*FACTOR- AND GUANOSINE 5′-TRIPHOSPHATE-DEPENDENT RELEASE OF DEACYLATED TRANSFER RNA FROM 70S RIBOSOMES**

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Since the discovery of a guanosine 5′-triphosphate (GTP) requirement for protein synthesis,[1] its role in this process has been studied in various laboratories. Despite much information available on the possible involvement of GTP in various steps of protein biosynthesis,[2–7] its exact mechanism of action has remained obscure. As a continuation of our series of studies on the specific binding of transfer RNA (tRNA) to ribosomes,[8–14] we focused our attention on the mechanism by which deacylated tRNA is released from ribosomes. It was found that release of deacylated $tRNA_{phe}$ (transfer RNA specific for phenylalanine) was dependent on the addition of GTP and soluble factor from *Escherichia coli*.

Materials and Methods.—E. coli extract and other materials: In this communication, sRNA refers to a commercially available mixture of soluble ribonucleic acid of *E. coli* obtained from General Biochemicals. Preparation of ribosomes and aminoacyl tRNA from *E. coli* has been reported.[12] The ribosomes were washed three times and fraction A containing aminoacyl tRNA synthetase and aminoacyl tRNA transfer factor was prepared as described previously.[15] The specific activities of radioisotopes used in this study were C^{14}-phenylalanine, 369 $\mu c/\mu mole$, and H^3-phenylalanine, 1000 $\mu c/\mu mole$. The Millipore filters used had a pore size of 0.45 μ and were obtained from the Millipore Filter Corporation.

Isolation of the complex of $tRNA_{phe}$, ribosomes, and poly U: The reaction mixture (0.9 ml) for the formation of the complex of $tRNA_{phe}$, ribosomes, and poly U contained the following in μmoles: 126 tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) (pH 7.8), 60 KCl, 6.3 β-mercaptoethanol, and 18 magnesium acetate. In addition, it contained 2.5 mg of *E. coli* sRNA, 0.36 mg of polyuridylic acid (poly U), and 4.5 mg of *E. coli* ribosomes. The mixture was incubated at 30°C for 30 min and chilled to 3°C. Three 0.25-ml portions of the mixture were separately placed on three 5-ml gradients of sucrose (5–20% in 0.14 *M* Tris-HCl, pH 7.8, 66 mM KCl, 7 mM β-mercaptoethanol, and 20 mM magnesium acetate). The tubes were centrifuged for 60 min at 37,000 rpm in a Beckman-Spinco SW50 rotor. After the centrifugation, 3-drop fractions were collected from the bottom. Polysome fractions containing bound $tRNA_{phe}$ were pooled (fractions 2–11 from the bottom of the tube). This pooled fraction was called the polysome fraction.

Assay of the release of $tRNA_{phe}$: The reaction mixture (0.7 ml) for the release of $tRNA_{phe}$ contained 98 μmoles of Tris-HCl (pH 7.8), 4.9 μmoles of β-mercaptoethanol, 46 μmoles of KCl, 14 μmoles of magnesium acetate, 2.03 OD_{260} units of polysome containing $tRNA_{phe}$, 0.2 μmole of GTP, and 80 μg of fraction A. The mixture was incubated at 30°C for 5 min, unless otherwise stated, and poured through a Millipore filter. The released $tRNA_{phe}$ went through the filter while the bound $tRNA_{phe}$ was retained. The filter was then carefully washed three times with Buffer 1 containing 5×10^{-2} *M* KCl, 2×10^{-2} *M* magnesium acetate, and 0.1 *M* Tris-HCl (pH 7.1). The first washing was performed with 0.3 ml, the second with 0.5 ml, and the last with 0.5 ml. The wash and the filtrate were combined (total 2 ml). Since the bound $tRNA_{phe}$ could not be eluted from ribosomes with Buffer 1, the washing is done merely to elute off the released $tRNA_{phe}$ mechanically stuck to the Millipore filter. The released $tRNA_{phe}$ in the filtrate and the wash was assayed. In the

case where the total bound $tRNA_{phe}$ was measured, the bound $tRNA_{phe}$ was eluted with Buffer 2, containing 5×10^{-2} *M* KCl, 1×10^{-4} *M* magnesium acetate, and 0.1 *M* Tris-HCl (pH 7.1), instead of washing the ribosomes with Buffer 1. The reaction mixture for the assay of $tRNA_{phe}$ contained the following in μmoles per 1.4 ml: 500 Tris-HCl (pH 7.8), 24 magnesium acetate, 20 β-mercaptoethanol, 10 adenosine 5′-triphosphate (ATP), and 20 phosphoenolpyruvate. In addition, it contained 50 μg of pyruvate kinase, 0.4 mg of fraction A, and 1.0 μc of C^{14}- or 10 μc of H^3-phenylalanine. The solution (0.5 ml or 1.0 ml) containing the above filtrate and the wash was mixed with 0.14 ml of the above reaction mixture for the assay and the total mixture was incubated at 30°C for 30 min. At the end of the incubation period, cold (2°C) trichloroacetic acid was added to a final concentration of 10%. The mixture was poured through a Millipore filter and the radioactivity on the filter was counted after several washings with 5% trichloroacetic acid. The background value in the absence of added $tRNA_{phe}$ was about 300 cpm, and the values expressed in this paper have been adjusted for this background.

Results.—Releasing of $tRNA_{phe}$ from the complex of $tRNA_{phe}$, poly U, and ribosomes: Table 1 shows the requirement for the maximum release of $tRNA_{phe}$ from the complex of $tRNA_{phe}$, ribosomes, and poly U. The release of $tRNA_{phe}$ was maximum when fraction A and GTP were added to the releasing medium. Since all the bound $tRNA_{phe}$ can be eluted by lowering Mg^{++}, it is released by washing the complex with Buffer 2 (containing 10^{-4} *M* magnesium acetate). The total amount of bound $tRNA_{phe}$ can be obtained by this procedure. It is clear that approximately 50 per cent of total bound $tRNA_{phe}$ was released under the condition of maximum release. In these experiments, the release of $tRNA_{phe}$ from the ribosomes was carried out in the absence of unbound $tRNA_{phe}$. Therefore, the released $tRNA_{phe}$ had very little chance to rebind to the ribosomes and thus we were able to detect the release. In a separate experiment, it was found that when the release reaction was carried out in the presence of nonbound $tRNA_{phe}$, the sites emptied by the release reaction were immediately filled by $tRNA_{phe}$, resulting in no change in the total amount of bound $tRNA_{phe}$.

The data in Figure 1 show the time course of the release of $tRNA_{phe}$. It is noted that at ten minutes after the onset of the releasing reaction, the release dependent on GTP and factor was almost six times as much as the release not dependent on GTP and factor. It is for this reason that most of the release reaction was carried out with a short (5-min) incubation. It should be pointed out that all the remainder of tRNA on the ribosomes can be removed by washing the ribosomes with Buffer 2 containing 10^{-4} *M* Mg^{++}.

TABLE 1. *Release of $tRNA_{phe}$ from $tRNA_{phe}$ poly U-ribosome complex.*

Reaction mixture	Released $tRNA_{phe}$ measured as C^{14}-phe tRNA in 0.5 ml of the filtrate (cpm)
Control	3,192
Plus fraction A	2,152
Plus GTP	4,048
Plus GTP and fraction A	8,148
Total bound $tRNA_{phe}$	17,226

The control reaction mixture (0.7 ml) for the release of $tRNA_{phe}$ was as described in the text, except that it lacked GTP and fraction A. The mixture was incubated for 40 min at 30°C. The $tRNA_{phe}$ released and total bound $tRNA_{phe}$ at zero time are expressed as C^{14}-phe tRNA formed in the assay mixture per 2 ml of the wash and the filtrate. The total bound $tRNA_{phe}$ was measured by eluting the bound $tRNA_{phe}$ with Buffer 2 containing 10^{-4} *M* Mg^{++}.

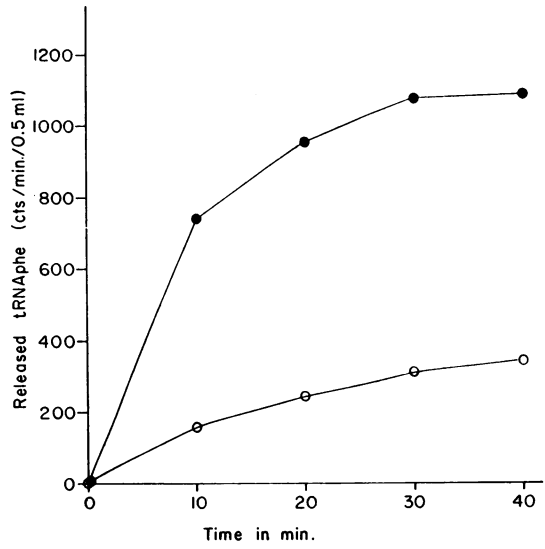


FIG. 1.—Time course of the release of $tRNA_{phe}$ from the complex of ribosome, $tRNA_{phe}$, and poly U. The complete mixture (3.5 ml) for release of $tRNA_{phe}$ was as described in the text, except that it contained 7.99 OD_{260} units of polysome containing $tRNA_{phe}$. The mixture was incubated at 30°C and 0.6-ml samples were taken at the indicated times and poured onto a Millipore filter. Of the filtrate and the wash (total 2 ml), 0.5 ml was used for the assay of $tRNA_{phe}$. The amount of $tRNA_{phe}$ in 0.5 ml of the filtrate and washing was expressed as C^{14}-phenylalanyl tRNA formed.
●—● Complete reaction mixture;
○—○ GTP and fraction A omitted.

The nature of the factor required for the release: Figure 2 shows the stimulation of the release reaction in the presence of various concentrations of fraction A. Under our conditions, the system was saturated at about 15 μg of fraction A. Up to this point, linear increase of the rate of release of $tRNA_{phe}$ was observed. It is known that at least three factors are required for polyphenylalanine formation, and of these factors, G and T_s are heat-stable.[16, 17] The data in Figure 3 show that the releasing factor is also heat-stable. In this experiment, fraction A was incubated at 50°C for various periods and its capacity to support polyphenylalanine formation as well as the release of $tRNA_{phe}$ was tested. One can conclude from this figure that the factor is relatively heat-stable, and its capacity to support release under the experimental conditions was not diminished, even after 20 minutes at 50°C, whereas the capacity to support polyphenylalanine formation was destroyed.

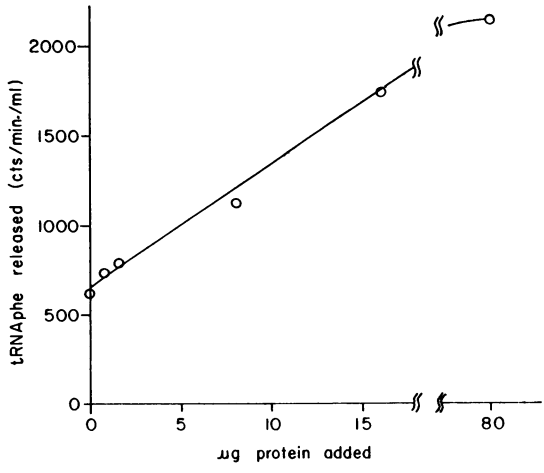


FIG. 2.—Release of $tRNA_{phe}$ in the presence of various amounts of fraction A. The reaction mixture (0.7 ml) for release of $tRNA_{phe}$ was as described in the text, except that it contained various amounts of fraction A and 1.69 OD_{260} units of the polysome fraction with bound $tRNA_{phe}$. After 5-min incubation at 30°C, the mixture was poured through a Millipore filter. Of the filtrate and the wash (2 ml), 1 ml was used for the assay of the $tRNA_{phe}$ released. The C^{14}-phenylalanyl tRNA formed in the assay mixture was plotted against the amount of fraction A in the mixture for $tRNA_{phe}$ release.

On the GTP requirement for the release: In order to test the nucleotide specificity of the GTP requirement, various nucleotides were tested for their stimulatory effect on the release of $tRNA_{phe}$ in the presence of fraction A. As shown in Table 2, the stimulation of the release was observed only with GTP and other nucleotide triphosphates, or GDP could not substitute for GTP. If the main function of GTP in the protein biosynthesis is for the release of deacylated tRNA, one should expect that the *Km* of GTP for the release reaction should be approximately the same as the *Km* of GTP for polyphenylalanine formation. The inset of Figure 4 shows the stimulation of the release reaction by various concentrations of GTP. From these data one can calculate a *Km* value of 6.7×10^{-5} *M* by the Lineweaver-Burk plotting method, as shown in this figure. The published value for the *Km* of GTP for polyphenylalanine formation is 1.6×10^{-5} *M* and that for GTPase is 1.8×10^{-4} *M*.[2] One can therefore conclude that the *Km* value of GTP for the release reaction is approximately the same order of magnitude as that for polyphenylalanine synthesis.

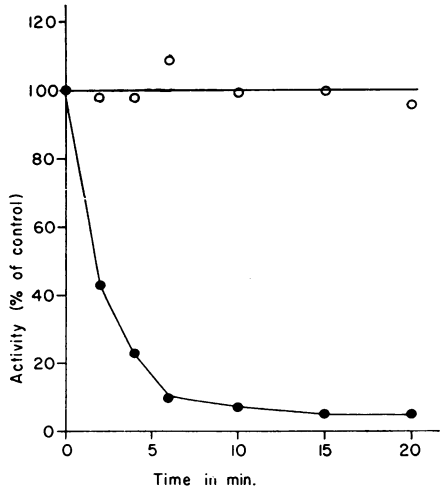
Effect of various antibiotics: Since the releasing reaction is a new reaction not previously discovered, and since it apparently represents a partial reaction of the very complex process of polypeptide synthesis, the effect of various antibiotics on this reaction was of interest. Table 3 shows that tetracycline and sparsomycin were the most effective inhibitors. It therefore appears that tetracycline not only inhibits the binding of tRNA but also inhibits the release of tRNA from ribosomes. The present observation is consistent with the previous finding that tetracycline could not remove a bound tRNA from ribosomes.[11] It has been found that sparsomycin inhibits the formation of polyphenylalanyl puromycin, indicating that sparsomycin inhibits the peptide bond formation *per se*.[18] The fact that this antibiotic inhibits the release of tRNA suggests a dual nature for sparsomycin action. Streptomycin inhibits the release of $tRNA_{phe}$ by about 30 per cent and chloramphenicol has only a slight inhibitory effect. Puromycin, on the other hand, had no inhibitory effect at all. When these antibiotics were tested on the polyphenylalanine formation, they all had an appreciable inhibitory activity under similar experimental conditions.

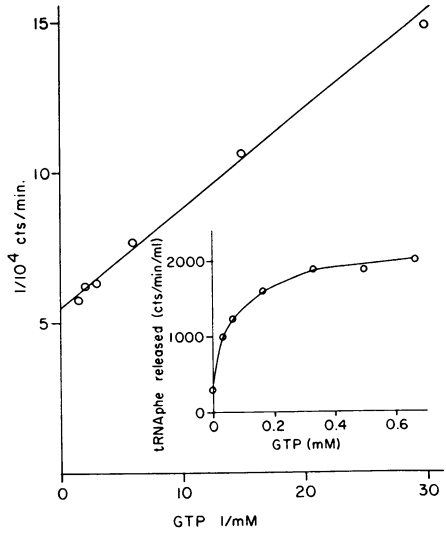
Release of $tRNA_{phe}$ from the complex of phenylalanyl tRNA, ribosomes, and poly U: All the preceding experiments in this communication dealt with the release of $tRNA_{phe}$ from the complex of $tRNA_{phe}$, poly U, and ribosomes. To test the

TABLE 2. *Specificity of nucleotide required for the factor-dependent release of $tRNA_{phe}$ from the complex of $tRNA_{phe}$, poly U, and ribosomes.*

Added nucleotide	$tRNA_{phe}$ released (cpm)
None	314
UTP	146
CTP	245
ATP	144
GTP	1672
GDP	221
GMP	280

The reaction mixture (0.60 ml) for the release of $tRNA_{phe}$ was as described in the text, except that it contained 1.25 OD_{260} units of polysome containing $tRNA_{phe}$, 80 μg of fraction A, and 0.2 μmole of various nucleotides. The mixture was incubated at 30°C for 5 min and poured on a Millipore filter. C^{14}-phenylalanyl tRNA formed per 1.0 ml of the filtrate and the wash is shown in the table.





(*Left*) FIG. 3.—Heat treatment of the releasing factor. Fraction A (16 mg/ml of buffer containing 10^{-2} *M* Tris-HCl (pH 7.8), 10^{-2} *M* magnesium acetate, 6×10^{-2} *M* KCl, and 6×10^{-3} *M* β-mercaptoethanol) was incubated at 50°C and 0.2-ml aliquots were removed after the indicated time intervals and chilled to 0°C. The reaction mixture (0.6 ml) for the release of $tRNA_{phe}$ was as described in the text, except that it contained 80 μg of fraction A treated at 50°C for various lengths of time and 1.40 OD_{260} units of the polysome fraction with bound $tRNA_{phe}$. The assay procedure for the released $tRNA_{phe}$ was the same as that of Fig. 2. The release of $tRNA_{phe}$ with untreated fraction A was 1752 C^{14}-phenylalanyl tRNA per 1.0 ml of the filtrate and the wash. The reaction mixture for polyphenylalanine formation contained the following in μmoles per 0.6 ml: 84 Tris-HCl (pH 7.8), 40 KCl, 12 magnesium acetate, 4.2 β-mercaptoethanol, and 0.2 GTP. In addition, it contained 200 μg of poly U, 80 μg of fraction A exposed to 50°C for various lengths of time, 170 μg of ribosomes, and 5.69×10^4 cpm of C^{14}-phenylalanyl tRNA in 0.3 mg of sRNA. The mixture was incubated at 30°C. After 30 min, a 0.1-ml sample was taken to count the C^{14}-polyphenylalanine formed. With the nontreated fraction, 784 cpm of C^{14}-polyphenylalanine was formed in 0.1 ml, which was regarded as 100%. Relative activity was plotted against the length of the heat treatment.

O—O Releasing activity; ●—● polyphenylalanine formation.

(*Right*) FIG. 4.—The Michaelis constant of GTP for the release of $tRNA_{phe}$ from the complex of ribosome, poly U, and $tRNA_{phe}$. The reaction mixture (0.6 ml) for the release of $tRNA_{phe}$ was as described in the text, except that it contained 80 μg of fraction A, various amounts of GTP, and 1.23 OD_{260} units of the polysome fraction with bound $tRNA_{phe}$. The assay of released $tRNA_{phe}$ was performed as described in the text, and C^{14}-phenylalanyl tRNA formed per 1 ml of the filtrate and the wash was plotted against the concentration of GTP used in the releasing reaction. The Lineweaver-Burk plot of the releasing reaction is shown in this figure.

possibility that $tRNA_{phe}$ or phenylalanyl tRNA may be released from the complex of phenylalanyl tRNA, poly U, and ribosomes, the experiments shown in Table 4 were performed. In these experiments the complex of phenylalanyl tRNA, poly U, and ribosome was incubated with various factors and the release of $tRNA_{phe}$ was measured. Again, both GTP and fraction A were necessary for maximum stimulation. When puromycin was added, the release was further stimulated. It should be emphasized that *only* $tRNA_{phe}$ was released and no appreciable amount of phenylalanyl tRNA was released. Thus, in a separate experiment, release of aminoacyl tRNA rather than $tRNA_{phe}$ was studied, and it

TABLE 3. *Effects of antibiotics on the factor-dependent release of $tRNA_{phe}$ from the complex of $tRNA_{phe}$, poly U, and ribosomes.*

Conditions for the release reaction	Released $tRNA_{phe}$ (cpm)	GTP-dependent release (cpm)
Complete	1668	1329
Omit GTP and fraction A	339	0
Plus sparsomycin	784	445
Plus puromycin	1798	1459
Plus streptomycin	1308	969
Plus tetracycline	837	498
Plus chloramphenicol	1542	1203

The reaction mixture (0.6 ml) for the release of $tRNA_{phe}$ was identical to that of Table 2. The rest of the procedure was also identical to that of Table 2. The value of the last column was obtained by subtracting the amount of $tRNA_{phe}$ released in the absence of GTP and fraction A from the values in the first column. The concentration of antibiotics used was 0.833 mM.

was found that less than 5 per cent of bound phenylalanyl tRNA was released as phenylalanyl tRNA. As has been shown previously,[19] formation of diphenylalanine takes place under these conditions in the presence of GTP and soluble factors. Thus, the release of $tRNA_{phe}$ must be subsequent to the formation of diphenylalanyl tRNA, which produces one $tRNA_{phe}$ on the ribosomes. The stimulation by puromycin can be understood as a result of increased production of $tRNA_{phe}$ due to formation of diphenylalanyl and phenylalanyl puromycin.

Discussion.—In polypeptide synthesis, each time the polypeptide is elongated by the addition of an amino acid, one tRNA is liberated from peptidyl tRNA. It is therefore reasonable to assume that there should exist in nature some kind of enzymatic mechanism that removes this tRNA from ribosomes. Our present data show that such a mechanism indeed exists and that this process is specifically GTP-dependent. The fact that the *Km* of GTP for the release of $tRNA_{phe}$ is approximately the same order of magnitude as that for polyphenylalanine formation suggests that the primary action of GTP is at this step. On the other hand, one should not disregard the possibility that the release of $tRNA_{phe}$ is a result of its movement from one binding site to another and that GTP is involved in this movement. Further experiments are necessary to elucidate the mode of action of GTP on the release of $tRNA_{phe}$. Whether splitting of

TABLE 4. *Release of $tRNA_{phe}$ from the complex of phenylalanyl tRNA, poly U, and ribosomes.*

Reaction mixture	Released $tRNA_{phe}$ (cpm, H^3)
Control	440
Plus GTP	368
Plus fraction A	336
Plus GTP and puromycin	402
Plus puromycin and fraction A	409
Plus GTP and fraction A	1068
Plus GTP, fraction A, and puromycin	1579

The polysome used in this experiment was prepared as described in the text, except that 8.53×10^5 cpm of C^{14}-phenylalanyl tRNA in 4.57 mg of sRNA was used instead of sRNA in the reaction mixture for making polysomes. The reaction mixture for the release of $tRNA_{phe}$ was identical to that of Table 2, except that it contained 1.37 OD_{260} units of the polysome fraction containing 4476 cpm of C^{14}-phenylalanyl tRNA. The assay of released $tRNA_{phe}$ was carried out as in Table 2, except that the assay mixture contained 1 μc of H^3-phenylalanine per 1.14 ml of the total reaction mixture. When puromycin was added, the final concentration was 0.833 mM.

terminal phosphate of GTP (GTPase) is involved in the release of tRNA remains to be determined. The earlier finding[20] that the inhibition of binding of phenylalanyl tRNA by $tRNA_{phe}$ was overcome by the addition of GTP may have close bearing on our present observation of GTP-dependent release of $tRNA_{phe}$. In addition to GTP, the release is largely dependent on a factor or factors present in a soluble fraction of *E. coli* extract. Since the fraction A used is almost free of RNA, the factor is most likely to be protein. Two of the three factors required for polyphenylalanine synthesis are heat-stable. Which one of these heat-stable factors, G[16] or T_s,[17] is responsible for the release will be determined subsequently.

Summary.—Release of $tRNA_{phe}$ from the complex of 70*S* ribosomes, phenylalanyl tRNA or $tRNA_{phe}$, and poly U is largely dependent on GTP and a soluble factor from *E. coli*. Sparsomycin and tetracycline were inhibitory to this release. It is suggested that the function of GTP is somehow related to the removal of deacylated tRNA from ribosomes.

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