STUDIES ON THE FORMATION OF TRANSFER RIBONUCLEIC ACID-RIBOSOME COMPLEXES, V. ON THE FUNCTION OF A SOLUBLE TRANSFER FACTOR IN PROTEIN SYNTHESIS

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Protein biosynthesis on polyribonucleotide templates involves a highly coordinated sequence of reactions. Requirements for the over-all process include ribosomes, supernatant factors, a polyribonucleotide template, magnesium and potassium ions, aminoacyl-tRNA, and GTP.[1-8] Furthermore, in bacterial extracts, distinct factors for initiation,[9-13] polymerization,[2, 6] and termination[14-17] have been described. The synthesis of the peptide bond appears to be an integral function of the 50*S* subunit of *E. coli* ribosomes.[18-23]

In studying the role of three polymerization factors[6] (T_u, T_s, and G), several groups have suggested that T_u and T_s may serve to bind aminoacyl-tRNA to ribosomes enzymatically.[24-26] Regarding the role of factor G, Nishizuka and Lipmann[3] have correlated its ribosomal-dependent GTPase activity with its stimulation of amino acid polymerization in the presence of factors T_u and T_s. They suggested that factor G may be involved in the translocation processes which occur during protein biosynthesis. In a previous report,[22] the formation of diphenylalanyl-tRNA on ribosomes containing a polyuridylic acid template in the absence of supernatant proteins was demonstrated. Under these conditions, little triphenylalanine was formed. Based on the hypothesis that the formation of triphenylalanine and larger peptides involve translocation processes, whereas diphenylalanine formation does not, the formation of triphenylalanine and larger peptides might be considered a measure of translocation. This communication describes a specific assay for factor G in the absence of additional supernatant proteins and suggests one possible role of factor G and GTP in protein biosynthesis.

Experimental Procedure.—Ribosomes and cell extracts: Ribosomes were prepared from *E. coli* A-19 by four washes in 1 *M* NH_4Cl as previously described.[22] *E. coli* 100,000 × *g* supernatant, ^{14}C-Phe-tRNA and tRNA were prepared as previously reported.[18, 22, 27]. Transfer RNA was from *E. coli* W3100 unless otherwise specified. Purified factors G, T_u, and T_s were generously supplied by Drs. Nathan Brot, Robert Ertel, and Herbert Weissbach.[26] Protein was determined by the method of Lowry *et al.*[28]

Chemicals, radioactive isotopes, and materials: Uniformly labeled ^{14}C-L-phenylalanine (351 and 333 mc per mmole) was obtained from New England Nuclear Corp. Cellulose nitrate membrane filters were type HA 25-mm diam. Millipore filters. *E. coli* B tRNA was obtained from General Biochemicals. Benzoylated DEAE-cellulose was prepared as reported by Gillam *et al.*[29]

Determination of di- and oligophenylalanine formation: Reactions for studying di- and oligophenylalanine formation contained, in a volume of 0.050 ml 0.05 *M* Tris-acetate (pH 7.2), 0.05 *M* potassium acetate and 0.02 *M* magnesium acetate, unless otherwise indicated. The presence and quantity of ribosomes, polynucleotide, and ^{14}C-Phe-tRNA as well as incubation conditions are specified in the table and figure legends. Reactions were stopped and tRNA was deacylated by adding 0.005 ml of 3 *N* KOH and incubating for 5 min at 37°. After neutralization with HCl, 0.5 ml of 0.05 *M* potassium acetate, pH 5.7, was added and the entire mixture was applied to a 2 cm × 0.5-cm diam. benzoylated

DEAE-cellulose column equilibrated with water.[30] Each tube was washed with an additional 0.5 ml of 0.05 *M* potassium acetate, which was applied to the same column ^{14}C-Phe was then eluted with an additional 2 ml of the buffer. Next, ^{14}C-diphenylalanine was eluted with 3 ml of solvent A (formamide/ethanol/water: 32/30/38 by volume). Lastly, ^{14}C-oligophenylalanine (consisting of peptides greater or equal to triphenylalanine in chain length) was eluted with 3 ml of solvent B (0.5 *N* NaOH in ethanol). A detailed account of the procedure will be described in a separate publication.[30] The eluted fractions were counted directly in a triton-toluene scintillation fluor[30, 31] with a Packard model 4300 scintillation spectrometer.

Determination of ^{14}C-aminoacyl-tRNA binding to ribosomes and amino acid polymerization: ^{14}C-Phe-tRNA binding to ribosomes was determined by washing the ribosomes on nitrocellulose filters as previously reported.[32] The details of the conditions and assays for amino acid polymerization have been described.[22]

Results.—Dependence of PHE$_{\geq 3}$ formation on factor G and GTP: Reaction mixtures containing ribosomes and ^{14}C-Phe-tRNA in the presence of poly U were incubated for 30 minutes at 0° to bind ^{14}C-Phe-tRNA to ribosomes. At 30 minutes, factor G and GTP were added to reactions where indicated and formation of PHE$_2$ and PHE$_{\geq 3}$ followed as a function of time at 24° (Fig. 1). As can be seen (Fig. 1*B*), formation of PHE$_{\geq 3}$ was dependent on the presence of both factor G and GTP. In the absence of factor G or GTP, little PHE$_{\geq 3}$ was formed. In contrast, diphenylalanine formation (Fig. 1*A*) was not dependent on the presence of GTP or factor G. The formation of PHE$_{\geq 3}$ was linear up to at least 20 minutes under conditions of the assay. PHE$_2$ and PHE$_{\geq 3}$ formation were measured as a function of factor G concentration (Fig. 2). Although

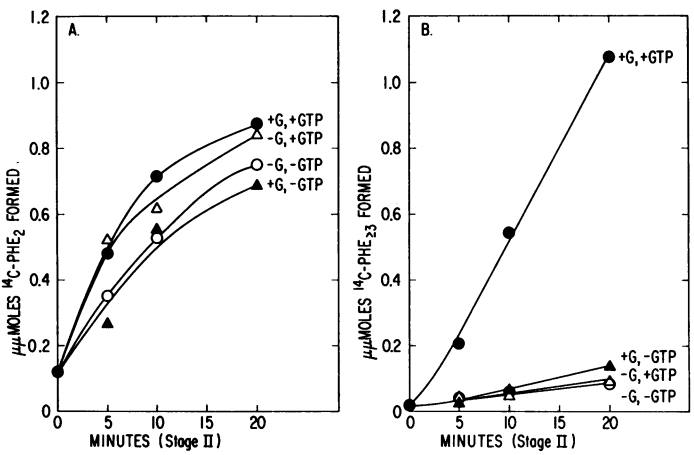

FIG. 1.—Formation of di- and oligophenylalanine as a function of time. Each 0.050-ml stage I reaction mixture contained the components indicated under *Experimental Procedure* in addition to: 0.38 A_{260} unit of ribosomes; 0.01 *M* ammonium chloride; 6.8 μμmoles of ^{14}C-Phe-tRNA (0.18 A_{260} unit); 46 mμ moles of poly U. Incubations were performed at 0° for 30 min (stage I). At the end of stage I, GTP (to a final concentration of 0.002 *M*) and 1.2 μg of factor G were added to reaction mixtures where indicated. Reactions were then performed at 24° (stage II) and assayed at the times indicated on the abscissa.

(*A*) Diphenylalanine formation.

(*B*) Oligophenylalanine formation.

●, Plus factor G, plus GTP; △, minus factor G, plus GTP; ○, minus factor G, minus GTP; ▲, plus factor G, minus GTP.

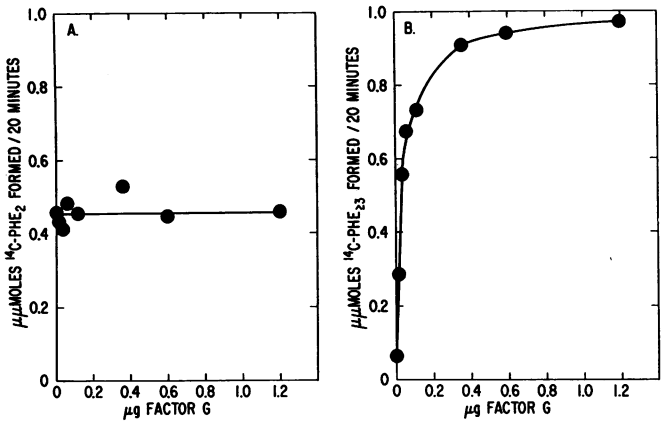

FIG. 2.—Di- and oligophenylalanine formation as a function of factor G concentration. Reaction conditions were similar to those described in the legend to Fig. 1 except for the following: 4.1 $\mu\mu$moles of ^{14}C-Phe-tRNA (0.19 A_{260} unit). Stage II incubations were performed at 24° for 20 min and GTP was present at a final concentration of 0.0018 *M*. The amount of factor G present in each stage II reaction is given on the abscissa. Factor G was diluted in a buffer consisting of 0.01 *M* magnesium acetate, 0.01 *M* Tris-acetate, pH 7.2, 1 mg/ml bovine serum albumin, and 0.001 *M* glutathione.
(*A*) Diphenylalanine formation.
(*B*) Oligophenylalanine formation.

$PHE_{\geq 3}$ formation was dependent on the addition of factor G (Fig. 2*B*), diphenylalanine formation was not (Fig. 2*A*).

Formation of PHE_2 and $PHE_{\geq 3}$ as a function of ribosome concentration: The concentration of ribosomes optimal for diphenylalanine formation was about one-third higher than the concentration optimal for $PHE_{\geq 3}$ formation (Fig. 3). The presence of excess ribosomes was inhibitory to formation of both.

Requirements for PHE_2 and $PHE_{\geq 3}$ formation: The data of Table 1 indicate effects of various additions and deletions on PHE_2 and $PHE_{\geq 3}$ formation. Maximal rate of $PHE_{\geq 3}$ formation was dependent on the presence of both factor G and GTP. Neither factor T_u nor T_s could substitute for factor G, although they slightly stimulated $PHE_{\geq 3}$ synthesis. Factor G was free of T_u and T_s activity; and factors T_u and T_s were each free of any G activity by the polymerization

TABLE 1. *Requirements for formation of di- and oligophenylalanine.*

Addition or deletion	$\mu\mu$Moles ^{14}C-PHE_2	$\mu\mu$Moles ^{14}C-$PHE_{\geq 3}$
Complete	1.0	1.14
−G	0.91	0.06
−G, + T_u	1.1	0.22
−G, + T_s	0.90	0.18
−G, + T_u + T_s	1.1	0.30
−Poly U	0.09	0.05
−Ribosomes	0.06	0.03
−GTP	0.83	0.15

Reaction mixtures were similar to those described in the legend to Fig. 1, with the following additions where designated in the table: 2.2 μg T_u, 5.6 μg T_s; stage II incubations were at 24° for 20 min. Di- and oligophenylalanine were determined as described under *Experimental Procedure*.

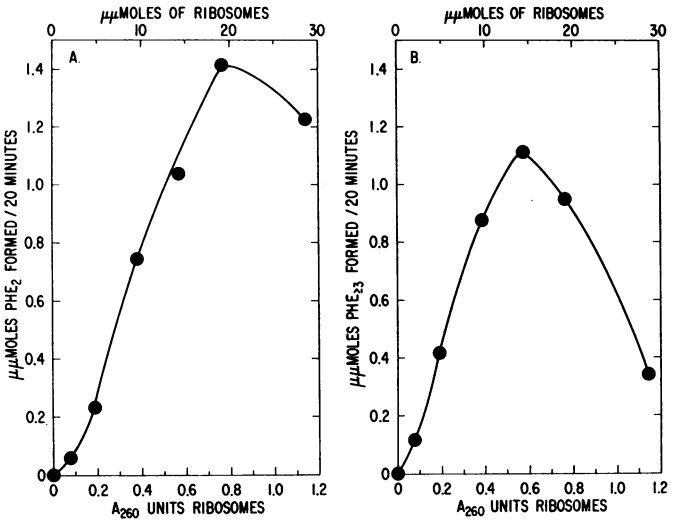

FIG. 3.—Di- and oligophenylalanine formation as a function of ribosome concentration. Reaction conditions were similar to those described in the legend to Fig. 1 except that ribosome concentration was varied and stage II reactions performed for 20 min.
(*A*) Diphenylalanine formation.
(*B*) Oligophenylalanine formation.

assay.[22, 26] GTP is apparently required as a substrate for the over-all reaction forming $PHE_{\geq 3}$ (Figs. 4*A* and *B*). The apparent K_m was found to be 3.7×10^{-6} *M* under the conditions given in the legend to Figure 4. In addition, the GTP analog guanyl-5′-methylene diphosphonate is clearly a competitive inhibitor of GTP for the formation of $PHE_{\geq 3}$. The apparent K_i calculated from the curves of Figure 4*B* is 2.5×10^{-6} *M*.

The nucleotide requirements for the formation of PHE_2 and $PHE_{\geq 3}$ are shown in Table 2. Deoxyriboguanosine triphosphate is almost as effective as GTP in supporting $PHE_{\geq 3}$ synthesis. However, neither GDP, GMP, CTP, UTP, ATP, nor GDPCP can substitute for GTP.* Diphenylalanine formation appeared to be independent of the presence of the protein factors, GTP, or any of the other nucleotides. The analog GDPCP slightly inhibited diphenylalanine formation as previously reported.[22] The formation of both PHE_2 and $PHE_{\geq 3}$ was dependent on the presence of ribosomes and poly U.

Di- and oligophenylalanine formation was examined using UpUpU, $(Up)_6G$, and $(Up)_9G$ templates. Diphenylalanine synthesis occurred with the latter two templates, but not with UpUpU. A small amount of $PHE_{\geq 3}$ was detected with the $(Up)_9G$ preparation, but not with the smaller templates.

Effect of antibiotics on PHE_2 and $PHE_{\geq 3}$ formation: The effect of chloramphenicol, fusidic acid, vernamycin A, and sparsomycin on PHE_2 and $PHE_{\geq 3}$ synthesis was studied (Table 3). Fusidic acid, sparsomycin, and vernamycin A inhibit $PHE_{\geq 3}$ formation substantially. Vernamycin A and sparsomycin appeared to inhibit both PHE_2 and $PHE_{\geq 3}$ formation; in contrast, fusidic acid inhibited $PHE_{\geq 3}$ formation preferentially. Chloramphenicol slightly inhibited $PHE_{\geq 3}$ formation and stimulated diphenylalanine synthesis reproducibly.

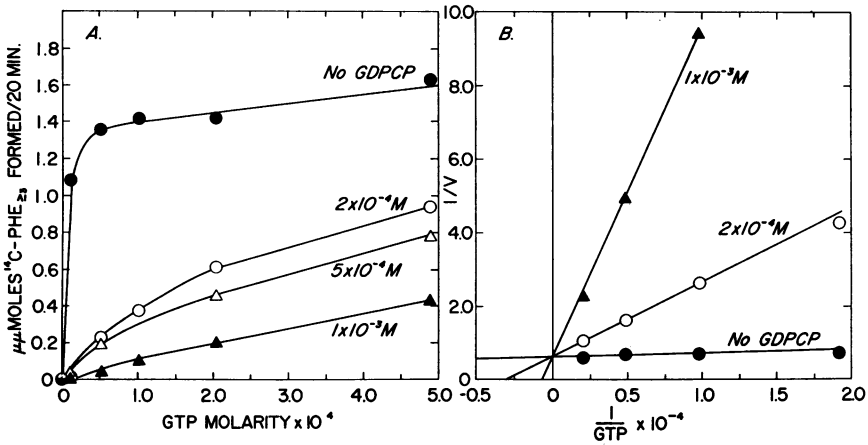

FIG. 4.—Competitive inhibition of oligophenylalanine formation by guanyl-5′-methylene diphosphonate. Each 0.050-ml reaction mixture contained 0.02 *M* magnesium acetate, 0.05 *M* potassium acetate, 0.05 *M* Tris-acetate (pH 7.2); 0.38 A_{260} unit of ribosomes; 25 mμmoles of base residues of poly U; 6.0 μμmoles of ^{14}C-Phe-tRNA (0.25 A_{260} unit); 0.6 μg of factor G; and GTP and guanyl-5′-methylene diphosphonate (GDPCP) as given on the figure. Reactions were performed in one stage only at 24° for 20 min.

(*A*) Rate of oligophenylalanine formation (μμmoles of ^{14}C-phenylalanine incorporated into peptides of chain length three and greater per 20 min) as a function of GTP concentration for several concentrations of GDPCP.

(*B*) Reciprocal plot of velocity as a function of the reciprocal of the GTP concentration.

●, No GDPCP; ○, 2 × 10^{-4} *M* GDPCP; △, 5 × 10^{-4} *M* GDPCP; ▲, 1 × 10^{-3} *M* GDPCP.

Discussion.—The results demonstrate that $PHE_{\geq 3}$ formation is dependent on the presence of both factor G and GTP. Of the nucleotides tested, only dGTP could substitute for GTP. Neither factors T_u nor T_s could substitute for factor G. In contrast, diphenylalanine formation was essentially independent of factor G or GTP; it was dependent on the presence of ribosomes and poly U only.[22] Thus, $PHE_{\geq 3}$ formation appears to differ significantly from dipeptide synthesis. Also, whereas diphenylalanine can be synthesized with oligo U templates containing six or more uridylic acid residues, the tripeptide requires at least a nona-

TABLE 2. *The effect of various nucleotides on di- and oligophenylalanine formation.*

Nucleotide	μμMole PHE_2	μμMole $PHE_{\geq 3}$
None	0.51	0.12
GTP	0.35	1.38
GDP	0.48	0.39
GMP	0.52	0.16
dGTP	0.50	1.27
CTP	0.50	0.13
UTP	0.50	0.14
ATP	0.51	0.21
GDPCP	0.38	0.05

Reactions and incubations were similar to those described in the legend to Fig. 4, with the following changes: GTP, GDP, GMP, CTP, UTP, ATP, dGTP, and GDPCP (guanyl-5′-methylene diphosphonate) concentrations were each 5 × 10^{-5} *M* where indicated. It should be noted that dGTP oxidized with the use of sodium metaperiodate gave results identical to those obtained when nonoxidized dGTP was used.

TABLE 3. *Effect of antibiotics on di- and oligophenylalanine formation.*

Antibiotic	^{14}C-PHE$_2$		C-PHE$_{\geq 3}$	
	μμMoles	Per cent	μμMoles	Per cent
None	0.65	100	1.39	100
Fusidic acid	0.73	112	0.13	9
Chloramphenicol	1.02	157	1.08	78
Sparsomycin	0.47	72	0.41	29
Vernamycin A	0.25	38	0.08	6

Reaction mixtures were similar to those described in the legend to Fig. 2, with the following additions: after stage I incubations, fusidic acid (5.7×10^{-4} *M*), chloramphenicol (5.7×10^{-4} *M*), sparsomycin (9.1×10^{-5} *M*), or vernamycin A (0.57 μg/ml) were added and the reactions incubated an additional 5 min at 0°. GTP and factor G (0.6 μg) were then added to each reaction, and the incubation performed at 24° for 20 min before assaying for PHE$_2$ and PHE$_{\geq 3}$. The column headed "Per cent" refers to the per cent of the value in the absence of any antibiotic. Fusidic acid, sodium salt, was donated by Leo Pharmaceutical Products, Copenhagen, and by the Squibb Institute for Medical Research, New Brunswick, N.J.; vernamycin A by Squibb; chloramphenicol by Parke Davis and Co., Detroit, Mich.; and sparsomycin by The Upjohn Co., Kalamazoo, Mich.

nucleotide.[33] Additionally, the formation of PHE$_{\geq 3}$, therefore, provides an assay for factor G activity independent of the presence of other supernatant factors.

The K_m for GTP in PHE$_{\geq 3}$ formation was calculated to be 3.7×10^{-6} *M* (Fig. 4). This compares to a K_m of 2×10^{-6} *M* for phenylalanine polymerization estimated by Nishizuka and Lipmann.[3] Guanyl-5′-methylene diphosphonate (GDPCP) was shown to be a competitive inhibitor of GTP for PHE$_{\geq 3}$ formation (Fig. 4), as suggested by Hershey and Monro[34] for protein synthesis. The K_i for GDPCP is 2.5×10^{-6} *M*. The effective competition of GDPCP with GTP in PHE$_{\geq 3}$ synthesis is in accord with the suggestion that GTP hydrolyzes to GDP during the formation of peptides greater than the dipeptide.

The above findings indicate that PHE$_{\geq 3}$ formation as measured represents processes which are dependent on GTP, factor G, and template size (nonanucleotide or greater). These observations are consistent with the view that these processes resulting in PHE$_{\geq 3}$ synthesis involve movements of the tRNA-ribosome-template complexes (translocation). Specifically, the translocation steps might include movement of the ribosome along the polynucleotide template, rejection of deacylated tRNA from the ribosome, and movement of peptidyl-tRNA from one location to another reactive site. Previous results of Seeds and Conway[35] suggested that GTP and a soluble factor may function to dislocate deacylated tRNA from the polynucleotide-ribosome complex. Recently, several other groups have implicated factor G and GTP in translocation.[36–38]

The data of Table 3 indicate that vernamycin A and sparsomycin inhibit peptide bond formation, since they inhibit both PHE$_2$ and PHE$_{\geq 3}$ synthesis. It has been reported that sparsomycin inhibits peptide bond synthesis[39] and vernamycin A, the polymerization reaction.[40] In contrast, chloramphenicol appears to stimulate diphenylalanine synthesis and inhibit PHE$_{\geq 3}$ formation. The total number of peptide bonds is the same in the presence or absence of chloramphenicol. This appears to be inconsistent with the suggestion that chloramphenicol only inhibits the peptide synthetase,[41] but seems to agree with the results of Bresler *et al.*[42] and Julian,[43] which suggest that chloramphenicol may affect processes other than peptide bond synthesis.

Fusidic acid appears to inhibit the formation of $PHE_{\geq 3}$ specifically rather than PHE_2 formation. It does not inhibit ^{14}C-PHE-tRNA binding to ribosomes in the presence of poly U.[33, 44] Thus, this antibiotic may specifically affect translocation as suggested by Tanaka *et al.*[36] and provide a probe into its mechanism.

Summary.—An assay is described for oligophenylalanine synthesis, which requires only one supernatant factor (G) and GTP (or dGTP), as well as ribosomes and a polyuridylic acid template. Guanyl-5′-methylene diphosphonate competitively inhibits GTP. The antibiotic fusidic acid specifically inhibits the synthesis of triphenylalanine and larger peptides without affecting dipeptide bond synthesis. The results suggest that factor G and GTP are necessary for the translocation steps and that fusidic acid specifically inhibits this process.

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The following abbreviations are used: Poly U and oligo U, poly- or oligouridylic acid; GDPCP, guanyl-5′-methylene diphosphonate; ^{14}C-Phe-tRNA, transfer ribonucleic acid acylated with ^{14}C-L-phenylalanine and 19 unlabeled amino acids; PHE_2, diphenylalanine; $PHE_{\geq 3}$, peptides of phenylalanine of chain length three and greater; $PHE_{\geq 3}$ is considered oligophenylalanine in the text. The $\mu\mu$moles of PHE_2 and $PHE_{\geq 3}$ refer to the $\mu\mu$moles of ^{14}C-Phe incorporated into those species. tRNA, transfer RNA; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-phosphate; UTP, uridine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, *O*-(diethylaminoethyl) cellulose.

* *Note added in proof:* Inosine 5′-triphosphate was about half as active as GTP in stimulating $PHE_{\geq 3}$ formation.[33]

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