INHIBITION OF THE PUROMYCIN RELEASE BY GUANOSINE TRIPHOSPHATE

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It has been shown that in protein synthesis the antibiotic puromycin can substitute for amino acyl-RNA.* In doing so, puromycin forms a peptide bond, at the free amino group of its p-methoxy-L-phenylalanyl-moiety, with the t-RNA-bound carboxy terminus of the growing polypeptide chain. This reaction (puromycin release or puromycin reaction) results in the release of peptidyl-puromycins from the peptidyl-RNA-ribosome-mRNA complex (Smith et al., 1965, and original references therein).

There have been conflicting reports concerning the effect of GTP on the puromycin reaction in cell free systems. In several instances GTP has been reported to have no effect on the puromycin release (Casjens and Morris, 1965; Zamir et al., 1966; Gottesman, 1967) whereas in other instances GTP was reported to stimulate the puromycin release (Hershey and Thach, 1967; Lucas-Lenard and Lipmann, 1967).

In an effort to resolve these discrepancies we have examined in detail the effects of GTP on the reaction between puromycin and polylysyl-RNA (Rychlik, 1966; Gottesman, 1967; Goldberg and Mitsugi, 1967; Coutsogeorgopoulos, 1967a). In this communication we show that, depending on the concentration of Mg^{++} and the presence of "factors washable from ribosomes" with NH_4Cl solutions (FWR), GTP stimulates, inhibits, or has no effect on the puromycin reaction. Furthermore, we show that when GTP inhibits the puromycin release, it antagonizes puromycin in a competitive manner.

Materials and Methods: Preincubated S-30 (30,000 x g supernatant) was prepared from

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*Abbreviations used: RNA, ribonucleic acid; t-RNA, transfer-RNA; mRNA, messenger RNA; poly A, polyadenylic acid; PCA, perchloric acid; GTP, guanosine triphosphate; GDP, guanosine diphosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; FWR, "factors washable from ribosomes"; cpm, radioactivity in counts per minute.

E. coli B cells according to Nirenberg and Matthaei (1961). After dialysis it was frozen in small aliquots at -70°C and thawed before use. "Washed ribosomes" were prepared from a non-preincubated S-30 fraction by washing the ribosomal pellet three times with: 0.5M NH_4Cl - 0.01M $MgCl_2$ - 0.01M Tris-HCl pH 7.4. The ribosomes were finally suspended in the latter solution, kept in ice and used directly in the assays without previous dialysis. "Factors washable from ribosomes" (FWR) were prepared from a separate batch of non-preincubated S-30 fraction by a procedure adapted from Lucas-Lenard and Lipmann (1967). After centrifugation of the S-30 fraction at 100,000 x g for 2 hrs the ribosomal pellet was suspended in: 1.0M NH_4Cl - 0.01M $MgCl_2$ - 0.01M Tris-HCl pH 7.4, and recentrifuged at 100,000 x g for 2 hrs. To the supernatant was added ammonium sulfate to 80% saturation and the resulting precipitate was dissolved in the "standard buffer" of Nirenberg and Matthaei (1961). After dialysis against the latter buffer, this fraction was frozen at -70°C in small aliquots and thawed before use. The FWR-fraction may contain what has recently been defined as "initiation factors" (see e.g. Salas et al., 1967). The sources of the other materials used and the assay for the puromycin release have been described previously (Coutsogeorgopoulos, 1967a). GTP and GDP were purchased from Pabst Laboratories.

Results and Discussion: The effect of added GTP (10^{-4}M) on the reaction between polylysyl-RNA and puromycin (10^{-4}M) was first examined with the crude preincubated S-30 fraction, which contains macromolecular factors not present in washed ribosomes. At low concentrations of Mg^{++} (3 - 5mM) the puromycin release was inhibited by GTP, whereas at higher concentrations of Mg^{++} (7 - 15mM) the reaction was stimulated by GTP. At 6mM Mg^{++} GTP neither inhibited nor stimulated the puromycin release. The optimal concentration of Mg^{++} for release was 8mM in the absence of GTP or 10mM in the presence of 10^{-4}M GTP. The time course of the GTP effect on the puromycin release at 10mM Mg^{++} and at 4mM Mg^{++} is given in Fig. 1. That the release of oligolysines from polylysyl-RNA in the presence or absence of puromycin is due to two different reactions was shown by including chloramphenicol. Only the release induced by puromycin was inhibited (97%) by chloramphenicol (10^{-3}M, data not shown), indicating that in the presence of puromycin the formation of oligolysyl-puromycins was followed

(Rychlik, 1966; Gottesman, 1967; Goldberg and Mitsugi, 1967; Coutsogeorgopoulos, 1967a). The cleavage of $[C^{14}]$-polylysyl residues from $[C^{14}]$-polylysyl-RNA in the absence of puromycin is probably due to esterases and is not influenced by the addition of GTP (Fig. 1) or chloramphenicol (10^{-3}M, data not shown).

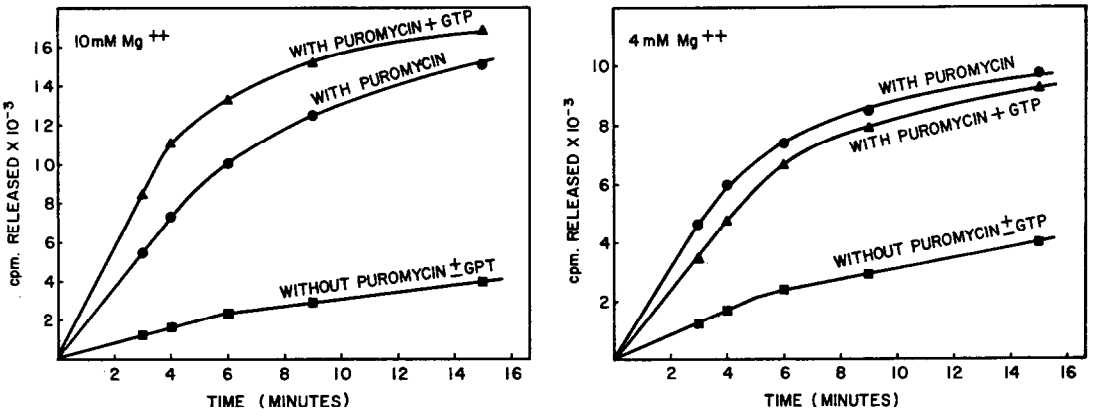

Fig. 1. Influence of the duration of incubation on the release of polylysines from polylysyl-RNA in the presence or absence of puromycin and GTP. The effect of 10^{-4}M GTP at two different concentrations of Mg^{++} is shown. The complete system contained in 0.25ml: 25μmoles of Tris-HCl buffer (pH 7.2), 25μmoles of ammonium chloride, magnesium acetate as defined, 40μg of poly A, 25mμmoles of puromycin where mentioned, 0.55 mg (protein) of preincubated S-30 and 1.2 ODU at 260 mμ of C^{14}-polylysyl-RNA (sp. act. of L-lysine 250μc/μmole), corresponding to 19,500 cpm per 0.1 ml of incubation mixture. Incubation at 37°. The reagents were added in the order cited. Where indicated, GTP, 10^{-4}M, was added after the magnesium acetate and before the poly A. At the end of the incubation period, two 0.1 ml aliquots were applied on 3MM Whatman paper discs and the radioactivity of the cold PCA precipitable material was determined as described elsewhere (Coutsogeorgopoulos, 1967b). The "radioactivity released", plotted on the ordinate, represents the difference of cpm of the cold PCA precipitable material before (0°, ice) and after incubation at 37°.

When the concentration of puromycin was lowered, the effects of GTP (stimulation or inhibition) on the puromycin release were much more pronounced (Table I). The fact that at a constant level of GTP the inhibition of the puromycin release was greater the lower the concentration of puromycin (Table I, 4mM Mg^{++}), in conjunction with the evidence that at a certain level of puromycin the inhibition by GTP was greater the higher the concentration of GTP (data not shown for experiments with S-30 fraction but given in Table III for experiments using washed ribosomes), suggests that there is competition between puromycin and GTP.

TABLE I

Influence of the Concentration of Puromycin on the Effect of GTP

Puromycin (M)	At 10mM Mg^{++} cpm released -GTP	+GTP	Stimulation %	At 4mM Mg^{++} cpm released -GTP	+GTP	Inhibition %
1.0×10^{-4}	5,678	9,556	68	4,360	3,097	29
4.8×10^{-5}	5,203	8,944	72	4,247	2,070	51
3.2×10^{-5}	4,972	8,693	75	3,482	1,520	56
2.4×10^{-5}	4,765	8,477	78	3,135	1,341	57
1.6×10^{-5}	3,931	7,682	95	2,663	1,010	62
1.0×10^{-5}	3,156	6,387	100	2,065	715	65

Conditions of incubation identical with those of Fig. 1 with the only difference that the concentration of puromycin was varied. GTP 10^{-4}M. Incubation at 37° for 4 min. "Cpm released" represents the difference of radioactivities in the cold PCA precipitable material in the absence and in the presence of puromycin.

The stimulation or inhibition caused by GTP on the puromycin release was also observed in a more purified system containing washed ribosomes. In the presence of washed ribosomes alone, GTP inhibited the puromycin release at all levels of Mg^{++} which could support the puromycin reaction (Table II). The inhibition was higher the lower the extent of release. In turn, the extent of release depended on the concentration of Mg^{++}. When FWR-fraction was also added, the inhibition by GTP was reversed. At 10mM Mg^{++} the inhibition by 10^{-3}M GTP was abolished or converted to stimulation depending on the amount of FWR added. At lower concentrations of Mg^{++}, in the presence of FWR, the inhibition by GTP (10^{-3}M) was also reversed but not to the point where stimulation could be observed (Table II).

With washed ribosomes it was observed that, in analogy with the findings in the S-30 system (Table I), the effects of GTP on the puromycin release (inhibition or stimulation according to the conditions, Table II) were more pronounced the lower the concentration of puromycin (data not shown).

TABLE II

Effect of Factors Washable from Ribosomes on the Puromycin Release

Conditions	Radioactivity released by 10^{-4}M puromycin cpm	GTP effect
Complete system (20mM Mg^{++})	1,325	—
Complete system (10mM Mg^{++})	4,417	—
plus FWR (0.1mg)	5,328	
" FWR (0.2mg)	4,136	
" 10^{-3}M GTP	2,347	47% inhibition (ref: 4,417)
" 10^{-3}M GTP + FWR (0.1mg)	4,438	no effect
" 10^{-3}M GTP + FWR (0.2mg)	6,301	42% stimulation
" 4×10^{-3}M GTP + FWR (0.2mg)	1,862	58% inhibition
Complete system (8mM Mg^{++})	3,597	
plus FWR (0.2mg)	3,618	
" 10^{-3}M GTP	1,661	54% inhibition (ref: 3,597)
" 10^{-3}M GTP + FWR (0.2mg)	2,954	18% inhibition
Complete system (6mM Mg^{++})	1,048	
plus FWR (0.2mg)	2,520	
" 10^{-3}M GTP	287	73% inhibition (ref: 1,048)
" 10^{-3}M GTP + FWR (0.2mg)	876	16% inhibition

The complete system was identical with that of Figure 1 except that 0.17mg (protein) of washed ribosomes (10μliter) were used instead of the preincubated S-30 fraction. Where mentioned, "factors washable from ribosomes" (FWR) were added after the ribosomes. No GTP was added to the complete system. Incubation at 37° for 4 min. "Radioactivity released by puromycin" represents the difference of radioactivities in the cold PCA material in the absence and in the presence of 10^{-4}M puromycin. When puromycin was not added, the difference of cpm in the cold PCA precipitable material before (0°, ice) and after incubation was: 152 cpm in the absence of FWR or 446 cpm in the presence of FWR (0.2mg); the addition of 10^{-3}M GTP did not change these figures.

The question of whether GTP or GDP caused the stimulatory and the inhibitory effects, was examined as shown in Table III. With washed ribosomes and in the absence of added FWR (Table III, first column) 10^{-4}M GTP inhibited the puromycin release to some extent. When higher concentrations of GTP or when "PEP + PK" were added, the inhibition became greater. Added GDP could not inhibit as efficiently as GTP unless "PEP + PK" were included. With washed ribosomes but in the presence of added FWR (Table III, last column) GTP stimulated the puromycin release. However, when GTP was added at concentrations exceeding 10^{-3}M (Table II) or when "PEP + PK" were

added along with 10^{-3}M GTP, the puromycin release was inhibited. GDP could replace GTP only partially in stimulating the puromycin release, even in the presence of "PEP + PK". It is possible that at high concentrations GDP may inhibit the stimulatory reaction in which GTP participates. Inhibition of the polypeptide forming machinery by GDP is known to occur (Nishizuka and Lipmann, 1966). The fact that 10^{-3}M GDP alone, in the presence or in the absence of added FWR, could mimic to some extent the effects of GTP (Table III) suggests that the washed ribosomes as well as the FWR-fraction may contain a system which converts some of the added GDP to GTP. It may be concluded that at optimal concentrations, GTP and not GDP is responsible for both the inhibition and the stimulation of the puromycin release observed upon addition of GTP.

TABLE III

Comparison Between GTP and GDP in Inhibiting or Stimulating the Puromycin Release

Conditions	Radioactivity (cpm) released by puromycin	
	In the presence of washed ribosomes	In the presence of washed ribosomes and FWR (0.2mg)
Complete	4,417	4,136
minus poly A	645	628
plus 10^{-4}M GTP	4,141	5,693
" 10^{-4}M GTP + PEP + PK	708	5,241
" 10^{-3}M GTP	2,347	6,301
" 10^{-3}M GTP + PEP + PK	104	3,638
plus 10^{-4}M GDP	4,253	4,213
" 10^{-4}M GDP + PEP + PK	1,222	5,517
" 10^{-3}M GDP	2,825	5,023
" 10^{-3}M GDP + PEP + PK	786	4,945

The complete system was identical with that of Table II and contained 10mM magnesium acetate and 10^{-4}M puromycin; incubation at 37° for 4 min. Where mentioned, 0.5μmole of trisodium phosphoenolpyruvate (PEP) and 10μg of pyruvate kinase (PK) (Boehringer-Mannheim Co.) were added after the addition of GTP or GDP.

One explanation for the observed results is that with the washed ribosomes used and in the absence of added FWR (Table II, complete system) the necessity for the GTP-promoted translocation of the peptidyl-RNA from the "amino acid site" to the "peptide site" is by-passed, because peptidyl-RNA may attach directly to the "peptide site" (the

translocation reaction has been reviewed by Schweet and Heintz, 1966). In that case, it is possible that GTP interferes with the participation of puromycin in the formation of a peptide bond with peptidyl-RNA bound to the "peptide site".

Irrespective of the exact mechanism involved, these results demonstrate that in reconstituted systems which utilize washed ribosomes, the overall effect of added GTP on the puromycin release consists of the sum of a stimulatory and an inhibitory effect both caused by GTP. The washed ribosomes prepared in various laboratories may contain different amounts of the "factors" that are removable from the ribosomes by washing with salt solutions. These "factors", the integrity of the washed ribosomes, the concentration of Mg^{++} employed in the puromycin reaction and the amounts of endogenous GTP or GDP present on the washed ribosomes may regulate the overall effect of externally added GTP. In this way, the contradicting reports concerning the effect of added GTP on the puromycin release can be explained.

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