Synthesis of Guanosine 5′-Di- and -Triphosphate Derivatives with Modified Terminal Phosphates: Effect on Ribosome–Elongation Factor G-Dependent Reactions†

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ABSTRACT: A series of GTP and GDP analogues modified in the terminal phosphate has been synthesized and their activities were investigated in elongation factor G dependent reactions. All of the analogues, with the exception of guanosine 5′-*O*-(3-thiotriphosphate), were not hydrolyzed by EF-G and ribosomes, but were competitive inhibitors of the ribosome-dependent EF-G GTPase. The most active inhibitors were P^3-fluoro P^1-5′-guanosine triphosphate and P^3-methyl P^1-5′-guanosine triphosphate with a K_i of 1.0×10^{-6} and 2.5×10^{-6} M, respectively. The activity of the GTP alkyl ester derivatives decreased with increasing number of carbon atoms in the side chain. GTP analogues were much more effective inhibitors than the corresponding GDP derivatives. This points out the necessity of the presence of at least three negative charges in the phosphate chain of the nucleotide for an effective interaction with the active site of the ribosomal EF-G GTPase. Guanosine 5′-*O*-(3-thiotriphosphate), which was hydrolyzed at one-third the rate of GTP, was able to support poly(U)-directed poly(phenylalanine) polymerization. Possible mechanisms of ribosome-EF-G GTP hydrolysis that arise from our results are discussed. Activity of the nucleotide analogues in EF-G·ribosome complex formation compared well with their ability to inhibit ribosome-dependent EF-G GTPase, P^3-fluoro P^1-5′-guanosine triphosphate and P^3-methyl P^1-5′-guanosine triphosphate being again the most effective ones. The stabilizing action of fusidic acid on the EF-G·ribosome complex formation induced by the various nucleotides could not be correlated to any of the structural modifications of the substrate. Guanylyl methylene diphosphonate was displaced more readily than GDP from the EF-G·ribosome complex by GTP analogues insensitive to fusidic acid.

The translation of the genetic message on the ribosome is catalyzed by various enzymatic reactions which utilize GTP as energy donor (for review, see Lucas-Lenard and Lipmann, 1971; Haselkorn and Rothman-Denes, 1973). A systematic investigation with GTP analogues seemed to be appropriate for a better understanding of the mechanism of GTP hydrolysis in these reactions. We approached this problem by trying to synthesize nonhydrolyzable GTP analogues other than GMPP(CH_2)P and GMPP(NH)P (Hershey arnd Monro, 1966; Eckstein et al., 1971). Our finding that P^3-methyl P^1-5′-guanosine triphosphate was not hydrolyzed by the ribosome-EF-G GTPase prompted us to synthesize a series of GTP γ- and GDP β-phosphate derivatives (Figure 1) and to study their properties in EF-G-dependent reactions. EF-G, an essential component of the elongation process, catalyzes a ribosome-dependent GTP hydrolysis which is involved in the advancement of mRNA and the simultaneous translocation of the polypeptide chain from the acceptor to the peptidyl site of the ribosome (Nishizuka and Lipmann, 1966; Haenni and Lucas-Lenard, 1968; Erbe et al., 1969). Ribosome-EF-G-dependent reactions have been extensively studied and represent an excellent tool to compare the effects of structural modifications of GTP and GDP; at the same time this approach allows the investigation of functional aspects of EF-G activities that are not yet fully understood.

Experimental Procedure

Materials. Alkaline phosphatase (calf intestine, 1 mg/ml, 350 U/mg), snake venom phosphodiesterase (1 mg/ml, 1.5 U/mg), GMPP(CH_2)P, GMPP(NH)P, GDP(βNH_2), GDP, GTP, ADP, ATP, and poly(U) were purchased from Boehringer. [^{3}H]GMPP(CH_2)P (6600 Ci/mol), [^{3}H]GDP (5000 Ci/mol), and [^{3}H]phenylalanine (20000 Ci/mol) were supplied by Amersham. [^{35}S]GTP(γS) was synthesized by the enzymatic phosphate exchange reaction described for ATP (Glynn and Chappell, 1964) using GTP(γS) (Goody and Eckstein, 1971) and [^{35}S]thiophosphate (F. Eckstein, unpublished). [^{32}P]GTP was prepared as described by Sander et al. (1972). Purity of commercial nucleotides was monitored by chromatography on PEI-cellulose. GMPP(NH)P was highly contaminated with GDP(βNH_2) and was purified by electrophoresis (see Other Methods). Dithiothreitol was from Calbiochem, Aquasol from New England Nuclear, and 2,5-diphenyloxazole from Packard. Fusidic acid was a gift of Leo Pharmaceutical Products, Denmark. Sucrose (Merck, für biochem-

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[1] Abbreviations used are: EF-G, elongation factor G; EF-T, the complex formed by elongation factors Tu and Ts; GMPP(CH_2)P, guanylyl methylene diphosphonate; GMPP(NH)P, guanylyl iminodiphosphate; GDP^{ox-red}, guanosine 5′-diphosphate with the C(2′)–C(3′) bond oxidized by periodate and reduced by borohydride; Cbz, benzyloxycarbonyl; poly(U), poly(uridylic acid). The modified GTP's, GDP's, and ATP's are abbreviated as, e.g., GTP(γX), where γ (or β) refers to the terminal phosphate residue (see Figure 1), and X is the substituent, expressed in standard symbols, except for Azph for 4-azidophenyl. With the exception of F, S, and NH_2, the substituents are attached to P through an oxygen.

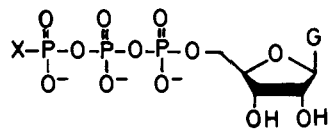

FIGURE 1: Representative structural formula for some GTP analogues. G = guanine; for GTP, X = OH; GTP(γF), X = F; GTP(γS), X = SH; GTP(γMe), X = OCH_3; GTP(γPh), X = OC_6H_5; GTP(γEtNHAc), X = $OC_2H_4NHCOCH_3$.

ische und mikrobiologische Zwecke) was treated with diethyl pyrocarbonate to inactivate RNase (Solymosy et al., 1968). All other chemicals were reagent grade.

Elongation factors and ribosomes were isolated from *Escherichia coli* BT2^r or A19. EF-G and EF-T were purified to homogeneity by modification of the published procedures (Parmeggiani, 1968; Parmeggiani et al., 1971): filtration on Sephadex G-200 was replaced by chromatography on DEAE-Sephadex A-50. The factors were stored at −25° in 50% glycerol, 50 m*M* Tris-HCl (pH 7.8 at 20°), and 2 m*M* dithiothreitol; 1 μg of EF-G and EF-T was taken to correspond to 12 and 15 pmol, respectively (Sander et al., 1975). For preparation of [^{3}H]EF-G, bacteria were grown in a medium containing in 4 l.: 20 g of NaCl, 42.8 g of KH_2PO_4, 8 g of $(NH_4)_2SO_4$, 0.8 g of $MgSO_4$, 2 mg of $FeSO_4$, 2 g of glutamic acid, and 40 g of glucose (adjusted to pH 7 with NH_4OH). 10 mCi of [^{3}H]glycine or [^{3}H]valine (Amersham, 2800 Ci/mol) was added during the early-log-phase to the medium. The cells were harvested as soon as the residual radioactivity of the medium, after centrifugation, indicated that most of the amino acid had entered the cells. [^{3}H]EF-G was extracted and purified as above. Ribosomes were prepared by suspending 0.5–1.0 g of crude ribosomes (Parmeggiani et al., 1971) in 23 ml of 0.5 *M* NH_4Cl, 20 m*M* Tris-HCl (pH 7.8), and 10 m*M* $MgCl_2$, and sedimented for 6 hr at 225000*g* in a Spinco 60 Ti rotor through 12 ml of 18% sucrose in the same buffer. This procedure was repeated two to three times; 1 A_{260} unit of ribosomes was assumed to equal 25 pmol (Hill et al., 1970). Protein concentrations were determined according to Lowry et al. (1951) using serum bovine albumin as a standard. Partially purified $tRNA^{Phe}$ from *E. coli*, accepting 152 pmol of phenylalanine/A_{260} unit of tRNA, was obtained from tRNA (Schwarz) by chromatography on benzoylated DEAE-cellulose (Gillam et al., 1967) and charged with [^{3}H]phenylalanine as described by Chinali and Parmeggiani (1973).

Synthesis of Analogues

P^3-Methyl P^1-5′-Guanosine Triphosphate. GTP (disodium salt, 0.57 g, 1 mmol) was converted into its pyridinium salt by passage over a Merck I ion exchange column (pyridinium form). For the conversion into the tri-*n*-octylammonium salt the pyridinium salt was dissolved in methanol containing tri-*n*-octylamine (0.84 ml, 2 mmol) by gentle heating with a fan, evaporated, and dried by repeated (three times) evaporation with pyridine. It was dissolved in dry dioxane (2 ml) and allowed to react for 2 hr at room temperature with diphenyl phosphorochloridate (0.3 ml) and tri-*n*-butylamine (0.45 ml). The solvents were then removed by evaporation, ether (30 ml) and petroleum ether (80 ml) were added, and the mixture was left at 0° for 30 min. The supernatant was decanted; the residue was dissolved in dioxane (3 ml) and evaporated. The residue was dissolved in pyridine (2 ml) and methanol (1 ml). After reaction for 2 hr at room temperature with stirring, the solvents were removed by evaporation. The residue was extracted with water and chromatographed on a DEAE-cellulose column (Whatman DE-52; 2.5 × 50 cm) with a linear gradient of water–0.3 *M* triethylammonium bicarbonate (1.5 l. each). The product was eluted at about 0.25 *M* buffer. The combined fractions were evaporated and the buffer was removed by repeated evaporation of the residue with methanol. Yield, 4000 A_{252} units, 0.29 mmol; ^{1}H NMR (D_2O) δ 4.36 ppm ($J_{P\text{-}O\text{-}C\text{-}H}$ = 12 Hz, d, CH_3; on ^{31}P-decoupling, s); ^{31}P NMR (D_2O, pH ~10, H-decoupled) δ 9.10 ppm (d, γ-P), 10.73 (d, α-P), 21.60 (t, β-P). Electrophoretic mobility at pH 3.5: R_{GMP} 0.47; R_{GDP} 0.82; R_{GTP} 1.0; $R_{GTP(\gamma Me)}$ 1.0. Anal. Calcd for $C_{29}H_{63}N_8O_{14}P_3$: N, 13.33; P, 11.05. Found: N, 14.10; P, 11.25.

The γ-phosphate ethyl, propyl, and butyl esters of GTP were prepared analogously as well as the γ-phosphate methyl ester of ATP. Electrophoretic mobility at pH 3.5: R_{AMP} 0.35; R_{ADP} 0.77; R_{ATP} 1.0, $R_{ATP(\gamma Me)}$ 1.0. ^{1}H NMR (D_2O) δ 3.58 ppm ($J_{P\text{-}O\text{-}C\text{-}H}$ = 12 Hz, d, CH_3; on ^{31}P-decoupling, s). Anal. Calcd for $C_{29}H_{63}N_8O_{13}P_3 \cdot 3H_2O$: N, 12.75; P, 10.57. Found: N, 12.52; P, 9.91. The β-phosphate methyl ester of GDP was synthesized in the same way using GDP instead of GTP [electrophoretic mobility at pH 3.5: R_{GDP} 1.05; R_{GTP} 1.15; $R_{GDP(\beta Me)}$ 1.0; ^{1}H NMR (D_2O) δ 4.36 ppm ($J_{P\text{-}O\text{-}C\text{-}H}$ = 12 Hz, d, CH_3; on ^{31}P-decoupling, s). Anal. Calcd for $C_{23}H_{47}N_7O_4P_2$: N, 14.19; P, 9.39. Found: N, 13.02; P, 8.80].

P^3-Aminoethyl P^1-5′-Guanosine Triphosphate. *N*-Cbz-aminoethyl Phosphate. *N*-Cbz-ethanolamine (Rose, 1947) (2 mmol, 400 mg) was dissolved in triethyl phosphate (5 ml) and $POCl_3$ (0.3 ml) added at 0°. After standing for 2.5 hr at 4°, aqueous barium acetate solution (10%, 20 ml) was added and after 5 min the material precipitated with triethylamine (2 ml). After addition of ethanol (60 ml) it was centrifuged; the residue was extracted with 70% aqueous ethanol (three times with 40 ml) and finally with water (5 × 50 ml). The aqueous solutions were combined and concentrated under reduced pressure to about 50 ml. The Ba^{2+} salt precipitated and was collected by filtration. Yield, 180 mg (23%). Anal. Calcd for $C_{10}H_{12}NO_5PBa$: N, 3.54; P, 7.85. Found: N, 3.15; P, 7.96.

P^3-(N-Cbz-aminoethyl) P^1-5′-Guanosine Triphosphate. The Ba^{2+} salt of *N*-Cbz-aminoethyl phosphate (250 mg, 0.63 mmol) was converted into the pyridinium salt by passage over a Merck I ion exchanger (pyridinium form). After evaporation to dryness this salt was converted to the tri-*n*-octylammonium salt by addition of tri-*n*-octylamine (0.26 ml) and methanol (ca. 50 ml), and gentle heating with a fan. After a clear solution had been obtained it was evaporated to dryness and dried by two evaporations with dry pyridine and one with dry $HCONMe_2$. The residue was taken up in dioxane (2 ml) and diphenyl phosphorochloridate (0.2 ml) and tri-*n*-butylamine (0.36 ml) were added. After 2 hr at room temperature, ether (20 ml) and petroleum ether (bp 40–60°, 40 ml) were added. After 30 min at 0° the supernatant was decanted, the residue dissolved in dioxane (5 ml) and evaporated, and the residue dissolved in $HCONMe_2$ (5 ml). This solution was added to the tri-*n*-octylammonium salt of GDP (1 mmol) which had been dried by repeated evaporations with pyridine and $HCONMe_2$. After 2 hr at room temperature the solution was evaporated; the residue was extracted with water and chromatographed on a DEAE-Sephadex A-25 column (2 × 30 cm) with a linear gradient of 1.5 l. each of water and 0.5 *M* triethylammonium bicarbonate (pH 7.5). The product

was eluted at about 0.3 *M* buffer. The fractions were pooled and evaporated to dryness and the buffer was removed by repeated evaporation with methanol. Yield, 4000 A_{252} units (31%). Electrophoretic mobility at pH 3.5: R_{GMP} 0.66; R_{GDP} 1.22; $R_{GTP(\gamma R)}$ 1.0.

P^3-Aminoethyl *P*^{1}-5′-Guanosine Triphosphate. *P*^{3}-(*N*-Cbz-aminoethyl) *P*^{1}-5′-guanosine triphosphate (2000 A_{252} units, 0.15 mmol) was dissolved in 5% aqueous acetic acid (5 ml) and 10% palladium on charcoal (60 mg) added. The vessel was filled with hydrogen to a slight excess above atmospheric pressure (50 cm of H_2O) and hydrogenation carried out for 2 hr. The catalyst was filtered off; the filtrate was evaporated to dryness and chromatographed on a DEAE-Sephadex A-25 column (1.5 × 20 cm) with a linear gradient of 500 ml each of H_2O and 0.5 *M* triethylammonium bicarbonate (pH 7.5). The product was eluted at about 0.25 *M* buffer. Yield, 1800 A_{252} units. Electrophoretic mobility at pH 3.5: R_{GMP} 0.77; R_{GDP} 1.39; $R_{GTP(\gamma EtNH_2)}$ 1.0.

P^3-*N*-*Acetylaminoethyl P*^{1}-5′-*Guanosine Triphosphate.* To a solution of the tri-*n*-octylammonium salt of *P*^{3}-aminoethyl *P*^{1}-5′-guanosine triphosphate (450 A_{252} units) in methanol (2 ml) a solution of acetyl-*N*-hydroxysuccinimide (157 mg, 1 mmol) (De Groot et al., 1966) in acetone (2 ml) was added. After reaction for 1 hr at room temperature the solution was evaporated; the residue was extracted with water and chromatographed on a DEAE-Sephadex A-25 column (1.5 × 20 cm) with a linear gradient of 500 ml each of water and 0.5 *M* triethylammonium bicarbonate (pH 7.5). The material was eluted at about 0.35 *M* buffer. Yield, 350 A_{252} units. Electrophoretic mobility at pH 3.5: R_{GDP} 0.95; $R_{GTP(\gamma EtNHAc)}$ 1.0; ^{1}H NMR (D_2O) δ 2.0 ppm (s, CH_3). Anal. Calcd for $C_{32}H_{68}N_9O_{15}P_3$: N, 13.82; P, 10.19. Found: N, 14.26; P, 10.78.

The corresponding ester of ATP was synthesized in the same way.

P^2-*Phenyl P*^{1}-5′-*Guanosine Diphosphate.* This compound was synthesized as described for *P*^{3}-(*N*-Cbz-aminoethyl) *P*^{1}-5′-guanosine triphosphate using phenyl phosphate (2 mmol) and GMP (1 mmol) as starting materials. Yield, 3900 A_{252} units. Electrophoretic mobility at pH 3.5: R_{GMP} 0.59; R_{GDP} 1.15; $R_{GDP(\beta Ph)}$ 1.0; $\lambda_{max}^{H_2O}$ 252 nm (ϵ 13300); ^{1}H NMR (D_2O) δ 7.65 ppm (s, 5 H); ^{31}P NMR (D_2O) δ 11.57 ppm (d) and 16.16 (d). Anal. Calcd for $C_{28}H_{47}N_7O_{11}P_2$: N, 13.62; P, 8.60. Found: N, 13.65; P, 8.47.

P^3-*Phenyl P*^{1}-5′-*Guanosine Triphosphate.* This compound was synthesized by activation of phenyl phosphate (2 mmol) with carbonyldiimidazole, in analogy to the work of Barker et al. (1972), and reaction with GDP (1 mmol). Purification was carried out by chromatography on DEAE-Sephadex as described for GTP(γMe). Yield, 300 A_{252} units. Electrophoretic mobility at pH 3.5: R_{GTP} 1.0; $R_{GTP(\gamma Ph)}$ 1.0; R_{GDP} 0.85; $R_{GDP(\beta Ph)}$ 0.85. ^{1}H NMR (D_2O) δ 7.65 ppm (s, 5 H); ^{31}P NMR (D_2O) δ 11.6 ppm (d); 16.2 (d); 23.3 (t). Anal. Calcd for $C_{34}H_{63}N_8O_{14}P_3$: N, 12.43; P, 10.31. Found: N, 11.98; P, 10.68. In this synthesis 330 A_{252} units of GDP(βPh) was produced as well.

P^3-*Fluoro P*^{1}-5′-*Guanosine Triphosphate and P*^{2}-*Fluoro P*^{1}-5′-*Guanosine Diphosphate.* These compounds were prepared following the procedure given for the synthesis of *P*^{3}-(*N*-Cbz-aminoethyl) *P*^{1}-5′-guanosine triphosphate with 1 mmol of GDP and using phosphorofluoridic acid instead of *N*-Cbz-aminoethyl phosphate. The reaction with GDP led to the di- as well as the triphosphate derivative. This method was also used for the synthesis of ATP(γF) by Haley and Yount (1972). Yield, 1320 A_{252} units of the diphosphate derivative and 1350 A_{252} units of the triphosphate derivative.

Diphosphate derivative: ^{31}P NMR (D_2O) δ 11.05 ppm (d, J = 19.7 Hz); 17.94 ppm (d, $J_{P\text{-}F}$ = 450 Hz; d, $J_{P\text{-}P}$ = 19.7 Hz). Anal. Calcd for $C_{22}H_{42}N_7O_{10}P_2F$: N, 15.56; P, 9.85; F, 2.70. Found: N, 15.14; P, 9.56; F, 2.93.

Triphosphate derivative: ^{31}P NMR (D_2O) δ 11.87 (d, J = 19.0 Hz) and 18.33 ppm (d, $J_{P\text{-}F}$ = 450 Hz; d, $J_{P\text{-}P}$ = 19.0 Hz). Anal. Calcd for $C_{28}H_{60}N_8O_{13}P_3F$: N, 13.52; P, 11.21; F, 2.29. Found: N, 14.06; P, 11.20; F, 2.2.

[^{3}H]GTP(γF) was synthesized in the same way starting with [^{3}H]GDP.

All of these analogues were stable against alkaline phosphatase and were degraded to GMP by snake venom phosphodiesterase. To eliminate contaminations of GTP or GDP, all modified nucleotides were treated with alkaline phosphatase. Approximately 1 μmol of substrate was incubated in 50 μl of 0.1 *M* Tris-HCl (pH 8.5) containing 5 μl of alkaline phosphatase for 2 hr at 37°. Recovery of the nucleotide was achieved by electrophoresis or column chromatography on DEAE-Sephadex A-25. All of these nucleotides were stable for several months when stored at −35° as frozen aqueous solutions even when repeatedly thawed and frozen.

Other Methods. Nuclear magnetic resonance spectra were recorded on a Bruker Physik HFX 60 spectrometer equipped with a Nicolet FT 1074 averaging system with Me_4Si as internal standard for ^{1}H spectra and dilute H_3PO_4 as external standard for ^{31}P spectra. Chemical shifts are given in δ units (ppm). Electrophoresis was carried out on paper, Schleicher & Schüll, 2043b (washed), at pH 3.5 (0.05 *M* ammonium formate) for 90 min and 30 V/cm. Chromatography on PEI-cellulose (Polygram CEL 300 PEI/UV, Macherery & Nagel) was done in 0.75 *M* KH_2PO_4 at pH 3.5 for 2 hr. For determination of labeled compounds the PEI-cellulose plates were cut in pieces (1.5 × 1.5 cm) and measured in a toluene scintillation fluid. Radioactivity was measured with a Packard 3380 scintillation spectrometer using either 5 ml of a toluene scintillation fluid containing 5 g of 2,5-diphenyloxazole/l. or 10 ml of Aquasol.

Assay of EF-G-Dependent Ribosomal Activities. Ribosome-dependent EF-G GTPase activity was tested by measuring the liberated inorganic phosphate (Parmeggiani et al., 1971). Stable EF-G·nucleotide·ribosome complexes were isolated by chromatography on a Sephadex G-200 column (25 × 0.7 cm) equilibrated with 50 m*M* Tris-HCl (pH 7.8), 20 m*M* $MgCl_2$, 80 m*M* NH_4Cl, and 1 m*M* dithiothreitol, or by sedimentation through 3 ml of a 10% sucrose cushion containing 50 m*M* Tris-HCl (pH 7.8), 20 m*M* $MgCl_2$, 80 m*M* NH_4Cl, and 1 m*M* dithiothreitol in a Spinco 50 Ti rotor. The centrifuge tubes were filled up with paraffin oil; centrifugation was done at 134000*g* for 150 min. The supernatant was rapidly removed and the small transparent ribosomal pellet rinsed with 0.1 ml of a solution containing 20 m*M* Tris-HCl (pH 7.8), 20 m*M* $MgCl_2$, 30 m*M* KCl, 30 m*M* NH_4Cl, and 1 m*M* dithiothreitol, and then suspended in 0.1 ml of the same solution by gentle shaking for 30–60 min. The amount of EF-G bound to ribosomes was calculated from the GTPase activity of the isolated ribosomal complex, corrected for the activity of ribosomes isolated under the same conditions in the absence of nucleotides. To test in a range linear to the amount of factor bound, the molar ratio of ribosomes to EF-G was at least 30

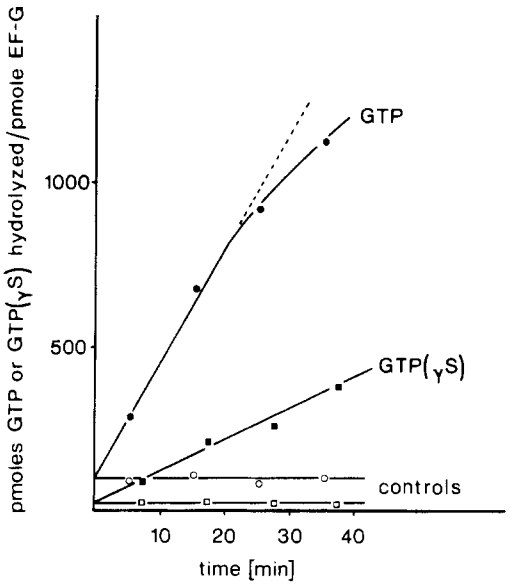

FIGURE 2: Rate of ribosome-EF-G-dependent hydrolysis of GTP and GTP(γS). The reaction mixture contained in 0.020 ml: 20 m*M* Tris-HCl (pH 7.8), 15 m*M* $MgCl_2$, 80 m*M* NH_4Cl, 1 m*M* dithiothreitol, 19 pmol of ribosomes, 29 pmol of EF-G when present, and 1.7 m*M* [^{32}P]GTP (1.5 Ci/mol) or 4.4 m*M* [^{35}S]GTP(γS) (1 Ci/mol). The reaction mixture was incubated at 30°. Aliquots of 4 μl were taken at the indicated time intervals and analyzed on PEI-cellulose (see Experimental Procedure). [^{32}P]GTP plus (●) and minus (○) EF-G; [^{35}S]GTP(γS) plus (■) and minus (□) EF-G.

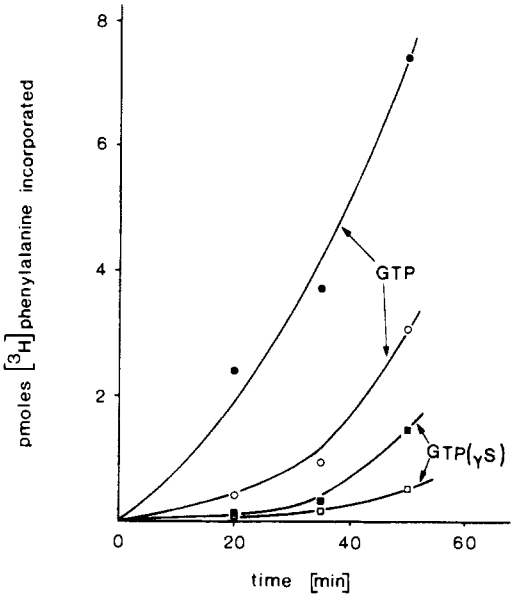

FIGURE 3: Rate of GTP(γS)- and GTP-catalyzed poly(phenylalanine) synthesis. The reaction mixture contained in a final volume of 0.10 ml: 50 m*M* Tris-HCl (pH 7.8), 9 m*M* $MgCl_2$, 53 m*M* KCl, 1 m*M* dithiothreitol, 28 pmol of ribosomes, 6.2 μg of poly(U), 200 pmol of [^{3}H]Phe-tRNA^{Phe} (8030 Ci/mol), 60 pmol of EF-T, and 7 pmol of EF-G in the presence of 6.6×10^{-5} (○) and 3.3×10^{-4} *M* (●) GTP or 6.6×10^{-5} (□) and 3.3×10^{-4} *M* (■) GTP(γS); 25-μl samples were taken from the reaction mixture at the indicated time intervals. The activity was determined by measuring the incorporation of [^{3}H]phenylalanine into hot trichloroacetic acid insoluble material using GFA filters (Haenni and Chapeville, 1966).

(Chinali and Parmeggiani, 1973). This was obtained by adding untreated ribosomes to the assay system.

Chromatography of [^{3}H]EF-G·ribosome complexes was done on Sepharose 6B columns (0.5 × 7 cm) equilibrated with 20 m*M* Tris-HCl (pH 7.8), 10 m*M* $MgCl_2$, 10 m*M* NH_4Cl, and, when present, 0.2 m*M* fusidic acid. For the calculation of the amount of nucleotide bound in the complex, radioactivity of 0.10-ml aliquots of the 0.15-ml fractions, containing about 15 pmol of ribosomes, was measured in 10 ml of Aquasol and the amount of bound EF-G calculated on the basis of a 1:1 EF-G/nucleotide ratio. The ability of the various nucleotide analogues to displace GMPP(CH_2)P or GDP bound to EF-G·ribosome complexes was assayed by determining the amount of [^{3}H]GMPP(CH_2)P or [^{3}H]GDP retained on nitrocellulose filters (Sartorius, 0.45-μm pore size) in the presence of different concentrations of the nucleotide analogues. For details of experimental conditions see legends.

Results

Properties of the γ-Phosphate Analogues of GTP. The γ-phosphate methyl ester of ATP has previously been prepared by Wehrli et al. (1964) by condensation of ATP and methanol with dicyclohexylcarbodiimide. For the synthesis of the γ-phosphate alkyl esters of GTP and ATP we preferred activation of the nucleotide with diphenyl phosphorochloridate (Michelson, 1964) and subsequent reaction with the corresponding alcohol. This method has also been found to furnish good yields in the synthesis of other nucleoside triphosphate derivatives (Goody, Fröhlich, Walter and Schirmer; personal communication). The ^{31}P NMR spectrum of the methyl ester of GTP only showed a significant shift of the γ phosphorus from δ 5.08 ppm for GTP to δ 9.10 ppm. Together with the resistance of this compound to alkaline phosphatase this indicates that esterification occurred at the terminal phosphate. This method of synthesis was only applicable to liquid alcohols. For the preparation of derivatives of the aminoethyl ester of nucleoside triphosphates an approach was chosen which had been successful in the synthesis of ATP(γS) (Goody and Eckstein, 1971) and the *p*-nitrophenyl ester of ATP (Berglund and Eckstein, 1972). *N*-Cbz-aminoethyl phosphate was activated with diphenyl phosphorochloridate and allowed to react with a nucleoside diphosphate. Removal of the NH_2-protecting group yields a compound which can be selectively acylated using the esters of *N*-hydroxysuccinimide (De Groot et al., 1966). For the synthesis of a similar compound, P^2-(6-amino-1-hexyl) P^1-5′-uridine diphosphate, Barker et al. (1972) gave preference to activation by carbonyldiimidazole while protecting the amino group as its trifluoroacetyl derivative.

P^3-Fluoro P^1-5′-adenosine triphosphate, ATP(γF), had been synthesized earlier by Haley and Yount (1972) as an ATP analogue resistant to alkaline phosphatase, myosin, and hexokinase. We found that in the synthesis of GTP(γF) substantial amounts of GDP(βF) were formed. This might be due to hydrolysis of the nucleoside diphosphate to the nucleoside monophosphate prior to the condensation reaction. Attempts to synthesize this analogue by reaction of the nucleoside triphosphate with dinitrofluorobenzene in analogy to a similar reaction with thymidine 5′-monophosphate (Wittmann, 1963) were not successful.

Ability of the γ-Phosphate Analogues of GTP to be Hydrolyzed by EF-G and Ribosome and to Support Poly(U)-Directed Poly(phenylalanine) Synthesis. The γ-phosphate analogues with the exception of GTP(γS) were found to be resistant to the hydrolytic action of EF-G and ribosomes as is the case with GMPP(CH_2)P and GMPP(NH)P. This was measured in a reaction mixture containing in a final volume of 0.020 ml: 20 m*M* Tris-HCl (pH 7.8), 13 m*M*

Table I: Inhibition of Ribosome-Dependent EF-G GTPase by Various Nucleotides.

Nucleotide	K_i [M]	Nucleotide	K_i [M]
GDP	3.6×10^{-5}	GTP(γBu)	7.5×10^{-5}
GMPP(CH_2)P	3.9×10^{-5}	GTP(γEtNH_2)	4.4×10^{-4}
GMPP(NH)P	4.3×10^{-5}	GTP(γEtNHAc)	4.6×10^{-4}
GTP(γS)	1.3×10^{-5}	GTP(γPh)	2.1×10^{-4}
GTP(γF)	1.0×10^{-6}	GDP(βNH_2)	2.4×10^{-4}
GTP(γMe)	2.5×10^{-6}	GDP(βF)	4.0×10^{-4}
GTP(γEt)	4.5×10^{-5}	GDP(βMe)	2.8×10^{-4}
GTP(γPr)	5.6×10^{-5}	GDP(βPh)	1.0×10^{-3}

[a] The reported K_i values were the average of several experiments. Under these conditions the K_m for GTP was $5.6-6.6 \times 10^{-5}$ M. ATP(γMe) and ATP(γEtHNAc) showed no inhibition at concentrations up to 1.0×10^{-3} M. The reaction mixture contained in 0.075 ml: 50 mM Tris-HCl (pH 7.8), 13 mM $MgCl_2$, 80 mM NH_4Cl, 1 mM dithiothreitol, 55 pmol of ribosomes, 14 pmol of EF-G, 0.1 μCi of [^{32}P]GTP, GTP in five different concentrations (0.02, 0.04, 0.08, 0.24, 1.20 mM), and nucleotide analogs in concentrations about twice their K_i. Incubation time: 15 min at 30°.

Table II: Nucleotide-Dependent Binding of [^{3}H]EF-G to Ribosomes in the Presence and Absence of Fusidic Acid.[a]

	pmol of [^{3}H]EF-G/pmol of Ribosome	
	− Fusidic Acid	+ Fusidic Acid
GDP	0.15	0.66[b]
GMPP(CH_2)P	0.72	0.67[b]
GMPP(NH)P	0.61	0.73
GTP(γS)	0.07	0.70[b]
GTP(γF)	1.04	1.05[b]
GTP(γMe)	0.81	0.77
GTP(γEt)	0.62	0.66
GTP(γPr)	0.38	0.49
GTP(γBu)	0.37	0.50
GTP(γEtNH_2)	0.05	0.62
GTP(γEtNHAc)	0.04	0.31
GTP(γPh)	0.00	0.32
GDP(βF)	0.11	0.35
GDP(βMe)	0.05	0.31
GDP(βPh)	0.08	0.11
GDP(βNH_2)	0.06	0.08
ATP(γMe)	0	0
ATP(γEtNHAc)	0	0

[a] Binding of [^{3}H]EF-G to ribosomes was performed in a mixture containing in 0.020 ml: 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM NH_4Cl, 1 mM dithiothreitol, 75 pmol of ribosomes, 188 pmol of [^{3}H]EF-G (66 Ci/mol), 1.5 mM fusidic acid when present, and 0.1 mM nucleotides. Incubation time: 10 min at 30°. For the isolation of the [^{3}H]EF-G·ribosome complex on Sepharose 6B and determination of the radioactivity see Experimental Procedures. [b] These results were confirmed by using ^{3}H-labeled nucleotides.

$MgCl_2$, 80 mM NH_4Cl, 1 mM dithiothreitol, 105 pmol of EF-G, 12 pmol of ribosomes, and 50 nmol of nucleotides. After incubation for 120 min at 30° no hydrolytic products were detected by thin-layer chromatography on PEI-cellulose, whereas GTP was already completely hydrolyzed after 15 min. As shown in Figure 2 the initial rate of GTP(γS) hydrolysis was found to be 28% of that of GTP. The activity of GTP(γS) was measured also in poly(U)-directed poly(phenylalanine) synthesis: initial rate of GTP(γS)-catalyzed incorporation of [^{3}H]phenylalanine was found to be 10–20% of that of the GTP-catalyzed reaction (Figure 3). This result does not differentiate between the EF-G- and the EF-Tu–GTPase activity. The effect of GTP(γS) on the latter activity is presently under investigation.

Inhibition of the Ribosome-Dependent EF-G GTPase Activity. All GTP analogues were found to be competitive inhibitors of the GTP hydrolysis catalyzed by EF-G and ribosomes. In Table I the K_i values for the different analogues, calculated from double reciprocal plots, are reported. GTP(γF) and GTP(γMe) are the most potent inhibitors even in comparison to GMPP(CH_2)P and GMPP(NH)P. The inhibitory activity of the γ-phosphate esters of GTP decreased with increasing length of the side chain. GTP(γEtNH_2) and GTP(γEtNHAc) showed a lower inhibition than the corresponding alcohol esters. Adenosine triphosphate analogues, tested to control the specificity of this reaction, did not show any inhibition. GDP, the product of the GTPase reaction, is an effective competitive inhibitor of the ribosome-dependent EF-G GTP hydrolysis (Conway and Lipmann, 1964) while the β-phosphate analogues of GDP, GDP(βMe), GDP(βF), GDP(βPh), and GDP(βNH_2) were found to be much less effective than GDP.

Activity of the Nucleotide Analogues in the Formation of the EF-G·Ribosome Complex. The nucleotide-dependent binding of EF-G to ribosomes was directly measured by using labeled elongation factor in the presence or absence of fusidic acid (Table II). GTP(γF) was the most effective nucleotide analogue inducing formation of a complex containing equimolar amounts of EF-G and ribosomes. In general the effect of most triphosphate analogues in the absence of fusidic acid followed a pattern similar to that observed in the inhibition of EF-G GTPase activity. In the presence of fusidic acid, GTP(γS) stimulated the binding of EF-G to the same extent as that induced by GDP. Parallel experiments using either ^{3}H- or ^{35}S-labeled GTP(γS) showed that 80–90% of the nucleotide bound to the complex was in this case represented by GDP. Of the other analogues the greatest effect of fusidic acid was observed with GDP(βF), GDP(βMe), GTP(γPh), and with the aminoalkyl ester derivatives of GTP. For comparison we studied also the effect of GMPP(CH_2)P and GMPP(NH)P in this system. The GMPP(CH_2)P-mediated binding of [^{3}H]EF-G to ribosomes was slightly inhibited by fusidic acid in line with observations of other authors while that of GMPP(NH)P showed a stimulation (Eckstein et al., 1971). The amount of [^{3}H]EF-G bound to ribosomes in the presence of GMPP(CH_2)P was the same as the amount of labeled GMPP(CH_2)P bound in the EF-G·ribosome complex (see Figure 4).

Nucleotide-dependent binding of EF-G to ribosomes was also investigated by isolation of stable EF-G·ribosome complexes on Sephadex G-200 and upon ultracentrifugation. The pattern of activity confirms the results obtained with [^{3}H]EF-G. A 50–65% yield of complex was obtained with ultracentrifugation when compared to filtration on Sephadex G-200 (Table III). This was likely due to the hydrostatic pressure, which dissociates the less stable part of EF-G·ribosome complexes.

The possibility that [^{3}H]GTP(γF) had been bound covalently to the EF-G·ribosome complex was excluded by the observation that addition of sodium dodecyl sulfate to a final concentration of 1% caused the release of the radioactivity otherwise retained on nitrocellulose filter (results not shown).

Ability of the Nucleotide Analogues to Displace GDP or GMPP(CH_2)P from EF-G·Ribosome Complexes. To study in more detail the affinity and specificity of the GTP and GDP analogues for EF-G and ribosomes we investigated the ability of a few representative nucleotide analogues to

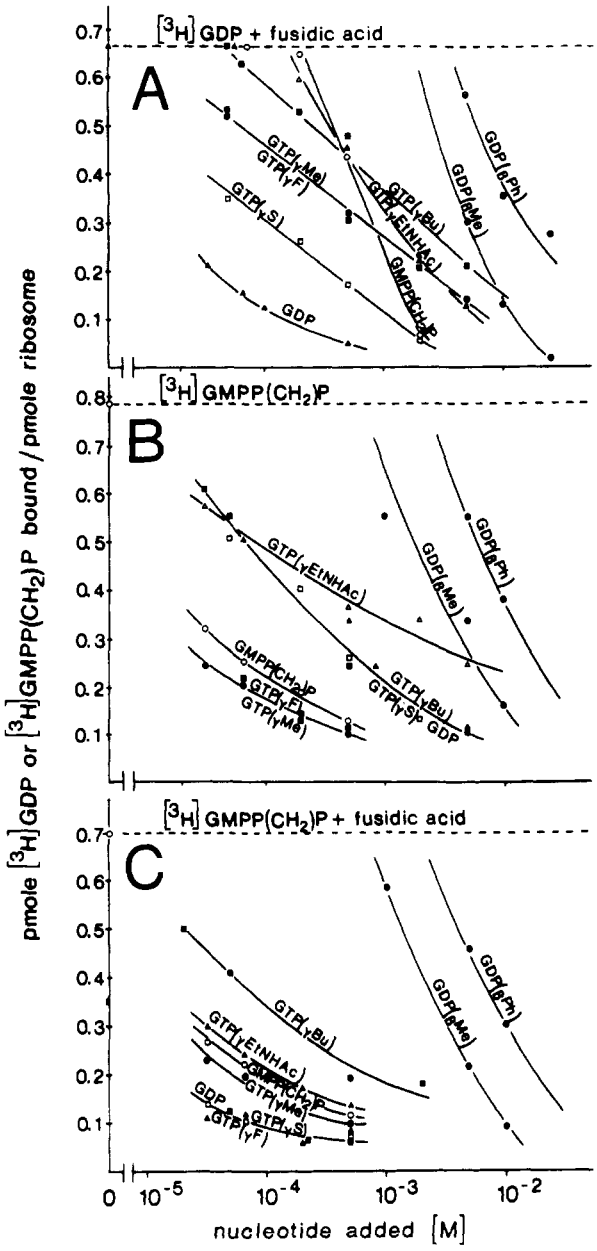

FIGURE 4: Ability of nucleotide analogues to displace GDP or $GMPP(CH_2)P$ from their respective EF-G·ribosome complexes. Complex formation was carried out in a reaction mixture of 0.030 ml containing in a final volume of 0.035 ml: 20 m*M* Tris-HCl (pH 7.8), 10 m*M* $MgCl_2$, 10 m*M* NH_4Cl, 1 m*M* dithiothreitol, 35 pmol of ribosomes, 125 pmol of EF-G, 1 m*M* fusidic acid when indicated, and 2×10^{-5} *M* [^{3}H]$GMPP(CH_2)P$ or [^{3}H]GDP (70 Ci/mol). After an incubation of 10 min at 30°, 5 μl of unlabeled nucleotides was added to the final concentration indicated. After a second incubation of 15 min at 30°, an aliquot of 25 μl was taken from the reaction mixture and pipetted under 10 ml of cold buffer (20 m*M* Tris-HCl, pH 7.8, 10 m*M* $MgCl_2$, and 10 m*M* NH_4Cl) onto the surface of a nitrocellulose filter. Suction was immediately applied so that filtration process lasted a few seconds. Filters were dried and radioactivity was measured in toluene scintillation fluid. The dashed lines indicate the level of [^{3}H]GDP or [^{3}H]$GMPP(CH_2)P$ bound to the EF-G·ribosome complex without addition of unlabeled nucleotide. The amount of [^{3}H]GDP (0.15 pmol/pmol of ribosome) or [^{3}H]$GMPP(CH_2)P$ (0.08 pmol/pmol of ribosome) bound in the absence of EF-G was subtracted. (▲) GDP; (○) $GMPP(CH_2)P$; (□) GTP(γS); (⊡) GTP(γF); (●) GTP(γMe); (■) GTP(γBu); (△) GTP(γEtNHOAc); (⊙) GDP(βMe); (⊖) GDP(βPh).

displace [^{3}H]GDP and [^{3}H]$GMPP(CH_2)P$ from their respective EF-G·ribosome complexes. To cover a wider range of conditions we have chosen both the fusidic acid-GDP and the $GMPP(CH_2)P$-dependent complexes, because observations indicate that they are functionally not equivalent (Modolell et al., 1973; Otaka and Kaji, 1974; Inoue-Yokosawa et al., 1974). The displacement was determined by measuring the amount of the radioactivity that remained bound to the EF-G·ribosome complexes in the presence of various concentrations of unlabeled nucleotide analogues. Figure 4A shows the results obtained with the fusidic acid·[^{3}H]GDP·EF-G·ribosome complex. GDP was the most active compound in this reaction. At a concentration of 2×10^{-5} *M*, 50% of the bound [^{3}H]GDP was displaced after 15 min of incubation. GTP(γS) was required in a somewhat higher concentration (5×10^{-5} *M*). With increasing length of the side chain, the γ-phosphate esters turned out to be increasingly less effective. It is interesting to note that $GMPP(CH_2)P$ showed as little activity as GTP(γEtNHAc). The GDP analogues, GDP(βMe) and GDP(βPh), were the least effective compounds: they required concentrations of 5×10^{-2} *M* and 10^{-2} *M*, respectively, to displace 50% of [^{3}H]GDP.

With the [^{3}H]$GMPP(CH_2)P$·EF-G·ribosome complex (Figure 4B), GTPγF, GTP(γMe), and $GMPP(CH_2)P$ were most active. Under the conditions chosen 50% displacement occurred in a concentration range of $1-2 \times 10^{-5}$ *M*. All triphosphate esters of GTP tested, as well as GTP(γF), were more effective in the displacement of [^{3}H]$GMPP(CH_2)P$ than of [^{3}H]GDP.

Additional presence of fusidic acid during formation of

Table III: Nucleotide-Dependent Binding of EF-G to Ribosomes Measured as Ribosome–EF-G GTPase Activity.

	pmol of GTP Hydrolyzed/pmol of Ribosome in 10 min at 30°	
	Sephadex G-200	Ultracentrifugation
—nucleotide	26.1	13.3
GDP	2.5	1.1
$GMPP(CH_2)P$	246.8	162.6
GMPP(NH)P	188.4	131.4
GTP(γS)	4.3	7.8
GTP(γF)	281.4	
GTP(γMe)	320.8	214
GTP(γEt)	75.1	52.4
GTP(γPr)	55.8	24.6
GTP(γBu)	46.6	26.1
GTP(γEtNH$_2$)	3.9	
GTP(γEtNHAc)	3.9	
GDP(βMe)	25	19.3
GDP(βPh)	7.1	19.3
ATP(γMe)	0	
ATP(γEtNHAc)	0	

[a] Nucleotide-dependent binding of EF-G to ribosomes was investigated by isolation of the nucleotide·EF-G·ribosome complexes either on Sephadex G-200 filtration or by ultracentrifugation (see Experimental Procedure). For filtration on Sephadex binding of EF-G to ribosomes was performed in a mixture containing in 0.020 ml: 50 m*M* Tris-HCl (pH 7.8), 20 m*M* $MgCl_2$, 80 m*M* NH_4Cl, 1 m*M* dithiothreitol, 250 pmol of ribosomes, 640 pmol of EF-G, and $2-10^{-4}$ *M* nucleotides. Incubation time: 10 min at 30°. GTPase activity of 7 pmol of EF-G·ribosome complex passed through Sephadex G-200 was measured in a reaction mixture containing in 0.075 ml: 50 m*M* Tris-HCl (pH 7.8), 13 m*M* $MgCl_2$, 80 m*M* NH_4Cl, 1 m*M* dithiothreitol, 0.1 μCi of [^{32}P] GTP (7 Ci/mol), and 200 pmol of 70S ribosomes. For ultracentrifugation binding mixture contained in 0.3 ml: 50 m*M* Tris-HCl (pH 7.8), 20 m*M* $MgCl_2$, 80 m*M* NH_4Cl, 1 m*M* dithiothreitol, 412 pmol of ribosomes, 960 pmol of EF-G, 8×10^{-5} *M* nucleotides. GTPase activity of the isolated ribosomes was measured as described for the EF-G·ribosome complex isolated on Sephadex G-200.

the [^{3}H]GMP(CH_2)·EF-G·ribosome complex significantly affected the patterns of the reaction (Figure 4C). GTP(γS) and GDP became more effective than GMPP(CH_2)P, while GTP(γEtNHAc) became nearly as active as GMPP(CH_2)P. The diphosphate analogues GDP(βMe) and GDP(βPh) showed little activity in all three systems.

Discussion

The introduction of different modifications at the terminal phosphate of GTP and GDP allowed us to obtain some general conclusions about the effect of such alterations on the interaction of the nucleotide with the active site of the ribosome-EF-G GTPase. Modification of the γ-phosphate results in compounds which, with the exception of GTP(γS), cannot be hydrolyzed by EF-G and the ribosome. These analogues are, however, competitive inhibitors of GTP hydrolysis and thus are able to interact with its catalytic center. Since only one hydroxyl group in the terminal phosphate of our nucleotide analogues was modified, these results suggest that both negative charges of the two hydroxyl groups of the γ-phosphate are essential for compounds to act as substrates, but one is sufficient for the binding to the active site. One has, therefore, to conclude that the second negative charge of the γ-phosphate does not significantly contribute to the binding energy. The need of two negative charges in the terminal phosphate of GTP for hydrolysis seems to be strengthened by the observation that among the analogs only GTP(γS) can be hydrolyzed and be active in poly(U)-dependent poly(phenylalanine) incorporation even if at a lower rate than GTP.

A nucleophilic attack in an SN2 mechanism on the γ phosphorus has recently been proposed as a mechanism for GTP hydrolysis in ribosome-EF-G GTPase (Rohrbach et al., 1974). However, an alternative reaction, the formation of metaphosphate in an elimination mechanism, which is operative in the hydrolysis of phosphate monoesters (Benkovic and Schray, 1973) has also to be considered for this GTPase. The GTP esters might be expected still to be able to undergo an SN2-type reaction, although one cannot exclude that the proper alignment of the functional groups necessary for the enzymatic reaction might be impaired by the ester group. If the hydrolysis of GTP, however, proceeds by the elimination mechanism one would expect the esters to be inert since there is no evidence for elimination of metaphosphate from diesters (Benkovic and Schray, 1973). The observed stability of GTP esters might therefore be an indication of an elimination type mechanism for this enzyme system. Because of the inherent difficulties in extrapolating from nonenzymatic to enzymatic reactions the possibility that the EF-G GTPase might follow an elimination rather than an SN2 mechanism cannot be more than a suggestion at the present time.

The rate of hydrolysis of GTP(γS) is unexpectedly high compared to that of GTP. The cleavage step of ATP(γS) in the myosin-catalyzed reaction is about 1000 times slower than that of ATP (Bagshaw et al., 1974). Also, the hydrolysis of ATP(γS) by alkaline phosphatase is extremely slow (Goody and Eckstein, 1971). On the other hand, the ATPase of the sarcoplasmatic reticulum (Gratecos and Fischer, 1974) seems to cleave ATP(γS) faster than ATP. Whether these variations in relative rates are due to different mechanisms for different enzymes has to await further study. The activity in ribosome-EF-G-dependent reactions of the alkyl ester derivatives of GTP could be correlated to the length of the alcohol: increasing the number of carbon atoms in the added side chain progressively decreased their inhibitory activity likely as a direct consequence of a reduced accessibility to the active site.

The same chemical modification in the terminal phosphate of GTP and GDP does not cause comparable inhibitory effects in the EF-G-dependent reactions: GDP(βF) and GDP(βMe) are about 100-fold less active than the corresponding GTP analogues. Since GDP can effectively compete with GTP for the active site, this indicates together with the results obtained with the GTP analogues that the presence of at least three negative charges in the phosphate chain of the nucleotide is needed for effective binding to the active site.

Since the actual hydrolysis of the γ-phosphate is preceded by the formation of a ternary complex with EF-G and ribosomes, it was to be expected that the activity of the different analogues in complex formation followed a pattern similar to their activity in the GTPase reaction. Substitution of one hydroxyl group of the γ-phosphate with a fluoride produced an effective GTP analogue, which could apparently enable all ribosomes to participate in the formation of the EF-G·ribosome complex. Thus the GTP(γF)-induced EF-G·ribosome complex would be most suitable for the identification of the ribosomal components neighboring EF-G by the use of cross-linking reagents in the absence of fusidic acid. A clear relationship between the action of fusidic acid and the structure of the different nucleotide analogues cannot be seen: only the aminoalkyl ester derivatives, GTP(γPh), as well as GDP(βF) and GDP(βMe), are considerably stimulated by this antibiotic. The different properties of the various GTP and GDP analogues relative to their sensitivity toward fusidic acid is likely to be the result of a different functional alignment of the components in the ternary complexes. Structural modifications in the β-γ-phosphate bond, as in GMPP(NH)P and GMPP(CH_2)P, also cause different sensitivity toward fusidic acid. Although these two nucleotide analogues inhibited GTPase activity to a similar degree, only the ability of GMPP(NH)P to stimulate complex formation was increased by fusidic acid. The observation that the spatial configuration of imidodiphosphate is closer to that of pyrophosphate than to methylene diphosphonate (Larsen et al., 1969) might explain the greater similarity of GMPP(NH)P to GTP. The molar amounts of [^{3}H]EF-G and labeled GDP, GTP(γF), GTP(γS), and GMPP(CH_2)P measured in the respective EF-G·ribosome complexes were equivalent. The possibility that two molecules of EF-G are required for each GMPP(CH_2)P bound to the ribosome as reported by Kuriki (1973) is not supported by our experiments. The displacement of GDP and GMPP(CH_2)P from their respective complexes with EF-G and ribosomes underlined the different properties of the nucleotide analogues and the difference between the GMPP(CH_2)P- and the GDP-fusidic acid-dependent complexes. In general, nucleotide analogues insensitive to fusidic acid, as GTP(γF) and GTP(γMe), displaced GMPP(CH_2)P more readily than GDP. Nucleotide analogues sensitive to fusidic acid were most effective in the GMPP(CH_2)P system when fusidic acid was present, indicating that the antibiotic increased their stability relative to that of the GMPP(CH_2)P-dependent EF-G·ribosome complex. The possibility to improve the accessibility of a nucleotide analogue to the active site by displacing a compound prebound in the EF-G·ribosome complex was tempting. In this regard it was of particular interest that GTP(γEtNHAc), a potential substrate for affinity labeling,

which was a poor inhibitor of the EF-G GTPase activity, dramatically increased its activity in the displacement reaction by going from the GDP to the GMPP(CH_2)P system in the presence of fusidic acid. Whether this increased activity corresponds to a specific binding to the EF-G·ribosome complex remains, however, to be proved.

The possibility of a covalent binding of bromo- or chloroacetylaminoalkyl ester derivatives of GTP to the active site of the EF-G–ribosome is presently under investigation. GTP(γF) was unfortunately found to be unable to react covalently with EF-G or the ribosome. ATP(γF) could also not be bound irreversibly to myosin (Haley and Yount, 1972). Similarly, attempts to use a ribose-modified GDP analogue, GDP^{ox-red}, as an affinity label for EF-G and ribosome were also not successful (Bodley and Gordon, 1974). GDP(βPh), the parent compound of the azidophenyl derivative of GDP, GDP(βAzph), which has been used for photoaffinity labeling of the 50S ribosomal subunit (Maassen and Möller 1974), showed little activity in the EF-G-dependent reactions we tested.

Nucleoside triphosphate analogues modified in the γ-phosphate have hardly been studied in enzymatic reactions and it is therefore difficult to say whether they will be generally useful. ATP(γF) (Haley and Yount, 1972) and ATP(γMe) (R. S. Goody, personal communication) have been found to be very weak inhibitors of the myosin-dependent ATPase. Also for DNA-dependent RNA polymerase from *E. coli* ATP(γMe) and ATP(γF) are only very poor inhibitors at best (Sternbach and Armstrong, personal communication). It could well be that only a certain class of ATP- or GTP-utilizing enzymes can tolerate such modifications at the γ-phosphate without appreciable loss of affinity to the active site.

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References

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., and Trentham, D. R. (1974), *Biochem. J. 141,* 351.

Barker, R., Olsen, K. W., Shaper, J. H., and Hill, R. L. (1972), *J. Biol. Chem. 247,* 7135.

Benkovic, S. J., and Schray, K. I. (1973), *Enzymes, 3rd Ed. 8,* 201.

Berglund, O., and Eckstein, F. (1972), *Eur. J. Biochem. 28,* 492.

Bodley, J. W., and Gordon, J. (1974), *Biochemistry 13,* 3401.

Chinali, G., and Parmeggiani, A. (1973), *Eur. J. Biochem. 32,* 463.

De Groot, N., Lapidot, Y., Panet, A., and Wolman, Y. (1966), *Biochem. Biophys. Res. Commun. 25,* 17.

Eckstein, F., Kettler, M., and Parmeggiani, A. (1971), *Biochem. Biophys. Res. Commun. 45,* 1151.

Erbe, R. W., Nau, N. M., and Leder, P. (1969), *J. Mol. Biol. 39,* 441.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6,* 3043.

Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.

Goody, R. S., and Eckstein, F. (1971), *J. Am. Chem. Soc. 93,* 6252.

Gratecos, D., and Fischer, E. H. (1974), *Biochem. Biophys. Res. Commun. 58,* 960.

Haenni, A.-L., and Chapeville, F. (1966), *Biochim. Biophys. Acta 114,* 135.

Haenni, A.-L. and Lucas-Lenard, J. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 1363.

Haley, B., and Yount, R. G. (1972), *Biochemistry 11,* 2863.

Haselkorn, R., and Rothman-Denes, L. B. (1973), *Annu. Rev. Biochem. 42,* 397.

Hershey, J. W. B., and Monro, R. E. (1966), *J. Mol. Biol. 18,* 68.

Hill, W. E., Anderegg, J. W., and van Holde, K. E. (1970), *J. Mol. Biol. 53,* 107.

Inoue-Yokosawa, N., Ishikawa, C., and Kaziro, Y. (1974), *J. Biol. Chem. 249,* 4321.

Kuriki, Y. (1973), *Biochem. Biophys. Res. Commun. 54,* 1470.

Larsen, M., Willett, R., and Yount, R. G. (1969), *Science 166,* 1510.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Lucas-Lenard, J., and Lipmann, F. (1971), *Annu. Rev. Biochem. 40,* 409.

Maassen, J. A., and Möller, W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1277.

Michelson, A. M. (1964), *Biochim. Biophys. Acta 91,* 1.

Modolell, J., Cabrer, B., and Vazquez, D. (1973), *J. Biol. Chem. 248,* 8356.

Nishizuka, Y., and Lipmann, F. (1966), *Arch. Biochem. Biophys. 116,* 344.

Otaka, T., and Kaji, A. (1974), *FEBS Lett. 44,* 324.

Parmeggiani, A. (1968), *Biochem. Biophys. Res. Commun. 30,* 613.

Parmeggiani, A., Singer, C., and Gottschalk, E. M. (1971), *Methods Enzymol. 20,* 291.

Rohrbach, M. S., Dempsey, M. E., and Bodley, J. W. (1974), *J. Biol. Chem. 249,* 5094.

Rose, W. G. (1947), *J. Am. Chem. Soc. 69,* 1384.

Sander, G., Marsh, R. C., and Parmeggiani, A. (1972), *Biochem. Biophys. Res. Commun. 47,* 866.

Sander, G., Marsh, R. C., Voigt, J., and Parmeggiani, A. (1975), *Biochemistry 14,* 1805.

Solymosy, F., Fedorcsák, J., Gulyás, A., Farkas, G. L., and Ehrenberg, L. (1968), *Eur. J. Biochem. 5,* 520.

Wehrli, W. E., Verheyden, D. L. M., and Moffatt, J. G. (1964), *J. Am. Chem. Soc. 86,* 1254.

Wittmann, R. (1963), *Chem. Ber. 96,* 771.