Synthesis of Deoxyguanosine Polyphosphates and Their Interactions with the Guanosine 5′-Triphosphate Requiring Protein Synthetic Enzymes of *Escherichia coli*[†]

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ABSTRACT: A chemical synthesis of deoxyguanosine analogs of the guanosine polyphosphates accumulated by bacteria during the stringent response is described. Both deoxyguanosine 3′-diphosphate 5′-triphosphate (d-pppGpp) and deoxyguanosine 3′-diphosphate 5′-diphosphate (d-ppGpp) were prepared, as well as the by-products deoxyguanosine 3′-monophosphate 5′-triphosphate (d-pppGp) and deoxyguanosine 3′-monophosphate 5′-diphosphate. A significant difference between d-(p)ppGpp and guanosine 3′-diphosphate 5′-tri- or 5′-diphosphate ((p)ppGpp) is that the 3′-pyrophosphate moiety is alkali stable in the deoxyguanosine and alkali labile in the guanosine polyphosphates. The new GTP analogs d-pppGp and d-pppGpp were compared to GTP, dGTP, and pppGpp in their ability to support reactions catalyzed by the *Escherichia coli* protein synthetic enzymes initiation factor 2, elongation factor Tu, and elongation factor G (EF-G). Like pppGpp, both d-pppGp and d-pppGpp showed substantial deficiency only in reactions requiring EF-G. While d-pppGpp closely resembled pppGpp in its very low activity with EF-G, d-pppGp was somewhat more active. Nevertheless, d-pppGp was a poor substrate in EF-G-dependent translocation. Qualitatively and quantitatively its support of translocation was very similar to the reaction driven by periodate-oxidized and borohydride-reduced GTP, a derivative of GTP in which the ribose ring has been cleaved between the 2′- and 3′-hydroxyl groups.

The stringent response in bacteria (Edlin and Broda, 1968) is accompanied by the simultaneous accumulation of ppGpp[1] and pppGpp (Cashel and Gallant, 1969; Cashel and Kalbacher, 1970; Que et al., 1973; Sy and Lipmann, 1973), and the synthesis of these compounds in vitro has been shown to involve codon-specific interaction of uncharged tRNA with the ribosomal acceptor site (Haseltine and Block, 1973; Pedersen et al., 1973). The degradative pathway and function of the guanosine polyphosphates in vivo remain obscure, however, although it has been suggested that degradation requires the conversion of ppGpp to pppGpp (Laffler and Gallant, 1974).

In vitro studies of a possible role for pppGpp in translation demonstrated that the nucleotide could readily substitute for GTP in reactions catalyzed by IF-2 and EF-Tu but was virtually inactive with EF-G, while dGTP was an effective substrate with all three enzymes (Hamel and Cashel, 1973, 1974). The slight activity of pppGpp with EF-G was particularly noticeable at 37° in both poly(Phe) synthesis and Ac-diPhe-puromycin formation, a partial reaction mimicking the translocation step of protein synthesis (Haenni and Lucas-Lenard, 1968; Ono et al., 1969). Since the 3′-pyrophosphate residue of ppGpp is relatively labile, with formation of a mixture of ppG2′p and ppG3′p (Cashel and Kalbacher, 1970), the apparent activity of pppGpp was somewhat suspect. It seemed possible that pppG2′p and pppG3′p were being formed nonenzymatically during the reaction and that they might have significant activity with EF-G. These isomers, however, are not yet available in pure form.

In this paper the synthesis of d-pppGpp and d-pppGp, as well as d-ppGpp and d-ppGp, is described, as are their interactions with IF-2, EF-Tu, and EF-G. As with pppGpp (Hamel and Cashel, 1973, 1974), the most significant effect was observed in reactions dependent on EF-G. Moreover, the 3′-pyrophosphate residue of d-pppGpp and d-ppGpp is much more stable than that of pppGpp and ppGpp. The findings reported here exclude breakdown of pppGpp to pppG2(3′)p during the reaction as the explanation of its sluggish activity with EF-G and demonstrate the marked reduction in activity observed when even a single phosphate residue is inserted at the 3′ position.

Experimental Procedure

Reactions Dependent on IF-2, EF-Tu, and EF-G. Materials, enzymes, and procedures used in examining IF-2-dependent formation of fMet-puromycin, EF-Tu-dependent formation of Ac-diPhe-tRNA, EF-G-dependent formation of Ac-diPhe-puromycin, and poly(Phe) synthesis have been

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[1] Abbreviations used are: ppGpp, guanosine 3′-diphosphate 5′-diphosphate; pppGpp, guanosine 3′-diphosphate 5′-triphosphate; d-pppGpp, deoxyguanosine 3′-diphosphate 5′-triphosphate; d-pppGp, deoxyguanosine 3′-monophosphate 5′-triphosphate; d-ppGpp, deoxyguanosine 3′-diphosphate 5′-diphosphate; d-ppGp, deoxyguanosine 3′-monophosphate 5′-diphosphate; ppG2′p, guanosine 2′-monophosphate 5′-diphosphate; ppG3′p, guanosine 3′-monophosphate 5′-diphosphate; pppG2′p, guanosine 2′-monophosphate 5′-triphosphate; pppG3′p, guanosine 3′-monophosphate 5′-triphosphate; pppG2′(3′)p, a mixture of pppG2′p and pppG3′p; ox-redGTP, the dialcohol derived from GTP by periodate oxidation and borohydride reduction; EF-G, elongation factor G; EF-Tu, elongation factor Tu; EF-T, elongation factor T, a mixture of EF-Tu and elongation factor Ts; IF-2, initiation factor 2; fMet-puromycin, *N*-formylmethionylpuromycin; Ac-diPhe-puromycin, *N*-acetylphenylalanylphenylalanylpuromycin; poly(U), poly(uridylic acid); poly(Phe), polyphenylalanine; PEI-cellulose, polyethylenimine-cellulose. IUPAC-IUB abbreviations are used for intermediates in the synthesis of d-pppGpp, d-pppGp, d-ppGpp, and d-ppGp, except in Figure 1, where structural formulas are used.

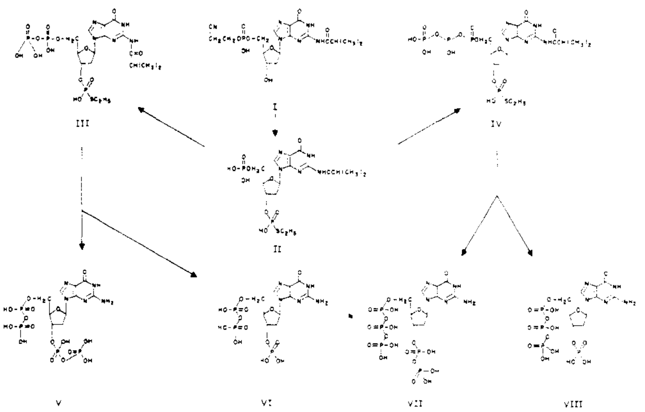

FIGURE 1: Reaction pathway for the synthesis of d-pppGpp, d-pppGp, d-ppGpp, and d-ppGp.

described in detail (Hamel and Cashel, 1973, 1974), and reaction components are described in the individual assays.

Nucleotides. GTP and dGTP were repurified by LiCl gradient elution from DEAE-Sephadex A-25, precipitation with ethanol, and thorough washing in ethanol to remove residual LiCl. The preparation of ox-redGTP will be described elsewhere. The preparation of pppGpp was as described by Cashel (1974), except that two sequential DEAE-Sephadex A-25 columns were used. The lability of the 3′-pyrophosphate of pppGpp has been found to be substantially greater in the lithium salt than in the triethylammonium salt of the nucleotide (M. Cashel, personal communication). Consequently, immediately after repurification of the lithium salt, the cation was exchanged by passage of the pppGpp through the triethylammonium form of Dowex 50-X1 (100-200 mesh).

Synthesis of d-pppGpp, d-pppGp, d-ppGpp, and d-ppGp. The reaction sequence is summarized in Figure 1.

d-pibuGpSEt (*II*). With exclusion of moisture, 10 mmol of d-CNEtpibuG (I) (Buchi and Khorana, 1972) and 70 mmol of *S*-ethyl phosphorothioate were dried by several coevaporations with dry pyridine in vacuo and finally dissolved in 100 ml of dry pyridine. After 140 mmol of dicyclohexylcarbodiimide was added, the suspension was left for 2 days. The suspension was filtered, the residue washed well with water and again filtered, and the combined filtrate made 1 *N* with NaOH. After 10 min, the solution was neutralized with Dowex 50-X8 (pyridinium form) and concentrated to a small volume. Gradient elution with triethylammonium bicarbonate (pH 7.2) from a DEAE-cellulose column (5 × 100 cm) resulted in purification of 4 mmol of II.

d-ppibuGpSEt (*III*). The procedure was that of Hoard and Ott (1965). A solution of 0.1 mmol of II in 1 ml of dry dimethylformamide was mixed with 80 mg (0.5 mmol) of carbodiimidazole in 1 ml of dimethylformamide, and the mixture was left overnight. After 35 µl of dry methanol was added, the solution was stirred for 0.5 hr. Then 0.6 mmol of tributylammonium phosphate in 2 ml of dimethylformamide was added, and the mixture was stirred overnight. III was obtained in a yield of 52% after triethylammonium bicarbonate (pH 7.2) gradient elution from a DEAE-Sephadex A-25 column (2.5 × 42 cm).

d-pppibuGpSEt (*IV*). II (0.1 mmol) in a mixture of hexamethylphosphoramidate (0.1 ml) and dimethylformamide (0.3 ml) was treated as above, except that 1 mmol of tributylammonium pyrophosphate was substituted for the phosphate. IV was obtained in 54% yield.

Table I: Properties of Deoxyguanosine Polyphosphates.

Compound	ELPHO[a] R_{dpG}	PC[b] R_{dpG}	CC[c] *M* Buffer	Phosphate Analysis[d]
d-pGpSEt	1.6	1.05		
d-pibuGpSEt (II)	1.5		0.30	0.97 (1)[e]
d-ppibuGpSEt (III)	1.9		0.38	1.78 (2)[e]
d-ppGpSEt	2.0	0.87		
d-ppibuGp			0.40	
d-ppGp (VI)	2.1	0.50		2.76 (3)[f]
d-pppibuGpSEt (IV)	2.2		0.45	3.06 (3)[e]
d-pppGpSEt	2.3	0.75		
d-pppibuGp			0.50	
d-pppGp (VIII)	2.2	0.35		4.00 (4)[f]
d-ppibuGpp			0.47	
d-ppGpp (V)	2.3	0.35		3.76 (4)[f]
d-pppibuGpp			0.58	
d-pppGpp (VII)	2.4	0.27		4.80 (5)[f]

[a] Paper electrophoresis using 50 m*M* sodium citrate (pH 5.0) on Whatman 3MM, at 26.5 V/cm for 1 hr. [b] Paper chromatography, descending, on Whatman No. 1. The solvent was isobutyric acid–NH_3–H_2O, 57:4:39 (v/v). Development was for 18 hr. [c] Column chromatography: buffer molarity of triethylammonium bicarbonate (pH 7.2) at which the center of the peak eluted from a DEAE-Sephadex A-25 or DEAE-cellulose column during preparative chromatography. [d] Each 55-µl reaction mixture contained 10 A_{260} units of nucleotide, 0.5 µmol of $MgCl_2$, 20 µmol of Tris-HCl (pH 8.0), and 100 µg of bacterial alkaline phosphatase. After 4 hr at 37° an aliquot was analyzed for inorganic phosphate as described by Itaya and Ui (1966). Values in parentheses are theoretical. [e] P/d-ibuGpSEt. [f] P/dG.

d-ppGpp (*V*) *and d-ppGp* (*VI*). In a drybox, 0.1 mmol of III was rendered anhydrous by codistillation once with dry pyridine and twice with dry picoline. The residue was taken up in 0.75 ml of dimethylformamide and 0.25 ml of hexamethylphosphoramidate. Tributylammonium phosphate (1 mmol) and iodine (25 mg) were added, and the mixture was left overnight at room temperature. It was then concentrated in vacuo and partitioned between 1 ml each of water and ether. The aqueous portion was extracted four times with 1 ml of ether, then charged directly onto a DEAE-Sephadex A-25 column (2.5 × 40 cm). Elution with a linear gradient of triethylammonium bicarbonate (pH 7.2) produced d-ppibuGpp in 56% yield. Concentration in vacuo, followed by hydrolysis (concentrated NH_3, 60°, 2 hr), gave V.

When the reaction was not carried out in a drybox, moisture caused partial abortive decomposition of III to d-ppibuGp. Three peaks were obtained on column chromatography, corresponding to d-ppibuGp, an unidentified compound, and d-ppibuGpp. The first peak was concentrated and hydrolyzed as above to give VI in a 36% yield. The third peak produced a 20% yield of V.

d-pppGpp (*VII*). Tributylammonium phosphate (1 mmol) and iodine (25 mg) were added to a solution of 60 µmol of IV in 0.75 ml of dimethylformamide and 0.25 ml of hexamethylphosphoramidate, and the resulting mixture was left overnight. After concentration in vacuo, the product was partitioned between water and ether and chromatographed on DEAE-Sephadex A-25 as described above. A 33% yield of d-pppibuGpp was obtained, and hydrolysis as described above gave VII. The d-pppGpp was further purified by successive gradient elutions from DEAE-Sephadex A-25 with sodium formate (pH 3.4) and triethylammonium bicarbonate (pH 7.6) until no contaminant could be detected on thin-layer chromatography on PEI-cellulose in 1.5 *M* KH_2PO_4 (pH 3.4) (Cashel, 1969).

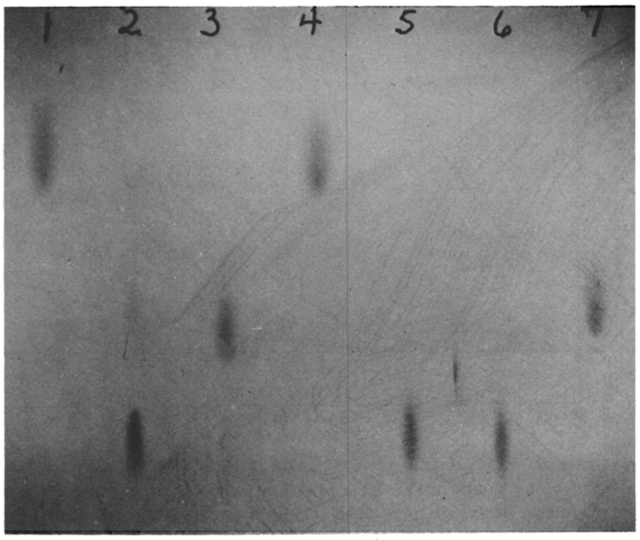

FIGURE 2: Alkali stability of d-pppGpp. (1) Untreated GTP (R_f 0.42); (2) untreated pppGpp (R_f 0.13); (3) pppGpp treated with KOH (product pppG2′(3′)p has R_f 0.23); (4) untreated dGTP (R_f 0.41); (5) untreated d-pppGpp (R_f 0.13); (6) d-pppGpp treated with KOH; (7) untreated d-pppGp (R_f 0.26). For samples 3 and 6, 18 nmol of nucleotide was treated with 0.3 *M* KOH and 1 m*M* EDTA in a 10-μl volume at 37°. After 20 hr, 3 μl of 1 *M* HCl was added, and the entire sample was spotted 1.5 cm from the edge of a 20-cm PEI-cellulose thin-layer chromatography sheet. For samples 1, 2, 4, 5, and 7 the KOH, HCl, and EDTA were first mixed and then 18 nmol of nucleotide was added. The entire sample was immediately spotted on PEI-cellulose, as above. The sample spots were dried, and the PEI-cellulose sheet was chromatographed in 1.5 *M* KH_2PO_4 (pH 3.4) (Cashel, 1969). The chromatogram was photographed during a 4-min exposure under short-wave ultraviolet light.

d-pppGp (*VIII*). This compound was obtained from an abortive synthesis of VII. The d-pppibuGp was initially isolated by triethylammonium bicarbonate (pH 7.2) gradient elution from DEAE-Sephadex A-25, and it was hydrolyzed as above. The d-pppGp was further purified by successive gradient elutions from DEAE-Sephadex A-25 with sodium formate (pH 3.4) and triethylammonium bicarbonate (pH 7.6) until no contaminant could be detected on PEI-cellulose thin-layer chromatography.

Results

Synthesis of d-ppGpp, d-pppGp, and d-pppGpp. The technical problem of generating pyrophosphate linkages at specific locations on the nucleoside molecule in a controlled sequence requires differential protection. Figure 1 illustrates how the use of Tener's cyanoethyl ester of nucleoside phosphate (Tener, 1961) can alternate with protection via phosphorothioate (Nussbaum and Tiberi, 1965) to operate at will on either side of the molecule. Thus, d-CNEtpibuG (I) (Buchi and Khorana, 1972) was condensed with *S*-ethyl phosphorothioate at the unprotected hydroxyl group in the 3′ position. When the resulting d-CNEtpibuGpSEt was treated with alkali, the protecting group at the 5′-phosphate was specifically removed, yielding d-pibuGpSEt (II) without affecting the phosphorothioate at the 3′ position. It was now possible to convert II, via the intermediacy of a 5′-phosphorimidazolidate (Hoard and Ott, 1965), to the pyrophosphate derivatives d-ppibuGpSEt (III) and d-pppibuGpSEt (IV). These phosphorothioates carry a hidden activating group which can be labilized, by mild oxidation, toward nucleophiles (Cook et al., 1969). Treatment of III with iodine and inorganic phosphate, followed by hydrolysis with alkali of the protecting group on the nucleobase, gave the ppGpp analog d-ppGpp (V) as well as d-ppGp (VI). The latter, normally present in smaller proportion, can become the major product if exclusion of moisture is not rigorous. Similar treatment of IV gave rise to d-pppGpp (VII), the presumed analog of pppGpp, and d-pppGp (VIII), an isomer of V. All compounds had ultraviolet spectra indicating unchanged chromophore, and were characterized by their diagnostically significant electrophoretic mobilities, phosphate content, charge as adjudged from ion exchange chromatography, and paper chromatography (Table I).

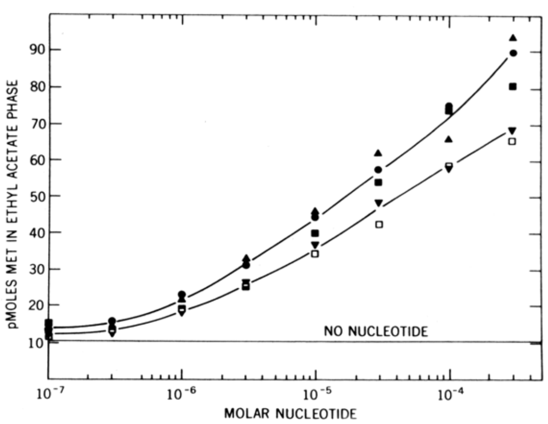

FIGURE 3: IF-2-dependent formation of fMet-puromycin. Each 100-μl assay contained 100 m*M* NH_4Cl (pH 7.4), 5 m*M* $MgCl_2$, 50 m*M* imidazole-HCl (pH 7.4), 12 m*M* 2-mercaptoethanol, 1 m*M* puromycin, 1 mg/ml of ribosomes, 0.004 A_{260} unit/ml of AUG codon, 0.4 mg/ml of [^{14}C]fMet-tRNA, 22.8 μg/ml of a mixture of IF-1 and IF-2, and the indicated amount of nucleotide, symbolized as follows: d-pppGpp, □; d-pppGp, ▼; pppGpp, ■; dGTP, ▲; and GTP, ●. Reaction mixtures were incubated for 3 hr at 8°. Data are expressed as picomoles/milliliter of reaction.

Since the synthesis of both d-ppGpp and d-pppGpp required a final hydrolysis in NH_4OH to remove the protecting isobutyryl group on the guanine nucleus, it is obvious that the 3′-pyrophosphate residue, like the phosphodiester bonds in DNA, is alkali stable. Both pppGpp and ppGpp, like RNA, are labile in alkali and even show significant degradation when stored frozen in solution. In KOH, a mixture of 2′- and 3′-monophosphates is produced, while in NH_4OH or in the slow degradation at neutrality there is also significant production of a cyclic 2′, 3′- monophosphate (the 5′-di- or -triphosphate remains unchanged) (Cashel and Kalbacher, 1970; M. Cashel, personal communication).

The contrast between the stability of d-pppGpp and the instability of pppGpp is demonstrated in Figure 2, both nucleotides having been subjected to 0.3 *M* KOH for 20 hr at 37°. The pppGpp preparation used in this experiment was about 6 months old, and two contaminants in the starting material resulting from degradation during storage can be observed. Since the upper spot disappears after KOH treatment, it is probably guanosine 2′,3′-cyclic monophosphate 5′-triphosphate.

Partial Reactions of Protein Synthesis Supported by d-pppGpp and d-pppGp. Figures 3–5 present concentration studies of three partial reactions of protein synthesis in which d-pppGpp and d-pppGp are compared to pppGpp, dGTP, and GTP. The reactions studied were IF-2-dependent formation of fMet-puromycin at 8° (Figure 3), EF-Tu-dependent formation of Ac-diPhe-tRNA at 0° (Figure 4), and EF-G-dependent formation of Ac-diPhe-puromycin at 37° (Figure 5) (for a review, see Lucas-Lenard and Lip-

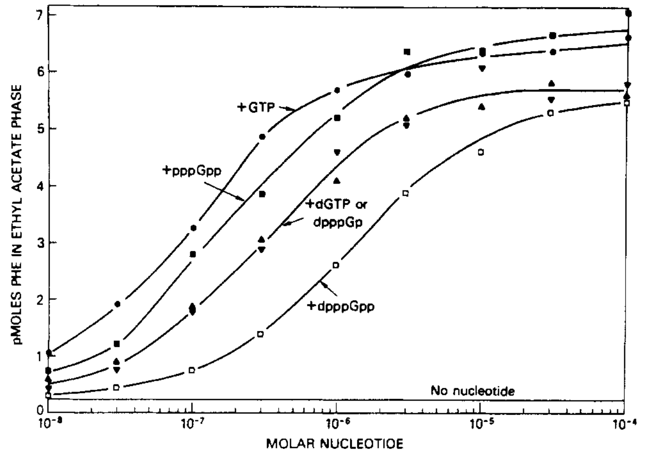

FIGURE 4: EF-Tu-dependent formation of Ac-diPhe-tRNA. Each 50-μl assay contained 20 μl of a ribosome-poly(U)-nonradioactive Ac-Phe-tRNA complex, prepared as described previously (Hamel and Cashel, 1974), 4 mM MgCl$_2$, 80 mM NH$_4$Cl (pH 7.4), 50 mM imidazole-HCl (pH 7.4), 12 mM 2-mercaptoethanol, 2 mg/ml of bovine serum albumin, 0.2 mg/ml of [^{3}H]Phe-tRNA, 2.5 μg/ml of EF-T, and the indicated amount of nucleotide, symbolized as in Figure 3. Reaction mixtures were incubated for 20 min at 0°. Data are expressed as picomoles/milliliter of reaction.

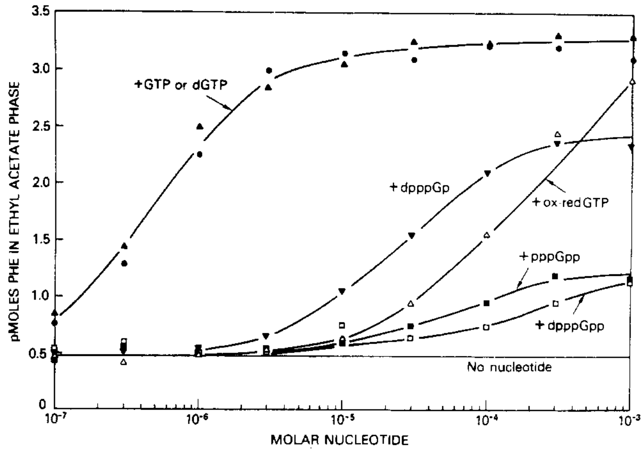

FIGURE 5: EF-G-dependent formation of Ac-diPhe-puromycin. Each 100-μl assay contained 5 A_{260} units/ml of pretranslocation complex prepared as described previously (Hamel and Cashel, 1973), 10 mM MgCl$_2$, 80 mM NH$_4$Cl (pH 7.4), 50 mM imidazole-HCl (pH 7.4), 12 mM 2-mercaptoethanol, 1 mM puromycin, 2 mg/ml of albumin, 25.6 μg/ml of EF-G, and the indicated amount of nucleotide, symbolized as in Figure 3 with the addition of ox-redGTP, △. Reaction mixtures were incubated for 20 sec at 37°. Data are expressed as picomoles/milliliter of reaction.

mann, 1971). As with pppGpp (Hamel and Cashel, 1973, 1974) the most significant differences were observed in the EF-G-dependent translocation reaction (Figure 5).

In IF-2-dependent formation of fMet-puromycin (Figure 3), d-pppGp and d-pppGpp were somewhat less active than the other nucleotides. Double reciprocal plots of these data provided estimates of apparent K_m values ranging from 4 to 8 × 10^{-6} M for the five nucleotides. The maximum velocities with d-pppGp and d-pppGpp were about ⅔ of the values obtained with the other nucleotides. The points at the higher concentrations deviate from linearity in the double reciprocal plots, probably as a result of disproportionate nucleotide hydrolysis at lower concentrations by IF-2 and ribosomes uncoupled from fMet-tRNA binding to ribosomes (data not presented).

The range of activities of the nucleotides was somewhat greater in EF-Tu-dependent formation of Ac-diPhe-tRNA (Figure 4). Double reciprocal plots yielded apparent K_m values ranging from 0.8 × 10^{-7} M for GTP to 11 × 10^{-7} M for d-pppGpp. The 3′-pyrophosphate moiety appears to reduce the activity of the deoxyribonucleotide somewhat more than it does the activity of the ribonucleotide, while a single phosphate has little effect.

By far the most substantially affected reaction was EF-G-dependent formation of Ac-diPhe-puromycin (Figure 5). In addition to GTP, dGTP, and pppGpp, d-pppGp and d-pppGpp were compared to the dialcohol ox-redGTP derived from GTP by periodate oxidation and borohydride reduction. Studies to be presented elsewhere have demonstrated that ox-redGTP has reduced activity with all three GTP-requiring enzymes in these partial reactions, but the most substantial effect was observed with EF-G.

Although both d-pppGp and d-pppGpp were far less active in supporting translocation than dGTP, d-pppGp had significantly more activity than d-pppGpp. Nevertheless, d-pppGp was little more active than ox-redGTP. Apparently a single phosphate at the 3′-hydroxyl is nearly as disruptive to the activity of a guanine nucleotide with EF-G as is cleavage of the pentose ring.

Double reciprocal plots of the data of Figure 5 provide estimates of apparent K_m values as follows: 6.5 × 10^{-7} M for GTP and dGTP, 3 × 10^{-5} M for d-pppGp, and 2 × 10^{-4} M for ox-redGTP. Reproducibly, the maximum velocity of the reaction with d-pppGp has been about 70% of the maximum velocity with ox-redGTP, despite the higher apparent K_m of the latter compound. The maximum velocity determined for ox-redGTP was essentially identical with that of GTP and dGTP (about 5.5 pmol per min per ml of reaction).

Studies with pppGpp had shown that its deficiency in Ac-diPhe-puromycin formation was still more pronounced, even at high nucleotide concentrations, if the incubation temperature was lowered to 8° (Hamel and Cashel, 1973). As Table II demonstrates, this is also true for ox-redGTP, d-pppGp, and d-pppGpp. At 0.5 min there was no significant reaction with these compounds, although the reaction supported by GTP or dGTP was 50% complete. At 20 min there was a slight reaction with all GTP analogs.

The ability of d-pppGp and d-pppGpp, as well as pppGpp and ox-redGTP, to inhibit GTP-supported formation of Ac-diPhe-puromycin at 8° was examined to determine whether they could participate in a nonfunctional interaction with EF-G and/or the ribosome. Although no detectable inhibition was observed with ox-redGTP, the other analogs were relatively weak competitive inhibitors of GTP. At 40-fold excess of inhibitor over GTP, translocation was reduced 53% with pppGpp, 42% with d-pppGpp, and 63% with d-pppGp (data not presented). The β-phosphate residue at the 3′ position significantly reduced the ability of the deoxyribonucleotide to inhibit translocation, just as the additional phosphate reduced the ability of the nucleotide to support the reaction. Although the inhibitory activity of d-pppGpp, pppGpp, and d-pppGp qualitatively paralleled their ability to support Ac-diPhe-puromycin formation, this relationship was not observed with ox-redGTP. The dialcohol did not inhibit GTP-supported translocation at all, even though it was comparable to d-pppGp in its ability to support the reaction.

Poly(Phe) Synthesis Supported by d-pppGpp and d-pppGp. The effects of reaction temperature and of different

Table II: EF-G-Dependent Formation of Ac-diPhe-puromycin at 8°.[a]

Nucleotide Added	pmoles of Phe in Ethyl Acetate Phase	
	0.5 min at 8°	20 min at 8°
None	0.3	1.7
GTP	5.6 (5.3)	11.6 (9.9)
dGTP	5.2 (4.9)	12.1 (10.4)
d-pppGp	0.4 (0.1)	3.1 (1.4)
d-pppGpp	0.3 (0)	1.9 (0.2)
pppGpp	0.4 (0.1)	2.1 (0.4)
ox-redGTP	0.5 (0.2)	4.1 (2.4)

[a] Conditions were identical with those described for Figure 5, except that assay volume was 50 μl and each reaction mixture contained 15 A_{260} units/ml of pretranslocation complex and the indicated nucleotide at 1 m*M*. Incubation was for the indicated times at 8°. Data are expressed as pmoles/ml of reaction. The values in parentheses have been corrected for the reaction occurring without nucleotide.

Table III: Poly(Phe) Synthesis Supported by d-pppGp and d-pppGpp.[a]

Nucleotide Added	pmol of Phe Polymerized			
	Saturating EF-G and EF-T[b] 37°	Saturating EF-G and EF-T[c] 8°	Saturating EF-G and Rate-Limiting EF-T[d] 37°	Rate-Limiting EF-G and Saturating EF-T[e] 37°
0.3 m*M* GTP	49.5	51.9	9.5	12.4
0.3 m*M* dGTP	46.6	51.6	11.8	11.0
0.3 m*M* pppGpp	1.6	0.4	7.5	0.6
0.3 m*M* d-pppGpp	1.3	0.2	6.8	0.5
0.3 m*M* d-pppGp	7.8	1.6	11.6	0.9
0.3 m*M* ox-redGTP	6.2	2.5	11.6	0.8

[a] Each 50-μl assay contained 80 μg/ml of ribosomes, 0.2 mg/ml of [^{3}H]Phe-tRNA, EF-G and EF-T as described below, 10 m*M* $MgCl_2$, 80 m*M* NH_4Cl (pH 7.4), 50 m*M* imidazole-HCl (pH 7.4), 12 m*M* 2-mercaptoethanol, 2 mg/ml of albumin, 0.1 mg/ml of poly(U), and the indicated nucleotide. After incubations as described below, reactions were stopped with 5% trichloroacetic acid, hydrolyzed at 90°, and filtered through Millipore membranes. Data are expressed as picomoles/milliliter of reaction. Representative points from time course studies are presented. [b] Each assay contained 25.6 μg/ml of EF-G and 25.2 μg/ml of EF-T. Reaction mixtures were incubated at 37° for 2 min. [c] Each assay contained 25.6 μg/ml of EF-G and 25.2 μg/ml of EF-T and was incubated for 2 hr at 8°. [d] Each assay contained 25.6 μg/ml of EF-G and 0.39 μg/ml of EF-T and was incubated for 20 min at 37°. [e] Each assay contained 0.07 μg/ml of EF-G and 25.2 μg/ml of EF-T and was incubated for 20 min at 37°.

amounts of EF-T and EF-G on poly(Phe) synthesis supported by pppGpp, ox-redGTP, d-pppGp, and d-pppGpp are presented in Table III. Reaction rates with analogs relative to the rate with GTP could be substantially reduced by lowering the reaction temperature or, with d-pppGp and ox-redGTP, using rate-limiting amounts of EF-G; these conditions maximized differences between GTP and its analogs. On the other hand, differences between nucleotides could be minimized if EF-T was made rate limiting, all nucleotides supporting reactions similarly. These are the results expected if the GTP analogs function well with EF-Tu and poorly with EF-G, as the partial reactions indicate (Figures 3-5, and manuscript in preparation).

Poly(Phe) synthesis supported by ox-redGTP drops very rapidly as the concentration of the dialcohol is lowered (data not presented). As a result, the relative activities of d-pppGp and ox-redGTP in poly(Phe) synthesis are strongly concentration dependent. It has been found that ox-redGTP is significantly more active than d-pppGp at 1 m*M* nucleotide, but much less active at 0.1 m*M* (unpublished observations). This result agrees well with the relative activities of d-pppGp and ox-redGTP in EF-G-dependent Ac-diPhe-puromycin formation (Figure 5), but probably also reflects the reduced activity of ox-redGTP with EF-Tu (Gordon and Bodley, 1974; Hamel, 1975).

Discussion

Earlier studies demonstrated the indifference of IF-2 and EF-Tu to pyrophosphorylation at the nucleotide 3′ pentose position as compared to the more substantial effect on the EF-G-catalyzed translocation reaction (Hamel and Cashel, 1973, 1974). This generalization can now be extended to d-pppGp and d-pppGpp, analogs of dGTP with phosphate and pyrophosphate residues at the 3′-hydroxyl.

The fact that d-pppGp is significantly more active in EF-G-dependent translocation than either d-pppGpp or pppGpp suggests that steric and/or ionic effects introduced by the 3′-pyrophosphate interfere with the utilization of the pentaphosphates by EF-G and/or the ribosome. Nevertheless, the 3′-hydroxyl itself also appears to be important for EF-G function because 3′-deoxyguanosine 5′-triphosphate, differing structurally from GTP only by the absence of the 3′-hydroxyl, is unable to substitute fully for GTP with EF-G (manuscript in preparation).

While more active than pppGpp and d-pppGpp, d-pppGp is still markedly deficient as a substrate in both Ac-diPhe-puromycin formation and poly(Phe) synthesis. It has, in fact, activity with EF-G comparable to that of ox-redGTP, derived from GTP by periodate cleavage of the ribose ring and subsequent borohydride reduction. While the apparent K_m of d-pppGp in Ac-diPhe-puromycin formation is somewhat higher than that of ox-redGTP, the dialcohol-supported reaction reaches a higher maximum velocity. These differences, initially observed with the EF-G-dependent partial reaction, were fully reflected in the relative activities of the two compounds in poly(Phe) synthesis.

There is one major distinction, however, between ox-redGTP and d-pppGp. Although both compounds can support Ac-diPhe-puromycin formation only very weakly at 8°, d-pppGp inhibits the GTP-supported reaction while ox-redGTP does not.

Although d-pppGpp appears to be even less active than pppGpp with EF-G, the two nucleotides show basically similar activity in Ac-diPhe-puromycin formation and poly(Phe) synthesis. In particular, both compounds support rates of polymerization approaching that observed with GTP and dGTP with rate-limiting amounts of EF-T, a finding initially made with pppGpp. Since this assay required a prolonged incubation, we suspected that partially active pppG2′(3′)p was being generated by nonenzymatic degradation of totally inactive pppGpp. The analogous reactions supported by d-pppGpp with its highly stable 3′-pyrophosphate residue, however, excludes this possibility as a significant cause for the residual activity of pppGpp with EF-G.

Enzymatic degradation of pppGpp, d-pppGpp, or d-pppGp to GTP or dGTP seems unlikely from studies with [5′-α-]-, [3′-β-]-, and/or [5′-γ-^{32}P]pppGpp (Hamel and Cashel, 1973, and unpublished experiments). Significant hydrolysis was found only at the 5′-γ position, yielding P_i and ppGpp, which is not only inactive but also inhibitory.

Nevertheless, the generation of trace amounts of GTP or pppG2′(3′)p has not been excluded, and, moreover, small amounts of pppG2′(3′)p can almost always be detected in preparations of pppGpp by thin-layer chromatography. While d-pppGp is not detectable in the d-pppGpp preparation (Figure 2), the lower limit of detectability is about 2% (unpublished observations). Contamination of d-pppGpp or d-pppGp by dGTP is extremely unlikely because of the route of synthesis (Figure 1).

In summary, studies with d-pppGpp and radioactive preparations of pppGpp make it highly unlikely that the residual activity of these nucleotides with EF-G is due either to contaminating GTP or dGTP or to breakdown in the course of the reaction. It remains possible that contaminating tetraphosphates initially present in the pppGpp and d-pppGpp preparations cause their low activity, but we have no reason to suspect such contamination in the d-pppGpp.

Acknowledgments

The authors would like to thank Dr. M. Cashel, in whose laboratory the enzymatic studies were performed by one of them (E.H.), for his encouragement and interest; Dr. Cashel and Dr. A. Furano for their critical readings of the manuscript; and Ms. C. Kunkle for her help in preparing the manuscript.

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