*A STEPWISE REACTION YIELDING A COMPLEX BETWEEN A SUPERNATANT FRACTION FROM E. COLI, GUANOSINE 5'-TRIPHOSPHATE, AND AMINOACYL-SRNA**

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We have recently reported[1] the binding of guanosine 5'-triphosphate (GTP) to some macromolecule, or macromolecules, requiring the presence of aminoacyl-sRNA and the partially purified supernatant fraction, T.[2] This was assayed by gel filtration on Sephadex G-50. Concurrently, Allende *et al.*[3, 4] found an sRNA-independent binding of GTP to a protein fraction, probably T_u. They used a Millipore adsorption assay. We undertook a detailed comparison of the two techniques in order to explain this apparent discrepancy. We found that the presence of aminoacyl-sRNA caused reversal of the ability of the GTP acceptor to bind to the Millipore, with a simultaneous appearance of T activity in the Millipore filtrate. These findings led us to formulate a stepwise reaction. First, GTP binds to T. Then there is a further addition of aminoacyl-sRNA to the complex. The evidence is the subject of this communication.

Materials and Methods.—All materials and methods were essentially unchanged from the previous study.[1] All GTP binding reactions were measures of amounts rather than rates. Reactions were routinely carried out for 30 minutes at 0°. Millipore filtration assays were based on the method used by Allende and Weissbach.[3] The Millipores (HA 0.45-μ pore size) were boiled in 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ (Millipore wash medium), and then decanted several times with water to remove dust. The GTP binding reaction mixtures (0.05–0.25 ml vol) were diluted with 7 ml of Millipore wash medium, passed through a Millipore, and washed four times with 7 ml at a flow rate of 20 ml per minute. The filter was not allowed to run dry between washes. Radioactivity was determined on the dried Millipores with 5 ml of Liquifluor (Pilot Chemicals, Inc.) in a Nuclear-Chicago scintillation counter. Gel filtration assays were as before.[1] The specific activity of H^3-GTP was 1 c/mmole, and that of C^{14}-phenylalanyl-sRNA was 360 mc/mmole of phenylalanine.

Results.—A direct comparison of the assay of GTP binding by the gel filtration and Millipore adsorption techniques was carried out. The results, obtained with aliquots from the same reaction mixtures, are given in Table 1. As seen previously[1] with the gel filtration method, the binding of GTP in the presence of T was stimulated by phenylalanyl-sRNA but not by stripped sRNA. However, there was a decrease in the level of GTP bound to the Millipore in the presence of phenylalanine-charged sRNA compared with stripped sRNA. This suggests that the two methods detected different kinds of complexes.

Furthermore, it seemed that the Millipore-bound complex, T-GTP, was labile under the gel filtration conditions. In the reaction mixture with stripped sRNA in Table 1, only a fraction of the original T-GTP complex was recovered

TABLE 1. *Comparison of Millipore adsorption and gel filtration assays for bound GTP.*

Incubation mixture	Millipore-bound ($\mu\mu$moles)	G-50 excluded ($\mu\mu$moles)	Millipore-bound after G-50 exclusion ($\mu\mu$moles)
T + stripped sRNA	142	35	13
T + phenylalanyl-sRNA	77	210	6

Two reaction mixtures each contained in 0.5 ml: 25 μmoles of Tris-HCl, pH 7.4, 5 μmoles of $MgCl_2$, 80 μmoles of NH_4Cl, 6 μmoles of dithiothreitol, 150 μg of the hydroxyapatite fraction of T,[1] 5 mμmoles of H^3-GTP, and 250 μg of stripped sRNA or C^{14}-phenylalanyl-sRNA. After 30 min at 0°, 0.05-ml aliquots were assayed by the Millipore technique. The remainder of each was subjected to the gel filtration assay. After collection of the void volume, 0.2-ml aliquots from the 0.5-ml fractions were assayed for total H^3 radioactivity, and further aliquots were subjected to the Millipore assay. The amount of isolated complex was integrated as total GTP and as Millipore-bindable GTP. All values were corrected back to the original 0.5-ml volumes. The timing of the experiment was as follows: the complex was isolated 6 min after unclamping the columns, and the Millipore assays were completed after a further 20 min. No C^{14} was detected in any of the Millipore assays.

from the column. When the material from the column was tested for its content of T-GTP in a Millipore assay, a further decrease was found. From the timing of the experiment (see the legend to Table 1), it was estimated that there was 10 per cent survival of T-GTP complex in 30 minutes. Previously,[1] we found a half life of four hours for the aminoacyl-sRNA–dependent complex. The difference in response to aminoacyl-sRNA and in stability suggests that the two techniques measured experimentally distinguishable kinds of complex, possibly in equilibrium with each other.

The equilibrium situation is illustrated in Figures 1 and 2. Addition of increasing amounts of phenylalanyl-sRNA to the reaction mixture caused the disappearance of complex from the Millipores (Fig. 1) and a concurrent appearance of complex in the filtrate (Fig. 2*A*). In the presence of a saturating amount of phenylalanyl-sRNA, the amount of complex in the filtrate (Fig. 2*A*) was the same as in the unfiltered material (Fig. 2*B*). The transformation of complex

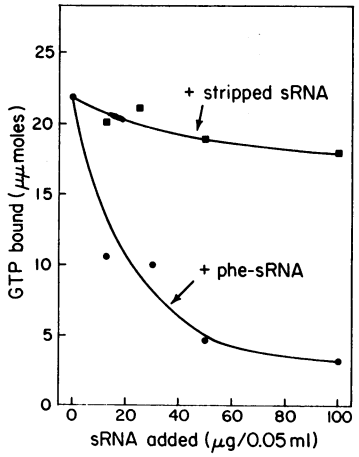

FIG. 1.—The effect of charged and uncharged sRNA on the T-GTP complex. Reaction mixtures of 0.05 ml each contained 2.5 μmoles of Tris-HCl, pH 7.4, 0.5 μmole of $MgCl_2$, 8 μmoles of NH_4Cl, 0.6 μmole of dithiothreitol, 15 μg of T, 0.5 mμmole of H^3-GTP, and C^{14}-phenylalanyl-sRNA (70 $\mu\mu$moles of phenylalanine/100 μg of sRNA (●—●) or stripped sRNA (■—■)). There was no Millipore-bound C^{14}.

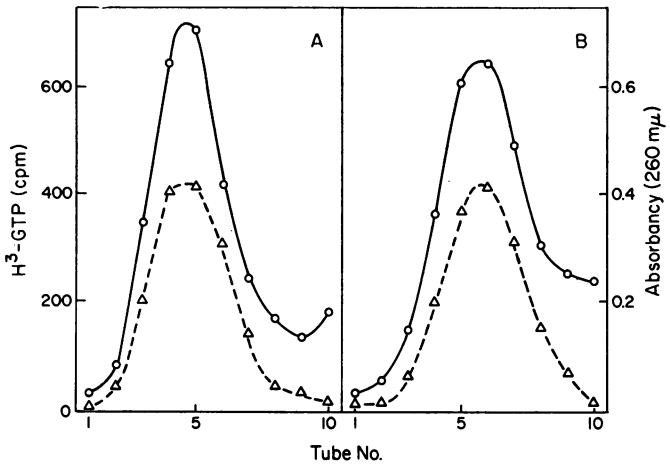

FIG. 2.—Appearance of complex in the Millipore filtrate after addition of excess phenylalanyl-sRNA. The two reaction mixtures (*A*) and (*B*) contained in 0.25 ml: 12.5 μmoles of Tris-HCl, pH 7.4, 2.5 μmoles of $MgCl_2$, 40 μmoles of NH_4Cl, 3 μmoles of dithiothreitol, 37.5 μg of T, 200 μg of C^{12}-phenylalanyl-sRNA, and 1.25 mμmole of H^3-GTP. After the binding reaction, 7 ml of Millipore wash fluid was added to (*A*) and (*B*). (*A*) was then passed through a Millipore and the filtrate was collected. A 1-ml aliquot of the filtrate was passed through a Sephadex G-50 column. A 1-ml aliquot of (*B*) was passed through a column without prior Millipore filtration. The excluded volumes were scanned for radioactivity (O—O) and for absorbancy (Δ---Δ).

from a Millipore-bindable state to a Millipore-filtrable state proceeded in two steps (Fig. 3). A reaction mixture containing T and radioactive GTP was incubated for 35 minutes. A portion was then added to phenylalanyl-sRNA. There followed a rapid disappearance of preformed Millipore-bindable complex.

That the transformation to a Millipore-filtrable state was a result of the addition of aminoacyl-sRNA to the preformed T-GTP complex was shown

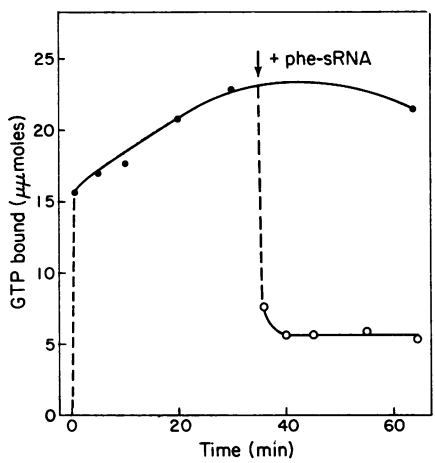

FIG. 3.—Displacement of the preformed T-GTP complex from the Millipore after addition of aminoacyl-sRNA. At intervals after the addition of H^3-GTP, 0.05-ml aliquots were taken from a reaction mixture (●—●). Each aliquot contained the same reagents as in Fig. 1, except that T, but no sRNA, was present. After 35 min, a portion of the reaction mixture was added to phenylalanyl-sRNA, and the sampling was continued (O—O). Each aliquot then contained 50 μg of C^{14}-phenylalanyl-sRNA.

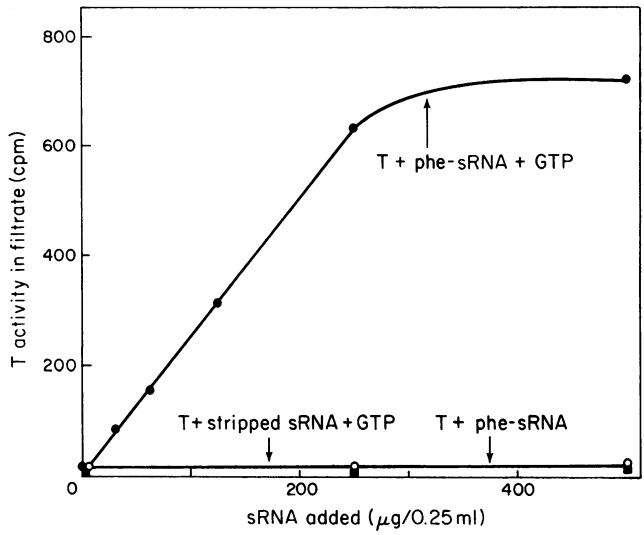

FIG. 4.—Appearance of T in the Millipore filtrate after addition of phenylalanyl-sRNA and GTP. Each reaction mixture contained the same concentrations of reactants as the experiment in Fig. 1, except that volumes were scaled up to 0.25 ml and nonradioactive GTP was used. Included were: phenylalanyl-sRNA (●—●), stripped sRNA (○—○), and phenylalanyl-sRNA without GTP (■—■). After completion of the binding reaction, 7 ml of Millipore wash medium, also containing 2 mM dithiothreitol, was added to each mixture and was passed through Millipores. The filtrates were collected and 0.2-ml aliquots were assayed for T activity in phenylalanine polymerization. The phenylalanine polymerization assays[2] contained in 0.25 ml: 12.5 μmoles of Tris-HCl, pH 7.4, 2.5 μmoles of $MgCl_2$, 40 μmoles of NH_4Cl, 0.5 μmole of dithiothreitol, 15 μg of G, 10 μg of poly U, 100 μg of C^{14}-phenylalanyl-sRNA, 100 μg of ribosomes, and 0.25 μmole of GTP. The assays were incubated for 10 min at 30°, and phenylalanine was determined as hot TCA-insoluble radioactivity.

by the disappearance of bound GTP from the Millipore (Figs. 1 and 3), paralleled by the appearance of T in the filtrate (Fig. 4). The T in the filtrate in Figure 4 was assayed by its ability to complement G and ribosomes in poly U-directed phenylalanine polymerization. Figure 4 also shows that the appearance of T activity in the filtrate depended on the presence of GTP. This highly specific transformation was taken to represent the formation of a complex between T, phenylalanyl-sRNA, and GTP.

Discussion.—The above experiments led to the formulation of the following two-step reaction:

$$T + GTP \rightleftharpoons T\text{-}GTP \quad (1)$$

$$\text{Aminoacyl-sRNA} + T\text{-}GTP \rightleftharpoons \text{Aminoacyl-sRNA}\text{-}T\text{-}GTP \quad (2)$$

where (−) represents an unknown labile linkage. From the experiments of Allende *et al.*,[4] T_u is probably active in (1), but it is possible that both T_u and T_s are active in (2) since they were both present in the complex in the filtrate

(Fig. 4). Allende[5] has found that RNase treatment of the complex in the filtrate restored its ability to bind to the Millipore. This is also consistent with (2). Further evidence for interaction between GTP and T_u came from the protection of the transfer activity of T_u against thermal inactivation in the presence of GTP.[6] In the mammalian system, Moldave and his co-workers have found that GTP inactivates one of their fractions.[7] This inactivation was prevented by aminoacyl-sRNA.[7] They also observed a binding between their factor and aminoacyl-sRNA.[8] In contrast to the *E. coli* system, this binding did not require GTP.

Our failure to detect release of inorganic phosphate from the GTP after isolation of the complex,[1] and also the recovery of the bound material as GTP,[9] suggest that there is no utilization of the phosphate bond energy in steps (1) and (2). It is possible, therefore, that this complex formation is preparatory to a role of GTP during interaction with the ribosome.

Note added in proof.—After the submission of this paper, J. M. Ravel, R. L. Shorey, and W. Shive (*Biochem. Biophys. Res. Commun.*, **29**, 68 (1967)) reported findings similar to some of those described here.

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[1] Gordon, J., these PROCEEDINGS, **58**, 1574 (1967).

[2] T was originally defined in Nishizuka, Y., and F. Lipmann, these PROCEEDINGS, **55**, 212 (1966). It is used here noncommittally to define the unseparated subfractions T_u and T_s defined by Lucas-Lenard, J., and F. Lipmann, these PROCEEDINGS, **55**, 1562 (1966).

[3] Allende, J. E., and H. Weissbach, *Biochem. Biophys. Res. Commun.*, **28**, 82 (1967).

[4] Allende, J. E., N. W. Seeds, T. W. Conway, and H. Weissbach, these PROCEEDINGS, **58**, 1566 (1967).

[5] Allende, J. E., personal communication.

[6] Seeds, N. W., and T. W. Conway, *Biochem. Biophys. Res. Commun.*, **28**, 1047 (1967).

[7] Ibuki, F., and K. Moldave, *J. Biol. Chem.*, in press.

[8] Rao, P., and K. Moldave, *Biochem. Biophys. Res. Commun.*, **28**, 909 (1967).

[9] Gordon, J., unpublished experiment.