysyl-tRNA is obtained when 50 S subparticles are added to 30 S subparticles. This stimulation is greater than that observed with lysyl tRNA. Whether this admixture creates an additional binding site that is preferred attachment locus of peptidyl-tRNA, or whether the effect requires a more subtle explanation, remains to be resolved. Peska and Nirenberg (32), with the use of a somewhat different ribosomal preparation, have demonstrated extensive binding of polylysyl-tRNA to 30 S ribosomal subparticles.

The experiments performed with chlortetracycline show that the binding of polylysyl-tRNA to the ribosomal peptidyl site is not affected by this antibiotic, whereas the binding of lysyl-tRNA to the aminoacyl site is almost entirely abolished. This confirms the conclusions of Suarez and Nathans (30) and Hierowski (31) that the ribosomal binding sites had different sensitivity to inhibition by chlortetracycline. Thus, the puromycin reaction is not inhibited by chlortetracycline but the limited addition reaction is prevented.

The ability of lysyl-tRNA to prevent the binding of polylysyl-tRNA suggests that lysyl-tRNA enters both ribosomal sites. Indeed, this must be the case if the synthesis of polylysine from lysyl-tRNA is to occur. Nonetheless, only polylysyl-tRNA is capable of reacting with puromycin. Similarly, in the phenylalanine system, N-acetylation triples the rate of reaction of phenylalanyl-tRNA with puromycin. Therefore, although both aminocarboxyl- and peptidyl-tRNA appear to be capable of binding to the peptidyl site, only the latter reacts to give a rapid rate of peptide bond formation.

Extensive analysis of this simplified system for a requirement for or a stimulation by GTP and the supernatant enzymes has yielded no evidence that factors other than ribosomes and template play a role in peptide bond formation per se. It appears, therefore, that the peptide synthetase or synthetases cannot be removed from E. coli ribosomes by extensive washing, and may form an integral part of the particle itself. The putative peptide synthetase is not affected by chlortetracycline or CMS, but is inhibited by chloramphenicol (33) and goerguerin.

It has been suggested that GTP and the supernatant enzymes are involved in the movement of messenger RNA on the ribosomes (12). Recent work in several laboratories has shown that at low Mg++ concentrations (34) GTP and factors extracted from the ribosomes (4) are required to bind N-acetylphenylalanyl-tRNA (35) or formylnmethionyl-tRNA (36, 37) to the peptidyl site. Repeated washing of ribosomes in 0.5 m NITCl removes the bulk of the initiation factors. It is likely that readjustment of these factors and reduction of the Mg++ concentration would render this system partially dependent on GTP; such results have been obtained with a similar system by Lucas-Lenard (Reference 35 and Footnote 3). The simplified system reported here circumvents the requirement for GTP, presumably by allowing the attachment of either aminocarboxyl-tRNA or peptidyl-tRNA to the peptidyl site. The observation that in the absence of GTP and factors apparently only a single lysine residue can be added to polylysyl-tRNA, supports the idea that the simplified system cannot move the elongated peptidyl-tRNA from the aminocarboxyl site back to the peptidyl site. In this respect, both systems are consistent and suggest that purified ribosomes alone cannot perform a translocation reaction.

Acknowledgments—I am indebted to Dr. Fritz Lipmann in whose laboratory and with whose support the bulk of this work was performed. I am grateful also to Dr. Daniel Nathans for his aid in the characterization of N-acetylphenylalanylpuromycin. I should also like to thank Dr. Clelia Ganoza and Dr. Julian Gordon for their help in the preparation of this manuscript.

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Reaction of Ribosome-bound Peptidyl Transfer Ribonucleic Acid with Aminoacyl Transfer Ribonucleic Acid or Puromycin

M. E. Gottesman


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