*ON THREE COMPLEMENTARY AMINO ACID POLYMERIZATION FACTORS FROM BACILLUS STEAROTHERMOPHILUS: SEPARATION OF A COMPLEX CONTAINING TWO OF THE FACTORS, GUANOSINE-5'-TRIPHOSPHATE AND AMINOACYL-TRANSFER RNA**

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Communicated by Norman H. Giles, April 1, 1968

The addition of an aminoacyl residue to the peptide chain is a multistep process. This process can be studied in amino acid-incorporating systems *in vitro*, in which poly U directs the formation of polyphenylalanine.[1] Such systems have been prepared from microbial and mammalian cell extracts. In addition to poly U, they include phe-tRNA, ribosomes, GTP, salts, and a high-speed supernatant fraction containing amino acid polymerization factors. Two such complementary factors were found in microbial and mammalian extracts.[2-5] The first of the two factors isolated from *E. coli*, designated G, is stable, and splits GTP into GDP and orthophosphate in the presence of ribosomes.[6] The second factor, designated T, purified from *Pseudomonas fluorescens*, was further separated into two components, Tu and Ts.[7] The splitting of T into Tu and Ts and the determination of their individual functions are made difficult by the fact that Tu becomes very unstable upon separation from Ts.[7]

Recent studies on peptide chain elongation have focused upon those steps in the process which might take place prior to peptide bond formation. Using a Millipore filtration technique, it was established that T factor from *E. coli* binds GTP.[8] Since heat inactivation of Tu activity (but not of Ts activity) in T factor also destroyed GTP binding, it was concluded that Tu is involved in this step.[8] Using a gel filtration technique, the binding of GTP to T factor depended upon aminoacyl-tRNA.[9] The results also suggested that aminoacyl-tRNA is bound to the T factor-GTP complex[10] (see also ref. 11). A complex of aminoacyl-tRNA with an amino acid polymerization factor purified from rat liver was isolated by gel filtration; its formation, however, did not seem to depend on GTP.[12] T factor was found to be also involved in the GTP-dependent binding of aminoacyl-tRNA to the ribosome-messenger complex[13, 14] (see also refs. 3, 15).

In an attempt to obtain stable amino acid polymerization factors that might enable us to study their functions, we selected *Bacillus stearothermophilus*, a thermophilic organism, as their source. Earlier, a highly active and stable amino acid-incorporating system was prepared from this organism.[16] We have now resolved the high-speed supernatant fraction of the cell extract into three complementary fractions designated as factors S_1, S_2, and S_3. All three factors are required for the synthesis of polyphenylalanine, as directed by poly U in a system also containing washed *E. coli* ribosomes and *E. coli* phe-tRNA. The separated and partially purified factors can be stored frozen for at least six months without a substantial loss of activity. Using the gel filtration technique, we have obtained direct evidence for the involvement of both S_1 and S_3 in a com-

plex including GTP and aminoacyl-tRNA. We have also found that both S_1 and S_3 are required for obtaining the maximal rate in the GTP-dependent binding of aminoacyl-tRNA to the messenger-ribosome complex. Some of these results were communicated earlier.[17]

Materials.—C^{14}-L-phenylalanine (SA 360 μc/μmole) was obtained from New England Nuclear Corp., Boston, Mass.; H^3-GTP (SA 1120 μc/μmole), H^3-GDP (SA 1270 μc/μmole), H^3-ATP (SA 3780 μc/μmole), and reconstituted H^3-protein-hydrolysate from Schwarz BioResearch, Inc., Orangeburg, N.Y.; *E. coli* B tRNA, sodium salt, and *E. coli* B cells from General Biochemicals, Chagrin Falls, Ohio.

Methods.—Preparation of three amino acid polymerization factors: The details of the partial purification and properties of the three factors will be described elsewhere. The outline of the procedure was as follows: The high-speed supernatant fraction of the cell extract of *Bacillus stearothermophilus* was fractionated with $(NH_4)SO_4$. The material precipitating between 45 and 60% saturation was further fractionated with acetone. The precipitate formed upon the addition of 650 ml of acetone per liter of $(NH_4)_2SO_4$ fraction was discarded and an additional 350 ml of acetone was added to the supernatant fraction. The resulting precipitate was resuspended and adsorbed to calcium phosphate gel. The active fraction was eluted with 30% saturated $(NH_4)_2SO_4$ (pH 8.5). This fraction was applied to DEAE-Sephadex (pH 6.4) and the three factors were separated by elution with a linear KCl concentration gradient between 0.15 and 0.55 *M*. S_1 and S_2 were further purified on DEAE-Sephadex (pH 7.4). S_3 was further purified on hydroxylapatite with a linear concentration gradient of potassium phosphate, between 0.01 and 0.15 *M*. S_1 and S_2 were purified 20-fold, S_3 100-fold. Each can be stored at -50°C for at least 6 months without substantial loss of activity. In all experiments these most highly purified factors were used. Protein was determined according to the method of Lowry *et al.*[18]

Preparation of ribosomes: Ribosomes from *E. coli* B cells harvested in early log phase were isolated according to methods given in reference 6.

Preparation of C^{14}-phe-tRNA: *E. coli* B tRNA was charged with C^{14}-phenylalanine (SA 120 μc/μmole).

E. coli K12 tRNA, enriched 16-fold for phenylalanine acceptor activity, was charged with C^{14}-phenylalanine (SA 360 μc/μmole).

Preparation of H^3-aminoacyl-C^{14}-tRNA: *E. coli* K12 AB 1861, a uracil-requiring strain, was grown on a medium including 46 μmoles/ml of C^{14}-uracil (SA 1.3 μc/μmole). C^{14}-tRNA (2×10^5 dpm/A_{260} unit) was prepared by phenol extraction of the high-speed supernatant fraction of the cell extract. After discharging in 0.2 *M* tris-HCl (pH 9.0) at 37°C for 90 min, the C^{14}-tRNA was charged with a reconstituted H^3-protein-hydrolysate. After charging, the preparation contained 1.8×10^5 dpm C^{14} and 8×10^5 dpm H^3/A_{260} unit.

All aminoacyl-tRNA was passed through Sephadex G-25 in order to remove low-molecular-weight materials.

Assay of polyphenylalanine formation: One ml of the reaction mixture contained the following components in μmoles (unless otherwise indicated): tris-HCl (pH 7.4), 40; NH_4Cl, 160; DTT, 10; Mg acetate, 10; GTP, 2; poly U, 40 μg; washed *E. coli* B ribosomes, 23 A_{260} units; 24.8 A_{260} units of *E. coli* B C^{14}-phe-tRNA (12,000 cpm) carrying 664 $\mu\mu$moles of phenylalanyl residues; S_1, S_2, and S_3 as indicated. The total volume of the reaction mixture was 0.125 ml. All components except GTP were mixed at 0°C and the reaction was started by adding GTP. The incubation period was 10 min at 30°C. The reaction was terminated with 5% TCA solution, and the hot TCA-insoluble material was filtered on Millipore filters and counted.

Assay for complex formation among S_1, S_3, GTP, and aminoacyl-tRNA: One ml of the reaction mixture contained the following components in μmoles (unless otherwise indicated): tris-HCl (pH 7.4), 40; NH_4Cl, 160; DTT, 10; Mg acetate, 10; H^3-GTP, 0.005 (SA 1120 μc/μmole); 0.39 A_{260} units of C^{14}-phe-tRNA (50,000 cpm, enriched 16-fold for phenylalanine acceptor activity), carrying 130 $\mu\mu$moles of phenylalanyl residues; S_1 and

S_3 as indicated. The total volume of the reaction mixture was 0.2 ml. All components except GTP were mixed at 0°C, and the reaction was started with GTP. The incubation period was 5 min at 30°C. The reaction was terminated by cooling at 0°C and the reaction mixture immediately applied to a 0.64 × 37-cm Sephadex G-100 column (total vol, 10 ml; void vol, 4 ml), which had been equilibrated with a solution of which 1 ml contained the following components in μmoles: tris-HCl (pH 7.4), 40; NH_4Cl, 160; Mg acetate, 10; DTT, 1. The column was eluted with the same solution at a flow rate of 6 ml/hr. Several identical columns were used simultaneously. The volume of the fractions varied between 0.40 and 0.45 ml. Aliquots of 0.3 ml from each fraction were counted for radioactivity in 10 ml of Bray's solution[19] in a scintillation counter. All operations with the column were performed at 4°C.

Assay of C^{14}-phe-tRNA binding to ribosomes: One ml of the reaction mixture contained the following components in μmoles (unless otherwise indicated): tris-HCl, (pH 7.4), 40; NH_4Cl, 160; DTT, 10; Mg acetate, 7.5; GTP, 0.002; poly U, 40 μg; 5.76 A_{260} units of washed ribosomes; 20.8 A_{260} units of C^{14}-phe-tRNA (16,000 cpm) carrying 632 μμmoles of phenylalanyl residues; S_1 and S_3 as indicated. The total volume of the reaction mixture was 0.125 ml. All components except ribosomes and poly U were incubated for 5 min at 30°C. At this time, ribosomes and poly U were added and the incubation was continued at 30°C for varying lengths of time as indicated. The reaction was terminated by adding 1.25 ml of an ice-cold solution, 1 ml of which contained 40 μmoles of tris-HCl (pH 7.4), 160 μmoles of NH_4Cl, and 7.5 μmoles of Mg acetate. Immediately thereafter, the reaction mixture was filtered through Millipore filters as described by Nirenberg and Leder.[20] The filters were counted in a gas-flow planchet counter.

Results.—The strict dependence of polyphenylalanine formation upon factors S_1, S_2, and S_3 is demonstrated in Figure 1. In each of the three experiments shown, various amounts of one single factor were added to a phenylalanine-polymerizing system containing a large amount of the other two complementary factors. That S_3 corresponds to the unstable Tu factor from *E. coli* is suggested by the following results. A partially purified fraction from *E. coli* (having Tu, Ts, and G activity) was heated under conditions required to destroy the activity of Tu, but not of Ts or G. This heated fraction could replace S_1 and S_2, but not S_3 in polyphenylalanine synthesis. That S_2 corresponds to factor G (from *E. coli*),

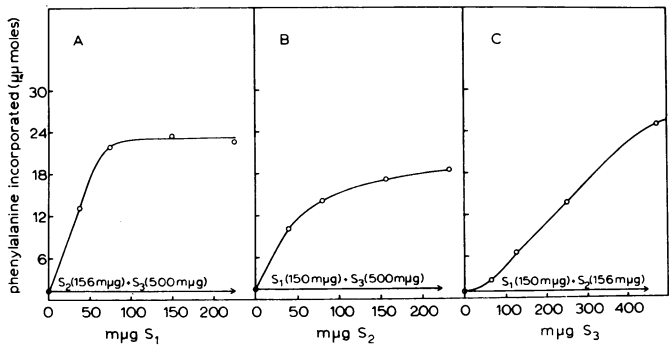

FIG. 1.—Requirement for three complementary factors in polyphenylalanine formation. Effects of increasing amounts of S_1 (*A*), S_2 (*B*), and S_3 (*C*) on polyphenylalanine formation in the presence of constant amounts of the other two complementary factors as indicated. Conditions are described in *Methods* under "Assay of polyphenylalanine formation." In the absence of S_1, S_2, and S_3, 0.55 μμmole of phenylalanine was incorporated into hot acid-insoluble product. This amount was subtracted from all experimental values.

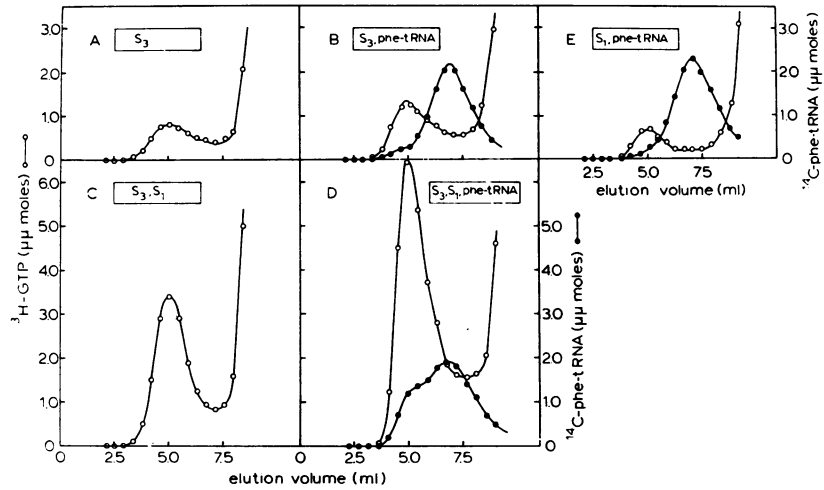

FIG. 2.—Examination of the interactions of S_1, S_3, GTP, and phe-tRNA by gel filtration. Interaction of: (*A*) GTP and S_3; (*B*) GTP, S_3, and phe-tRNA, (*C*) GTP, S_3, and S_1, (*D*) GTP, S_3, S_1, and phe-tRNA; (*E*) GTP, S_1, and phe-tRNA. The amounts of GTP and phe-tRNA indicated are those present in a total fraction. The conditions of the incubation and gel filtration are described in *Methods* under "Assay for complex formation among S_1, S_3, GTP, and aminoacyl-tRNA." When indicated, 3.4 μg of S_1 and 2.7 μg of S_3 were used.

which has ribosome-dependent GTPase activity, seems to be indicated by the following observation: In chromatography on DEAE-Sephadex, S_2 activity for polyphenylalanine formation coincided with a ribosome-dependent GTPase activity.

That both S_1 and S_3 can be components of a complex, also including GTP and phe-tRNA, was established by gel filtration on Sephadex G-100. The evidence for this conclusion is demonstrated in Figures 2 and 3. The elution volumes of S_1 and S_3 are at 4.5 and 5.0 ml, respectively (Fig. 3); in the absence of S_1 and S_3, the elution volume of GTP is at 11 ml (not shown), that of phe-tRNA at 7.0 ml (e.g., Fig. 2*B*). If S_1, S_3, GTP, and phe-tRNA are incubated together before the gel filtration, each of these four components seems to be eluted with approxi-

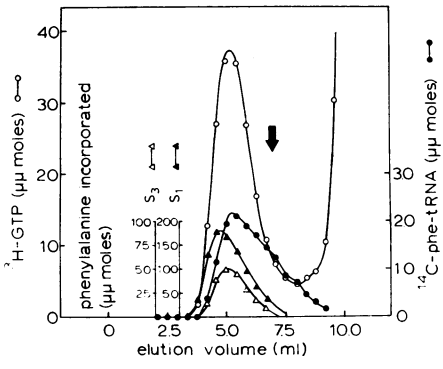

FIG. 3.—Determination of the elution volumes of S_1, S_3, GTP, and phe-tRNA—components of a complex—in the gel filtrate. The conditions of the incubation and gel filtration were the same as in Fig. 2, except that 10.1 μg of S_1 and 16.2 μg of S_3 were used in this experiment. S_1 activity (▲—▲) and S_3 activity (△—△) (expressed in μμmoles of phenylalanine incorporated) were determined by assaying for polyphenylalanine formation (see *Methods*) in aliquots of the fractions added to reaction mixtures, including an excess of the appropriate complementary factors. The arrow indicates the position of unbound phe-tRNA.

mately the same elution volume, suggesting the formation of a complex (see Fig. 3).[21] That the amount of phe-tRNA bound in the complex depends on that of S_1 and S_3 can be seen by comparing Figures 3 and 2*D*: there are three times more S_1 and S_3 in the experiment shown in Figure 3 than in that shown in Figure 2*D*; whereas most of the phe-tRNA is bound as shown in Figure 3, less than half of it is bound as illustrated in Figure 2*D*. That S_3 alone binds much less GTP and (even in the presence of GTP) very much less phe-tRNA than S_1 and S_3 together—under otherwise identical conditions—can be noticed by comparing Figures 2*A* and *B* with *D*. The amount of S_1 used in the experiment shown in Figure 2*E* is in sixfold excess compared to that of S_3 (see other parts of Fig. 2) in terms of activity in phenylalanine polymerization. In view of this, it can be concluded (from Fig. 2*E*) that an equivalent amount of S_1 binds much less GTP and phe-tRNA (if any) than S_3. Furthermore, since the S_1 used might be slightly contaminated with S_3, it is also possible that the binding of the small amount of GTP is a consequence of this contamination. It can also be seen that the amount of GTP bound in the complex is much larger in the presence of phe-tRNA than in its absence (compare Fig. 2*C* with *D*). All these observations then are consistent with the view that S_1, S_3, GTP, and phe-tRNA can all be parts of the same complex.

Binding of phe-tRNA in the complex strictly depends on GTP (unpublished data). GDP or ATP (equimolar with GTP) do not substitute for GTP in promoting the binding of phe-tRNA. Whereas ATP itself is not bound in the complex, GDP is (see also refs. 8, 9). As in the case of GTP, the amount of GDP bound in a complex is several times higher in the presence of both S_1 and S_3 than in that of S_3 alone or S_1 alone. Whereas the amount of GTP bound in the complex is increased by phe-tRNA, that of GDP is not (unpublished data).

The curves in Figure 4 indicate that aminoacyl-tRNA is bound in the complex, whereas uncharged tRNA is not (cf. with similar conclusions of Gordon[10]). Thus, C^{14}-tRNA (previously discharged) is eluted from Sephadex G-100 in two fractions, none of which is bound in the complex (Fig. 4*A*). The lack of interaction between S_1, S_3, and tRNA is also shown by the fact that the elution profile of tRNA remains essentially unchanged when the amount of S_1 and S_3 used is increased threefold, and consequently the amount of GTP in the complex also increases about threefold (compare *I* and *II* in Fig. 4*A*). For the experiments shown in Figures 4*B* and *C*, the C^{14}-tRNA was charged with a mixture of H^3-amino acids. Part of this aminoacyl-tRNA did bind in the complex (Fig. 4*B*). That the peak of H^3-aminoacyl residues, which coincides with the position of the complex, is not an artifact of charging is shown in Figure 4*C*. The lack of a peak in the C^{14} curve (reflecting tRNA shown in Fig. 4*B*) coinciding with the peak in the H^3 curve (reflecting aminoacyl residues) might be explained by the low efficiency (less than 20%) of charging the tRNA with amino acids. That tRNA does not interact with the complex is further documented by experimental results indicating that adding a threefold excess of tRNA (over aminoacyl-tRNA) to the reaction mixture has no effect upon the amount of aminoacyl-tRNA bound in the complex.

The curves in Figures 5*A* and *B* demonstrate that the effects of S_1 and S_3 in

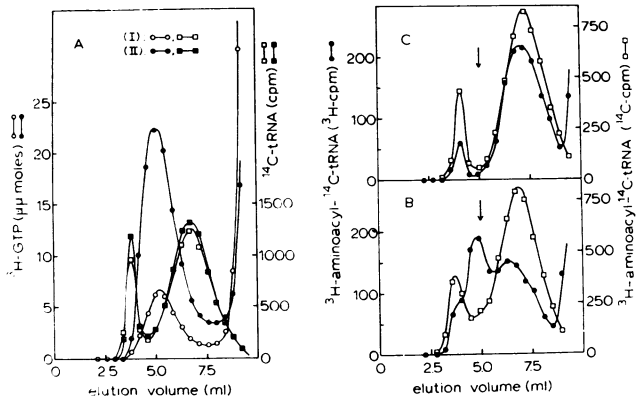

FIG. 4.—Gel filtration patterns indicating that tRNA (uncharged) is not bound to the S_1, S_3, GTP complex, whereas aminoacyl-tRNA is.

(*A*) Gel filtration of S_1, S_3, GTP, and C^{14}-tRNA; lack of binding of C^{14}-tRNA to the S_1, S_3, GTP complex. The components of the reaction mixture include the following: (*I*): 1 mμmole of H^3-GTP, 0.088 A_{260} units of C^{14}-tRNA (9,140 cpm), 3.36 μg S_1, 5.4 μg S_3. The points on the curves indicate μμmoles of GTP (○—○) and cpm of tRNA (□—□) per fraction. (*II*): The composition of the reaction mixture is the same as in (*I*), with the exception that the amounts of S_1 and S_3 are increased threefold. The points of the curve indicate μμmoles of GTP (⊕—⊕) and cpm of tRNA (⊞—⊞) per fraction.

(*B*) Gel filtration of S_1, S_3, GTP, and H^3-aminoacyl-C^{14}-tRNA; binding of H^3-aminoacyl-C^{14}-tRNA to the S_1, S_3, GTP complex. The components of the reaction mixture include the following: 1 mμmole of GTP (unlabeled), 3.36 μg S_1, 5.4 μg S_3, 0.065 A_{260} units of H^3-aminoacyl-C^{14}-tRNA (H^3, 5,900 cpm; C^{14}, 6,350 cpm). The points on the curves in (*B*) and (*C*) indicate cpm in aminoacyl residues (●—●) and in tRNA (□—□) (to which the aminoacyl residues are esterified) per fraction. Arrows in (*B*) and (*C*) indicate the position of GTP bound in the S_1, S_3, GTP complex.

(*C*) Gel filtration of H^3-aminoacyl-C^{14}-tRNA. The amount of H^3-aminoacyl-C^{14}-tRNA used is the same as in (*B*). For conditions, see the legend to Figure 2.

increasing the rate and the amount of phe-tRNA bound to the ribosome in the presence of poly U and GTP are much more than additive. This suggests that both factors might function in this step. These results are consistent with the view that the S_1, S_3, GTP, phe-tRNA complex might be an intermediate in the binding of phe-tRNA to ribosomes. It is of some interest that in the S_1, S_3-catalyzed binding of phe-tRNA to the ribosome, GDP does not substitute for GTP; furthermore, in the presence of GTP, GDP inhibits the binding. The majority of the product bound to ribosomes was identified as diphenylalanyl-tRNA and phe-tRNA; no TCA-insoluble product was found.

Discussion.—The availability of three stable, partially purified amino acid polymerization factors from *Bacillus stearothermophilus* has enabled us to start studies on their individual functions. Using the technique of gel filtration on Sephadex G-100, we separated a complex consisting of factors S_1, S_3, GTP, and phe-tRNA from unbound GTP and phe-tRNA. We believe that the binding of GTP in the complex precedes that of phe-tRNA for the following reasons: Though phe-tRNA increases the amount of GTP bound in a complex, nevertheless GTP is bound in a complex (with S_1 and S_3) in the absence of phe-tRNA.

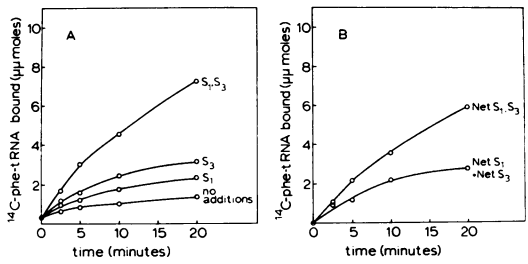

FIG. 5.—Effect of S_1 and S_3 on the kinetics of phe-tRNA binding to ribosomes. Conditions are described in *Methods*.

(*A*) Kinetics of binding of phe-tRNA in reaction mixtures not containing S_1 and S_3 (*no additions*) and in reaction mixtures supplemented with either 1.34 μg S_1 (S_1), or 0.5 μg S_3 (S_3), or 1.34 μg S_1 and 0.5 μg S_3 (S_1, S_3).

(*B*) The values on the two curves were calculated from values in (*A*) in the following way: *Net* S_1 + *Net* S_3 − μμmoles of phe-tRNA bound in the absence of factors (*no additions*) were subtracted from those bound in the presence of S_1 (S_1), as well as from those bound in the presence of S_3 (S_3). The resulting two numbers were added. *Net* S_1, S_3 − μμmoles of phe-tRNA bound in the absence of factors (*no additions*) were subtracted from those bound in the presence of both factors (S_1, S_3).

However, the binding of phe-tRNA in the complex is strictly dependent upon GTP (see also ref. 10). It is of some interest that, although binding of GTP occurs at 0°C, binding of phe-tRNA requires incubation at 30°C. The ratio of GTP to phe-tRNA in the complex under our conditions was about 6:1. However, this might be a consequence of not using a saturating concentration of phe-tRNA.[22] The individual roles of S_1 and S_3 in the complex remain to be determined. S_3 alone binds some GTP and phe-tRNA; it is possible that S_1 alone does not. Preliminary results suggest that GTP is not cleaved in the complex. S_2 alone does not seem to bind either GTP or phe-tRNA in our experiments. The possible contamination of our factors with initiation factors remains to be tested (for a review see ref. 23).

Summary.—Three stable, complementary amino acid polymerization factors (S_1, S_2, and S_3) have been isolated from *Bacillus stearothermophilus*, a thermophilic organism. S_1 and S_2 were purified 20-fold and S_3 100-fold. S_1 and S_3 form a complex with GTP and phe-tRNA. The complex was separated from unbound GTP and phe-tRNA by gel filtration. Binding of phe-tRNA in the complex depends on GTP. Uncharged tRNA is not bound. Both S_1 and S_3 seem to be involved in promoting the GTP-dependent binding of phe-tRNA to the messenger-ribosome complex.

Note added in proof: After this paper had been submitted for publication, Ertel *et al.* (these PROCEEDINGS, **59**, 861 (1968)) reported finding that two protein fractions from *E. coli* are involved in binding GTP. The same fractions also stimulate the binding of phenylalanyl-tRNA to ribosomes in the presence of GTP and poly-U.

We thank Dr. D. Söll (from this department) for 16-fold-purified phenylalanine-specific tRNA from *E. coli* K12, and Dr. E. Adelberg (Yale University School of Medicine) for *E. coli* K12 (AB 1861), a uracil-requiring strain.

Abbreviations: ATP, adenosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; GTP, guanosine-5′-triphosphate; poly U, polyuridylic acid; DTT, dithiothreitol; TCA, trichloroacetic acid; tRNA, transfer RNA; DEAE, *O*-(diethylaminoethyl); tris-HCl, tris(hydroxymethyl)aminomethane-HCl.

* This study was supported by grant no. GM 13707 from the U.S. Public Health Service.

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[21] The slight difference among the elution volumes of bound GTP, bound phe-tRNA, and S_3 might be due to experimental error. The elution volume of S_1, however, seems to be smaller than those of the other three components of the same complex. The cause of this is not clear. Since S_1 was in a threefold excess above S_3 in this experiment, it is possible that S_1 alone forms aggregates. As a matter of fact, the elution volume of S_1 alone was found to be the same as that of S_1 in this figure.

[22] The amount of phe-tRNA we could use was limited; the resolution was not sufficient to permit the determination of a small amount of bound phe-tRNA in the presence of a large excess of free phe-tRNA.

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