Studies on the Formation of Transfer Ribonucleic Acid-Ribosome Complexes

III. THE FORMATION OF PEPTIDE BONDS BY RIBOSOMES IN THE ABSENCE OF SUPERNATANT ENZYMES*

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SUMMARY

Peptide bond formation from aminoacyl transfer RNA and ammonium chloride-washed ribosomes can occur extensively in the absence of supernatant enzymes. More than 50% of the L-phenylalanine-tRNA can be converted into oligophenylalanine-tRNA. Although diphenylalanine is the major product, the oligophenylalanine formed consisted of 73, 16, and 9.7% of di-, tri-, and tetraphenylalanine, respectively. Oligophenylalanine synthesis in the absence of supernatant factors (peptide bond synthesis) and amino acid polymerization into long polypeptides in the presence of supernatant (protein synthesis) differ in magnesium and temperature optima and other characteristics. Guanosine triphosphate is not required for the former as it is for the latter; the sulfhydryl inhibitor p-chloromercuribenzenesulfonate has little effect on the former while completely abolishing the latter. In addition, an absolute requirement for ammonium or potassium ions was shown for peptide bond synthesis itself. The results suggest that peptide bond synthesis from aminoacyl-tRNA is a property of ribosomes and is not mediated by supernatant protein factors.

Amino acid incorporation by cell-free extracts directed by synthetic or natural polynucleotides (3) has been used extensively to study the mechanism of protein biosynthesis. With the use of cell-free extracts from Escherichia coli (4-7) and mammalian systems (8-10), several laboratories have shown that protein synthesis in vitro required guanosine triphosphate and supernatant enzymes. In addition, potassium or ammonium ions have been found to be necessary for protein biosynthesis in vitro (11) and in vivo (12), as well as divalent estions (13).

* A preliminary report of a part of this investigation has been published (1). The preceding paper of this series is listed as Reference 2.

Although several supernatant enzymes and cofactors have been implicated in protein biosynthesis, the specific role of each has yet to be defined. In a previous report (2), peptide bond synthesis from unfractionated transfer RNA acylated with 14C-phenylalanine in the absence of supernatant factors was found in the presence of washed ribosomes and a polyuridylic acid template. This paper is an elaboration of the above observation. It describes the requirements and characteristics of peptide bond synthesis and compares peptide bond synthesis with amino acid polymerization (protein synthesis).

EXPERIMENTAL PROCEDURE

Ribosomes and Cell Extracts—Ribosomes and cell extracts were prepared from E. coli cells grown to mid-log phase in 0.9% nutrient broth, 1% glucose, and 0.02 M sodium phosphate-potassium phosphate buffer, pH 7.3. The procedures in the preparation of cell fractions (including ribosomal subunits) were carried out at 0° and have been described in detail elsewhere (2, 14, 15).

Ribosomes were prepared by two different procedures from the endoribonuclease I-deficient strain E. coli A-19 (16). Ribosomes were washed once in 0.014 M MgCl2, 0.01 M Tris-HCl (pH 7.2), and 0.05 M KCl (Buffer A) and four times in 1 M NH4Cl, 0.01 M Tris-HCl (pH 7.2), and 0.01 M MgCl2 (Buffer B) by suspending the particles in the solution for 3 to 16 hours at 0° between sedimentations. After each resuspension of the ribosomes, aggregates were removed by centrifugation in the Sorvall SS-34 rotor at 15,000 rpm for 10 min. To sediment the ribosomes, suspensions were centrifuged in the Ty 65 rotor of the Spinco model L2-65B centrifuge at 50,000 rpm for 1 hour. After each wash, the ribosomes were resuspended in Buffer B and portions were stored at 0°. Unless otherwise indicated, these five times washed ribosomes were used for all experiments. They were stored at 5° and retained their original activity for at least 6 months.

Sucrose-washed ribosomes were prepared as follows. Unwashed ribosomes (500 A260 units in 2 ml) were layered on a 56-ml, linear, 5 to 30% sucrose gradient in 0.01 M magnesium acetate and 0.01 M Tris-acetate, pH 7.2 (Buffer G), and cen-
trifuged in the Spinco model L-2-65B centrifuge with an SW 25.2 rotor at 25,000 rpm for 5 hours at 5°. Gradients were analyzed and collected as previously reported (11). The contents of the tubes comprising the 70 S peak were pooled and centrifuged in a Spinco No. 30 rotor at 29,500 rpm for 24 hours to sediment the ribosomes. The ribosome pellet was resuspended in a small volume of buffer containing 1 x 10^{-4} M magnesium acetate and 0.001 M Tris-acetate, pH 7.2, and stored in liquid nitrogen.

E. coli tRNA and 14C-Aminoacyl-tRNA—Transfer RNA and 14C-aminoacyl-tRNA were prepared as previously described (2, 17). Transfer RNA was prepared from E. coli W3100 unless otherwise noted. 14C-Aminoacyl-tRNA was prepared with 1 14C-amino acid and 19 12C-amino acids. The presence of 14C-Aminoacyl-tRNA was determined by precipitating it with cold 10% trichloroacetic acid, filtering it through a nitrocellulose filter, and washing the filters with 5% trichloroacetic acid as previously described in detail (2).

Chemicals, Radioactive Isotopes, and Materials—Uniformly labeled 14C-l-phenylalanine (351 mCi per mmole) and 14C-lysine (240 mCi per mmole) were obtained from New England Nuclear. [3H]GTP (1.12 Ci per mmole) was obtained from Schwarz BioResearch. Nitrocellulose membrane filters were type HA (0.55 μm pore size) Millipore filters, 25 mm in diameter. E. coli B tRNA was obtained from General Biochemicals, Inc. (Chagrin Falls, Ohio). Diphenylalanine and polylysine were obtained from Miles-Yeda, Ltd. (Rehovoth, Israel). Tris-acetate, pH 7.2, and stored in 1.0 ml would yield a value of 1.0 for the absorbance measured at 260 nm in a cuvette with a path length of 1.0 cm.

For further analyses of the oligophenylalanes, two other systems were used: descending chromatography in Solvent B, which contained n-butyl alcohol-concentrated ammonia-water (100:3:18, by volume); and electrophoresis in 1.52 M (7%) formic acid with a voltage gradient of 47 volts per cm for 100 min (Miles Hivolt Electrophorator). The distribution of radioactivity was determined as described above.

For analysis of lysine peptides, the procedure was similar except that the solvent system used was n-butyl alcohol-glacial acetic acid-pyridine-water (90:6:20:24, by volume) and the developing time was 64 hours (19). A hydrobromic acid hydrolysate of polylysine was prepared as a standard chromatographic marker, which contained lysine as well as di-, tri-, and tetraside.

Determination of 14C-Phenylalanine Incorporation into Polypeptides—Each 0.050-ml reaction mixture contained the following components unless otherwise specified: 0.05 M potassium acetate; 0.05 M Tris-acetate, pH 7.2; 0.02 M magnesium acetate; 0.15 A260 unit of ribosomes; 0 x 10^{-4} M GTP; 0 x 10^{-4} M phosphoenolpyruvate; 0.5 μg of phosphoenolpyruvate kinase; 11.5 μmols of base residues of poly U; 23 μg of E. coli W3100 100,000 x g supernatant protein; and about 5 μmols of 14C-Phe-tRNA. Incubations were performed at 37° for the time indicated in each legend. Reactions were stopped by adding 2.0 ml of 10% trichloroacetic acid. The tubes were next heated at 80°-95° for 15 min, then cooled in an ice bath, and finally filtered through a nitrocellulose filter, which was washed three times with 3-ml portions of cold 5% trichloroacetic acid. The filters were dried under an infrared lamp and counted in a liquid scintillation spectrometer as previously described (2). For some experiments, purified transfer factors (T1, T2, and G), prepared from E. coli B, were added (20).

Carboxypeptidase A Treatment of Peptides—Reaction mixtures contained, in a volume of 0.03 ml, 0.1 M Tris-acetate (pH 7.2), 0.36 M NH4HCO3 (brining pH to 7.6), 50 μg of carboxypeptidase A, about 3 μmols of 14C-phenylalanine as oligophenylalanine, and nonradioactive carrier. The carrier consisted of mono-, di-, tri-, and tetraphenylalanine (15, 25, 50, and 50 μmols, respectively) and some L-phenylalanyl-L-phenylalanine diketopiperazone. Incubations were performed at 25° for 30 min. At 30 min, an additional 25 μmols of carrier diphosphorylalanine were added to the reaction mixture, and incubation was continued for another 30 min at 25°. Chromatographic analysis was performed as indicated under “Determination of Oligopeptide Formation” by means of chromatography with Solvent A and paper electrophoresis. The carrier diketopiperazone was added, for occasionally after alkaline

The abbreviations used are: tRNA, unfraccionated transfer ribonucleic acid; 14C-Phe-tRNA, tRNA acylated with 14C-phenylalanine and 19 12C-amino acids (and analogously 14C-Lys-tRNA); poly U, polyuridylic acid; poly A, polyadenylic acid; CMS, p-chloromercuribenzenesulfonate. One A260 unit is the amount of material which in 1.0 ml would yield a value of 1.0 for the absorbance measured at 260 nm in a cuvette with a path length of 1.0 cm.
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Oligophenylalanine Formation in Relation to Washing of Ribosomes—Ribosomes prepared by different procedures and number chromatographic analysis. The percentages of the counts found in the various areas of the chromatograms were as follows:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>46</td>
<td>96</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>Oligophenylalanine</td>
<td>51</td>
<td>3.7</td>
<td>1.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

PHE, PHE3, PHEa, and PHE4, mono-, di-, tri-, and tetraphenylalanine, respectively.
of washes were compared for ability to form oligophenylalanine in the absence of exogenous supernatant factors and GTP. Reaction mixtures contained ribosomes, $^{14}$C-Phe-tRNA, and poly U. The data of Table I indicate that, under conditions in which ribosomes were limiting, oligophenylalanine formation increased after the ribosomes were washed in 1 M NH₄Cl. Ribosomes prepared by washing through a sucrose gradient (G) or those washed in Buffer A only (Table I) were about half as active as those washed with 1 M NH₄Cl in extent of oligophenylalanine formation and in percentage of bound $^{14}$C-Phe-tRNA converted into oligopeptides. Increasing the number of washes in 1 M NH₄Cl to four did not significantly alter the extent of oligopeptide formation; also, the ratio of oligophenylalanine to $^{14}$C-Phe-tRNA bound was essentially constant after the first 1 M NH₄Cl wash.

Requirements for Oligophenylalanine Formation—Various components were added to or deleted from reaction mixtures containing five times washed ribosomes, $^{14}$C-Phe-tRNA, and poly U. As the data of Table II indicate, virtually no oligophenylalanine synthesis occurred in the absence of poly U, ribosomes, or magnesium ion. The addition of GTP or guanylyl-5'-methylene diphosphonate slightly inhibited oligopeptide formation. Addition of puromycin (10⁻⁴ M) at 15 min was able to cause release of about two-thirds of the counts incorporated into oligophenylalanine in the absence of exogenous supernatant factors and GTP (data not shown), and addition of supernatant factors with GTP was able to cause release of almost all the counts incorporated into oligophenylalanine. Also, oligophenylalanine formation occurred when the purified phenylalanine-accepting tRNA species (2) was used instead of unfractionated tRNA.

### Table I

<table>
<thead>
<tr>
<th>No. of washes</th>
<th>Buffer</th>
<th>$^{14}$C-Phe-tRNA bound</th>
<th>$^{14}$C-Phe-tRNA</th>
<th>Oligophenylalanine/$^{14}$C-Phe-tRNA bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>2.76</td>
<td>1.25</td>
<td>0.45</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>3.12</td>
<td>1.01</td>
<td>0.32</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>3.26</td>
<td>2.03</td>
<td>0.62</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>3.03</td>
<td>2.25</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>2.68</td>
<td>2.07</td>
<td>0.77</td>
</tr>
</tbody>
</table>

### Table II

**Requirements for oligophenylalanine formation**

Unless otherwise noted in the table, each 0.050-ml reaction mixture contained the components described under “Experimental Procedure” in addition to the following: 0.1 M ammonium chloride; 5.2 μmoles of $^{14}$C-Phe-tRNA (0.18 A₂₆₀ unit); 23 μmoles of base residues of poly U; and 0.70 A₂₆₀ unit of 70 S ribosomes. The concentration of ribosomes was limiting for diphenylalanine formation. Gradient washed ribosomes were washed only once through a sucrose gradient, and the other ribosomes used were portions taken during the preparation of ribosomes washed five times as described under “Experimental Procedure.” The column headed “Buffer” refers to the buffer and inorganic salt mixture which was used for the last ribosome wash. Incubations were performed at 37° for 15 min.

### Table III

**Monovalent Cation Requirement for Oligophenylalanine Formation**

The binding of $^{14}$C-Phe-tRNA to ribosomes and the formation of oligophenylalanine were studied in the presence and absence of various monovalent cations as shown in Table III. The data show that potassium or ammonium ions were an absolute requirement for oligophenylalanine formation. Neither sodium nor lithium could substitute for ammonium or potassium. The binding of $^{14}$C-Phe-tRNA to ribosomes, however, showed no absolute requirement for these monovalent cations as previ-
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TABLE III

Monovalent cation requirement for oligophenylalanine formation

<table>
<thead>
<tr>
<th>Monovalent cation</th>
<th>14C-Phenylalanine incorporated into oligophenylalanine</th>
<th>14C-Phe-tRNA bound to ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.06</td>
<td>3.30</td>
</tr>
<tr>
<td>Lithium acetate, 0.05 M</td>
<td>0.07</td>
<td>3.24</td>
</tr>
<tr>
<td>Sodium acetate, 0.05 M</td>
<td>0.02</td>
<td>3.17</td>
</tr>
<tr>
<td>Potassium acetate, 0.05 M</td>
<td>2.30</td>
<td>4.33</td>
</tr>
<tr>
<td>Ammonium acetate, 0.05 M</td>
<td>2.20</td>
<td>4.33</td>
</tr>
</tbody>
</table>

TABLE IV

Oligophenylalanine formation from ribosomal subunits

Each 0.050-ml reaction mixture contained the components described under "Experimental Procedure" in addition to the following: 5.5 μmole of 14C-Phe-tRNA (0.18 A260 unit); 23 mmole of base residues of poly U; 0.83 A260 unit of 30 S ribosomes (72 μmole) where indicated; and 2.13 A260 unit of 50 S subunits (79 μmole) where designated. Incubations were carried out at 37° for 15 min.

<table>
<thead>
<tr>
<th>Subunits</th>
<th>14C-Phenylalanine as oligophenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>μmoles</td>
</tr>
<tr>
<td>30</td>
<td>0.37</td>
</tr>
<tr>
<td>50</td>
<td>1.76</td>
</tr>
<tr>
<td>30 + 50</td>
<td></td>
</tr>
</tbody>
</table>

Provincially reported (15, 23, 24), although some stimulation by ammonium or potassium is evident.

Oligophenylalanine Formation from Ribosomal Subunits—The data of Table IV show that, although little or no oligopeptide formation occurred with purified 30 S or 50 S subunits, the combination of 30 and 50 S particles was effective in producing oligophenylalanine from 14C-Phe-tRNA. The 50 S particle preparation was contaminated with 2% 30 S subunits based on absorption at 260 mp; the 30 S ribosome preparation contained 1.6% 50 S subunits (15).

Formation of Dilyne in Absence of Supernatant Enzymes—By use of five times washed ribosomes, the formation of dilysine was examined in the absence of added supernatant factors. The data of Table V indicate that, in the presence of poly A but in the absence of supernatant enzymes, 1.81 μmole of 14C-Lys-tRNA were converted into dipeptide. More than 93% of the oligolysine detected was dilysine. In the absence of poly A, a small amount of dilyse was formed. In each case, the 0.1 μmole of oligolysine greater than dilyse did not correspond to tri- or tetralysine but was spread over the entire oligolysine region, suggesting that this might be the result of nonspecific adsorption to the paper.

Kineti of 14C-Phe-tRNA Binding to Ribosomes and Oligophenylalanine Formation—14C-Phe-tRNA binding to ribosomes and oligophenylalanine formation were examined in duplicate reaction mixtures as a function of time at 37°. As shown by the data of Fig. 3, binding of 14C-Phe-tRNA to ribosomes in the presence of poly A was examined in the absence of added supernatant factors. Each 0.050-ml reaction mixture contained the components described under "Experimental Procedure" in addition to the following: 8.2 μmole of 14C-Lys-tRNA (0.27 A260 unit), with tRNA from E. coli B; 27 mmole of base residues of poly A; 0.1 M ammonium chloride; and 2.9 A260 units of ribosomes. Incubations were performed at 37° for 15 min. On deacylation of the 8.2 μmole of 14C-Lys-tRNA, 0.15 μmole was found scattered over the oligolysine region, 0.02 μmole of which was found over the dilyse region.

TABLE V

Formation of di- and oligolysine from 14C-Lys-tRNA

Each 0.050-ml reaction mixture contained the components described under "Experimental Procedure" in addition to the following: 8.2 μmole of 14C-Lys-tRNA (0.27 A260 unit), with tRNA from E. coli B; 27 mmole of base residues of poly A; 0.1 M ammonium chloride; and 2.9 A260 units of ribosomes. Incubations were performed at 37° for 15 min. On deacylation of the 8.2 μmole of 14C-Lys-tRNA, 0.15 μmole was found scattered over the oligolysine region, 0.02 μmole of which was found over the dilyse region.

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>14C-Lys-tRNA bound to ribosomes</th>
<th>14C-Lysine as dilyse</th>
<th>14C-Lysine as oligolysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.59</td>
<td>0.31</td>
<td>0.41</td>
</tr>
<tr>
<td>Poly A</td>
<td>5.93</td>
<td>1.81</td>
<td>1.94</td>
</tr>
</tbody>
</table>

Fig. 3. The kinetics of formation of oligophenylalanine (OLIGO-PHE) from 14C-Phe-tRNA and of binding 14C-Phe-tRNA to ribosomes. Each 0.050-ml reaction mixture contained the components indicated under "Experimental Procedure" in addition to the following: 5.2 μmole of 14C-Phe-tRNA (0.18 A260 unit); 23 mmole of base residues of poly U where designated; 0.1 M ammonium chloride; and 2.9 A260 units of ribosomes. Incubations were performed at 37° for the times indicated. —— , μmoles of 14C-Phe incorporated into oligophenylalanine in the presence of poly U; - - - - - - - - - , μmoles of 14C-phenylalanine (14C-PHE) incorporated into oligophenylalanine in the absence of poly U; ∆, μmoles of 14C-Phe-tRNA bound to ribosomes in the presence of poly U.
presence of poly U was rapid, being almost maximal in 2 min; its extent gradually decreased after 5 min. Oligopeptide formation occurred at a much slower rate; its extent gradually increased during the 60 min studied. The initial rate of $^{14}$C-Phe-tRNA binding to ribosomes was about 5-fold that of $^{14}$C-phenylalanine incorporation into oligophenylalanine.

Comparisons of $^{14}$C-Phe-tRNA Binding to Ribosomes and Oligophenylalanine Formation Both $^{14}$C-Phe-tRNA binding to ribosomes and oligopeptide formation were studied as a function of ribosome concentration. The data of Fig. 4 show that at low ribosome concentrations the extent of binding of $^{14}$C-Phe-tRNA and formation of oligophenylalanine were similar. At higher ribosome concentrations, binding of $^{14}$C-Phe-tRNA to ribosomes was about twice the $^{14}$C-phenylalanine incorporated into oligopeptide. In addition, the maximal oligopeptide formation occurred at lower ribosome concentrations than did maximal $^{14}$C-Phe-tRNA binding to ribosomes. Also, the data of Fig. 4 indicated that a level of ribosomes in excess of that producing maximal oligopeptide formation caused a small but reproducible inhibition of oligopeptide formation. However, binding of $^{14}$C-Phe-tRNA was not inhibited by the presence of excess ribosomes.

Binding of $^{14}$C-Phe-tRNA to ribosomes and oligophenylalanine formation were examined as a function of magnesium concentration. The data presented in Fig. 5 indicate a sharp maximum at 0.02 M Mg$^{++}$ for oligopeptide formation. The magnesium curve for binding $^{14}$C-Phe-tRNA to ribosomes resembles that previously reported (17, 18). On the other hand, $^{14}$C-phenylalanine incorporation into a hot trichloracetic acid-precipitable product (amino acid polymerization) is markedly inhibited by magnesium concentrations larger than 0.04 M as shown in Fig. 6. Amino acid polymerization required supernatant enzymes and GTP.

The formation of oligopeptide as a function of temperature is shown by the data of Fig. 7. From 24° to 37°, the extent of oligophenylalanine formation in 15 min is approximately constant. Substantial formation of oligophenylalanine proceeds at 0° and 15°. In contrast, amino acid polymerization into hot trichloracetic acid-precipitable material is negligible from 0° to 15° as shown in Fig. 8.

**Effect of p-Chloromercuribenzenesulfonate on Oligophenylalanine Formation** Since it has been reported that sulphydryl reagents inhibit amino acid polymerization (6) and that thiols stabilize the transfer enzymes (7, 25), it was of interest to evaluate the
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Fig. 6. ^14C^-Phenylalanine (^14C-PHE) incorporation from ^14C^-Phe-tRNA into polypeptides (hot trichloracetic acid-precipitable material) as a function of magnesium concentration. Each 0.050-ml reaction mixture contained the following components: 0.05 M potassium acetate; 0.095 M ammonium chloride; 0.05 M Tris-acetate, pH 7.2; magnesium acetate concentration as specified on the abscissa; 9 X 10^-4 M GTP; 9 X 10^-3 M phosphoenolpyruvate; 0.5 μg of phosphoenolpyruvate kinase; 0.15 A_260 unit of ribosomes; 11.5 mmoles of base residues of poly U; 23 μg of E. coli W3100 100,000 X g supernatant protein; and 5.2 ppmoles of ^14C^-Phe-tRNA (0.18 A_260 unit). Incubations were performed at 37° for 10 min.

Fig. 7. ^14C^-Phenylalanine (^14C-PHE) incorporation from ^14C^-Phe-tRNA into oligophenylalanine as a function of temperature. Each 0.050-ml reaction mixture contained the components described under “Experimental Procedure” in addition to the following: 5.2 μmoles of ^14C^-Phe-tRNA (0.18 A_260 unit); 23 μmoles of base residues of poly U; 2.9 A_100 units of ribosomes; and 0.1 M ammonium chloride. Incubations were performed at the temperature indicated for 15 min.

FIG. 8. ^14C^-Phenylalanine (^14C-PHE) incorporation from ^14C^-Phe-tRNA into polypeptides as a function of temperature. Each 0.050-ml reaction mixture contained the components indicated in the legend to Fig. 4 and 0.02 M magnesium acetate. Incubations were performed at the temperature indicated on the abscissa for 15 min.

Effect of Sulfhydryl Reagents on Oligophenylalanine Formation.

The data of Table VI show the effect of CMS on ^14C^-Phe-tRNA binding to ribosomes, oligophenylalanine formation, and polyphenylalanine synthesis. Oligophenylalanine formation is essentially unaffected by the sulfhydryl inhibitor at concentrations of CMS greater than 3.3 X 10^-4 M which virtually completely inhibit amino acid polymerization. In the absence of CMS, 58% of the ^14C^-Phe-tRNA bound to ribosomes was converted to oligophenylalanine, in the presence of CMS, the amount of ^14C^-Phe-tRNA bound to ribosomes was decreased, but the percentage of that bound which was converted to oligophenylalanine was increased to 86% and 83% in the presence of 0.00033 M and 0.001 M CMS, respectively. In fact, in the presence of 0.00033 M CMS, although there is less total ^14C^-Phe-tRNA bound to ribosomes, there is more oligophenylalanine formed than in the absence of CMS.

Effect of Trinucleoside Diphosphates on Oligophenylalanine Formation.

Trinucleoside diphosphates have been used extensively in studying codon recognition by aminoacyl-tRNA binding to ribosomes and other aspects of protein synthesis (26, 27). Because of their extensive use, the possibility of their stimulating oligopeptide synthesis was evaluated. The data in Table VII represent the results of analyses of reactions for
oligophenylalanine formation in the presence and absence of UpUpU. As can be seen from the data, UpUpU stimulated no significant oligophenylalanine formation under the conditions studied. Similarly, as can be seen from the data of Table VIII,

| TABLE VI |
| Comparison of effect of p-chloromercuribenzenesulfonate on $^{14}$C-Phe-tRNA binding to ribosomes, oligophenylalanine formation, and amino acid polymerization |

Reactions were carried out as described under "Experimental Procedure" and in detail in the legends to Figs. 3 and 4, where applicable. Magnesium concentration was 0.02 M. All incubations were at 37° for 15 min. Where indicated, CMS was present in the reaction mixtures.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Binding of $^{14}$C-Phe-tRNA to ribosomes</th>
<th>Oligophenylalanine formation</th>
<th>$^{14}$C-Phe-tRNA incorporated into polyphenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>4.53</td>
<td>2.63</td>
<td>2.90</td>
</tr>
<tr>
<td>CMS, 0.00003 M</td>
<td>3.74</td>
<td>1.17</td>
<td>0.04</td>
</tr>
<tr>
<td>CMS, 0.001 M</td>
<td>1.30</td>
<td>0.08</td>
<td>0.02</td>
</tr>
</tbody>
</table>

| TABLE VII |
| Oligophenylalanine synthesis in presence of UpUpU |

Each 0.10-ml reaction mixture contained the components described under "Experimental Procedure" in addition to the following: 0.05 M potassium acetate; 0.04 M ammonium chloride; 0.05 M Tris-acetate, pH 7.2; 0.05 M magnesium acetate; 2.34 $A_{260}$ units of ribosomes; 122 mpmoles of base residues of UpUpU where indicated; and 11 gpmoles of $^{14}$C-Phe-tRNA (0.36 $A_{260}$ unit). Reactions were incubated at 37° for the times indicated.

<table>
<thead>
<tr>
<th>Template</th>
<th>Time of incubation</th>
<th>$^{14}$C-Phenylalanine as W-Phe-tRNA bound to ribosomes</th>
<th>Ubio-Phe-tRNA bound to ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15 min</td>
<td>0.07</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>UpUpU</td>
<td>15 min</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| TABLE VIII |
| Oligolysine synthesis in presence of ApApA |

Each 0.10-ml reaction mixture contained the components described under "Experimental Procedure" in addition to the following: 12.1 mpmoles of $^{14}$C-Lys-tRNA (0.40 $A_{260}$ unit), with tRNA from E. coli B; 30 mpmoles of base residues of ApApA where indicated; 2.34 $A_{260}$ units of ribosomes; 0.04 M ammonium chloride; 0.05 M potassium acetate; 0.02 M magnesium acetate; and 0.05 M Tris-acetate, pH 7.2. Each incubation was performed at the temperature and time indicated in the table.

<table>
<thead>
<tr>
<th>Template</th>
<th>Conditions of incubation</th>
<th>$^{14}$C-Lys-tRNA as oligolysine</th>
<th>$^{14}$C-Lys-tRNA bound to ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature</td>
<td>Time</td>
<td>$^{14}$C-Lys-tRNA bound to ribosomes</td>
</tr>
<tr>
<td>None</td>
<td>24°</td>
<td>15</td>
<td>0.21</td>
</tr>
<tr>
<td>ApApA</td>
<td>24°</td>
<td>15</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>24°</td>
<td>60</td>
<td>0.13</td>
</tr>
<tr>
<td>ApApA</td>
<td>37°</td>
<td>15</td>
<td>0.09</td>
</tr>
</tbody>
</table>

ApApA does not stimulate any significant formation of oligolysine under the several conditions evaluated.

Binding of $^{3}$H-Guanosine Triphosphate to NH$_2$Cl-washed Ribosomes—Since binding of $^{3}$H-GTP is a relatively simple method for detection of Factor T (probably $T_s$ and $T_a$) on ribosomes (20, 22), binding of $^{3}$H-GTP to 10 times the amount of ribosomes (12 $A_{260}$ units) used for oligophenylalanine formation was studied. No detectable binding of $^{3}$H-GTP was noted at 0° or 37° as a function of ribosome concentration. The binding of 0.1 $\mu$ mole of $^{3}$H-GTP would have been easily detectable.
Polypeptide Synthesis with Purified Transfer Factors from E. coli—Purified Factors T₀, Tₛ, and G were used to determine the requirements for peptide synthesis with five times washed ribosomes. The data of Table IX show that ¹⁴C-phenylalanine incorporation into polypeptide was dependent on the presence of all three factors for maximum rate of polymerization. Little or no polymerization occurred in the absence of Factor G. However, a small amount of polymerization occurred in the absence of Factors T₀ or Tₛ. Independent studies by Ertel et al. (20) have indicated that these preparations of Factors T₀ and Tₛ are slightly contaminated with each other. This can account for the slight polymerization detected when either one was omitted.

Protection of ¹⁴C-Phe-tRNA from Deacylation—In a previous report (2), protection of ribosome-bound ¹⁴C-Phe-tRNA from alkaline hydrolysis was shown; however, the formation of some diphenylalanyl-tRNA complicated interpretation of the results. Therefore, in the presence of 0.05 M sodium acetate and in the absence of any ammonium or potassium ions (conditions not permitting oligophenylalanine formation), the deacylation of ¹⁴C-Phe-tRNA was studied as a function of time in the presence and absence of ribosomes and poly U. As can be seen from the data of Fig. 9, ¹⁴C-Phe-tRNA bound to ribosomes in the presence of poly U hydrolyzed at a slower rate than unbound ¹⁴C-Phe-tRNA. Ribosomes alone slightly inhibited deacylation of the ¹⁴C-Phe-tRNA. The protection from hydrolysis, therefore, occurred in the absence of any peptide bond formation.

DISCUSSION

The present results seem to provide a method for separating amino acid polymerization and peptide bond formation itself. The former is measured by incorporation of ¹⁴C-phenylalanine into hot trichloracetic acid-precipitable material, and the latter by the incorporation of ¹⁴C-phenylalanine into oligophenylalanine by ribosomes. Peptide bond formation is merely one step in the over-all process of amino acid polymerization into proteins. The results indicate that peptide bond formation from Phe-tRNA to form diphenylalanine chiefly and smaller amounts of tri- and tetraphenylalanine did not require supernatant enzymes and differed significantly in several other respects from amino acid polymerization. The data of Figs. 8 to 10 indicate substantial differences in the formation of oligophenylalanine and polyphenylalanine as a function of magnesium acetate concentration and as a function of temperature. Furthermore, the data of Table II show that GTP was not required for oligophenylalanine formation and that both GTP and guanylyl-5'-methylene diphosphonate inhibited its formation slightly. In contrast, protein synthesis requires GTP (8), and guanylyl-5'-methylene diphosphonate has been shown to be a competitive inhibitor of GTP in protein synthesis (28).

Several of the results indicate that the supernatant factors, T₀, Tₛ, and G, did not participate in peptide bond synthesis. As the data of Table I show, washing the ribosomes in 1 M NH₄Cl, which removes many attached proteins (29), increased the capacity of ribosomes to form oligophenylalanine. In fact, increasing the number of washes in 1 M NH₄Cl appeared to increase the ability of the ribosomes to form oligophenylalanine in the absence of supernatant enzymes. Washing the ribosomes in 0.5 M ammonium chloride should essentially remove the transfer factors (7, 21). Supporting their removal is the finding that the ammonium chloride-washed ribosomes were dependent on all three transfer factors for polymerization activity as indicated in Table IX. Also, the fact that no H⁺-GTP binding to these ribosomes was detectable is good evidence that the ribosomes contain little or no Factor T (that is, T₀, Tₛ, or both, is limiting) (20, 21). In addition, because oligopeptide bond formation occurred extensively in the presence of CMS which inhibits Factor G (6), it is probable that peptide bond formation does not involve this factor. Furthermore, very little GTPase activity was detected on these ribosomes (data not presented). All of these results indicate that these ammonium chloride-washed ribosomes are substantially free of transfer factors. Therefore, the peptide bond synthesis observed probably occurred on ribosomes in the absence of these factors.

Purified 50 S particles do not support amino acid polymerization (15, 23, 30). However, using a T₁ ribonuclease formylmethionyl-tRNA fragment, CAACCA-formylmethionine (31), Monro (32) has shown that 50 S subunits can catalyze the formation of formylmethionylpyromycin. The present results and those of Monro (32) and Gutierrez (23) support the conclusion that supernatant factors are not required for peptide bond synthesis. The peptidyl synthetase appears to be an integral part of the 70 S ribosome, specifically a part of the 50 S subunit.

The discussed differences between peptide bond synthesis and amino acid polymerization are summarized in Table X. They support the hypothesis that oligophenylalanine formation on ribosomes represents peptide bond synthesis, whereas amino acid polymerization involves an entire spectrum of reactions, including, of course, peptide bond synthesis.

The data of Table V establish that oligophenylalanine formation or peptide bond synthesis from ¹⁴C-Phe-tRNA required the monovalent cations potassium or ammonium. In the absence of these cations or in the presence of the cations sodium or lithium, little or no oligophenylalanine formation was detectable. Lubin and Ennis (12), using a mutant strain of E. coli unable to concentrate potassium, were able to show that potassium was required for protein synthesis in E. coli cells. Similarly, Conway and Monro (11) showed a requirement for ammonium or potassium ion for protein biosynthesis by E. coli extracts. The present results localize the requirement for K⁺ or NH₄⁺ in protein synthesis to the synthesis of the peptide bond itself. Previously, Traut and Monro (34) have shown that formation of polyphenylalanyl-puromycin with 70 or 50 S ribosomes containing polyphenylalanyl-tRNA was dependent on K⁺ or NH₄⁺, and Monro and Marcker (31) have reported that the reaction of the CAACCA-formylmethionine fragment with puromycin requires K⁺ as well as Mg²⁺, ribosomes, and ethanol.

Since ¹⁴C-Phe-tRNA was protected from hydrolysis in the
absence of peptide bond formation (Fig. 9), it appears that the aminoacyl end of 14C-Phe-tRNA interacts with the 50 S subunit, which provides this protection (2) in the absence of potassium or ammonium ions. Thus, although either NH4+ or K+ is required for peptide bond synthesis, they are not necessary for the interaction of aminoacyl-tRNA with the 50 S subunit, and since interaction of aminoacyl-tRNA with the 50 S subunit in the absence of K+ or NH4+ does not assure peptide bond formation, it is possible that the necessity of K+ or NH4+ for peptide bond synthesis is a specific requirement for the peptide synthetase residing on the 50 S subunit.

The results of experiments with the sulfhydryl inhibitor (Table VI) suggest that CMS inhibits aminoacyl-tRNA binding preferentially to formation of oligophenylalanine. Traut and Haeni (35) reported that sulfhydryl inhibitors interfere with function of the 50 S ribosomal subunit, the site of codon recognition (15, 23, 36, 37). This would account for the depression of 14C-Phe-tRNA binding to ribosomes observed in the presence of CMS. Moreover, the results indicate that the peptide synthetase is not inhibited by CMS as also found by Monro (32).

The mechanism of formation of oligophenylalanine from 14C-Phe-tRNA by ribosomes in the absence of added supernatant enzymes is relevant to the question of the number and type of enzymes is relevant to the question of the number and type of enzymes that participate in the synthesis of proteins from amino acids. The use of ultracentrifugal analysis of the reaction mixtures by sucrose density gradient centrifugation as described previously (16) indicated that most of the radioactivity was bound to a complex with a sedimentation constant of 70 S. Similarly, analytical ultracentrifugation of 40-fold-diluted reaction mixtures indicate that almost all of the material absorbing at 260 nm had a sedimentation constant of 74 S or less. Furthermore, dilution of the reaction mixtures after the 14C-Phe-tRNA was bound did not sufficiently inhibit oligophenylalanine formation (data not shown), suggesting that inter-ribosomal interactions of 14C-Phe-tRNA are unlikely. The set of ribosomes containing 1 14C-Phe-tRNA molecule would be expected to predominate over those carrying 2 at high ribosome to 14C-Phe-tRNA ratios if each 14C-Phe-tRNA molecule were bound randomly. However, since diphenylalanine synthesis (which probably requires 2 14C-Phe-tRNA molecules on 1 ribosome) occurs well at high ribosome to 14C-Phe-tRNA ratios, it is likely that binding of the second 14C-Phe-tRNA to ribosomes is more rapid than binding of the first.

The reaction of puromycin with peptidyl-tRNA or aminoacyl-tRNA with a substituent on the α-amino group can be considered an analogue of the formation of peptide bonds during protein biosynthesis. The requirements for formation of peptidyl-puromycin are analogous to the requirements for the formation of oligophenylalanine (34, 38, 40). Ribosomes and a peptidyl-tRNA (or an α-amino-substituted aminoacyl-tRNA) are required for maximal reaction with puromycin. However, neither GTP nor supernatant factors are required for the reaction of ribosome-bound peptidyl-tRNA with puromycin (34, 38, 41). After peptidyl-tRNA reacts with puromycin, peptidylpuromycin is formed, releasing the peptide from tRNA (42, 46). In the case of oligopeptide formation, the peptide is attached to tRNA (34, 38-40). Ribosomes and peptidyl-tRNA are required, releasing the peptide from tRNA (42-46). In the case of oligopeptide formation, the peptide is attached to tRNA (34, 38-40). Ribosomes and peptidyl-tRNA are required, releasing the peptide from tRNA (42-46). In the case of oligopeptide formation, the peptide is attached to tRNA (34, 38-40). Ribosomes and peptidyl-tRNA are required, releasing the peptide from tRNA (42-46). In the case of oligopeptide formation, the peptide is attached to tRNA (34, 38-40). Ribosomes and peptidyl-tRNA are required, releasing the peptide from tRNA (42-46).

Nakamoto et al. (3) observed an induction period in the poly U-directed synthesis of polyphenylalanine. This induction period was eliminated by preincubation of ribosomes, poly U, and 14C-Phe-tRNA in high NH4Cl concentration (about 0.1 M) for a short time prior to the addition of GTP and the transfer factors. The time necessary for formation of a 14C-Phe-tRNA-ribosome-poly U complex was suggested to be responsible for the induction period. Nakamoto and Kolakofsky (47) reported that under appropriate conditions diphenylalanyl-tRNA is an efficient initiator of polypeptide synthesis. Therefore, it should be considered that the rate-limiting step in polyphenylalanine synthesis may be the formation of the initial diphenylalanyl-tRNA rather than the binding of 14C-Phe-tRNA to the ribosome-template complex (cf. Fig. 3).

The present studies of peptide bond synthesis by ribosomes from aminoacyl-tRNA indicated that the capacity to form peptide bonds from aminoacyl-tRNA molecules resides in the ribosome. The reaction required K+ or NH4+, Mg++, a template, and ribosomes. Not required were GTP or any supernatant factors. The multitude of factors thus far reported probably participate in the orderly sequence of events required for the synthesis of proteins from amino acids. The use of oligopeptide-tRNA-ribosome complexes may provide a convenient method for studying the stepwise process of translocation uncoupled from extensive amino acid polymerization.

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