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Guanosine 5′-Diphosphate, 3′-Diphosphate: Assignment of Structure by ^{13}C Nuclear Magnetic Resonance Spectroscopy

(*E. coli*/stringent control/Magic Spots)

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ABSTRACT **Guanosine tetraphosphate, recently discovered to mediate the regulatory relationship between protein synthesis and RNA accumulation in various bacteria, has been synthesized *in vitro* in large quantities and analyzed by natural-abundance ^{13}C nuclear magnetic resonance spectroscopy in order to confirm its structure and establish the positions of phosphate attachment. These studies have established its structure as guanosine 5′-diphosphate, 3′-diphosphate.**

Accumulating evidence suggests that various microorganisms, ranging from *Escherichia coli* to *Bacillus subtilis*, can produce large amounts of a class of unusual and presumably regulatory nucleotides (1–4). These nucleotides were first observed in *E. coli* and were called Magic Spots I and II (MS I and MS II) because of their mysterious appearance on autoradiograms when control of RNA synthesis was occasioned by amino-acid starvation (1, 2). Happily for the indexers of scientific publication titles (5), these compounds were later isolated and characterized, and their structures were proposed as guanosine 5′-diphosphate, 3′- or 2′-diphosphate (ppGpp), and the related nucleoside 5′- triphosphate derivative (pppGpp) (6, 7).

A regulatory function for these nucleotides is presumed for several reasons. First, the cellular concentrations of ppGpp and pppGpp are rather tightly controlled as a function of the physiological state of the cell. Basal levels of the compounds (roughly equivalent to the more abundant of the deoxyribonucleoside 5′-triphosphates) are inversely correlated with cellular growth rates, suggesting perhaps a function in normally growing cells (8). During growth restriction due to limitation in sources of carbon (8), nitrogen (9), amino acids (1, 2), or even of aminoacylated tRNA in the presence of free amino acids (2), the levels of ppGpp and pppGpp can markedly increase. This accumulation persists until these nucleotides can become amongst the most abundant of the acid-extractable cellular nucleotides (2, 10). Restoration of the source of nutritional deficiency leads to the immediate disappearance of these compounds (half-lives about 10–20 sec) and resumption of cellular growth (2, 10, 11).

The second reason implicating these nucleotides as regulators is that regulation of ppGpp and pppGpp concentrations can *itself* be a function of a regulatory gene. The product of the *rel* gene mediates the regulatory relationship between protein synthesis and RNA accumulation, which normally are coordinately controlled processes (12–14). Strains with this mutation show persisting unbalanced RNA accumulation when protein synthesis is restricted for lack of available amino acids. Such relaxed mutants have been isolated in *E. coli* (11), *Salmonella typhimurium* (15), and *B. subtilis* (16); the mutant allele leads to loss of the capacity to accumulate ppGpp and pppGpp during amino-acid deprivation. Further studies in *E. coli* have revealed that such mutants are nevertheless able to accumulate ppGpp during other, more severe, starvation conditions such as carbon-source starvation (8, 17) and plasmolysis in hypertonic salt solutions (18). Although no mutant has been isolated that is absolutely deficient in ppGpp and pppGpp, apparently normal strains do exist, such as EA-1 and NF-161, which show persistent accumulation of only ppGpp and not pppGpp during prolonged amino-acid starvation (2, 8). This has led, briefly perhaps, to focusing attention on the role of ppGpp.

Third, there are *in vitro* indications that some of the many cellular processes restricted by amino-acid starvation (in addition to protein and RNA accumulation) might be regulated by the inhibitory effects of ppGpp accumulation. These range from inhibition of inosine monophosphate dehydrogenase and adenylosuccinate synthetase (19) to inhibition of purine transport by membranes (20) and possible modification of the transcriptional specificity of RNA polymerase (21, 22). Finally, it must be admitted that abysmal ignorance of possible biosynthetic or catabolic roles of ppGpp and pppGpp has reinforced notions that these compounds probably act as regulator compounds.

Work in several laboratories has borne out indications of an intimate relationship between ppGpp and pppGpp production and protein synthesis. An *in vitro* measure of this relationship was discovered by Haseltine and coworkers (7), who found requirements for *in vitro* ppGpp synthesis to include ribosomes, elongation factor G, and a factor released from ribosomes by high salt concentration. This last factor has its activity specified by the allelic state of the *rel* gene (7). In addition to the obvious interest in this reaction for dissecting the regulatory intricacies of ppGpp and pppGpp biosynthesis, it also provides a preparative source for sizeable quantities of ppGpp, using GDP as the phosphate-accepting substrate

Abbreviations: ppGpp, guanosine 5′-diphosphate, 3′-diphosphate; pppGpp, guanosine 5′-triphosphate, 3′-diphosphate; NMR, nuclear magnetic resonance.

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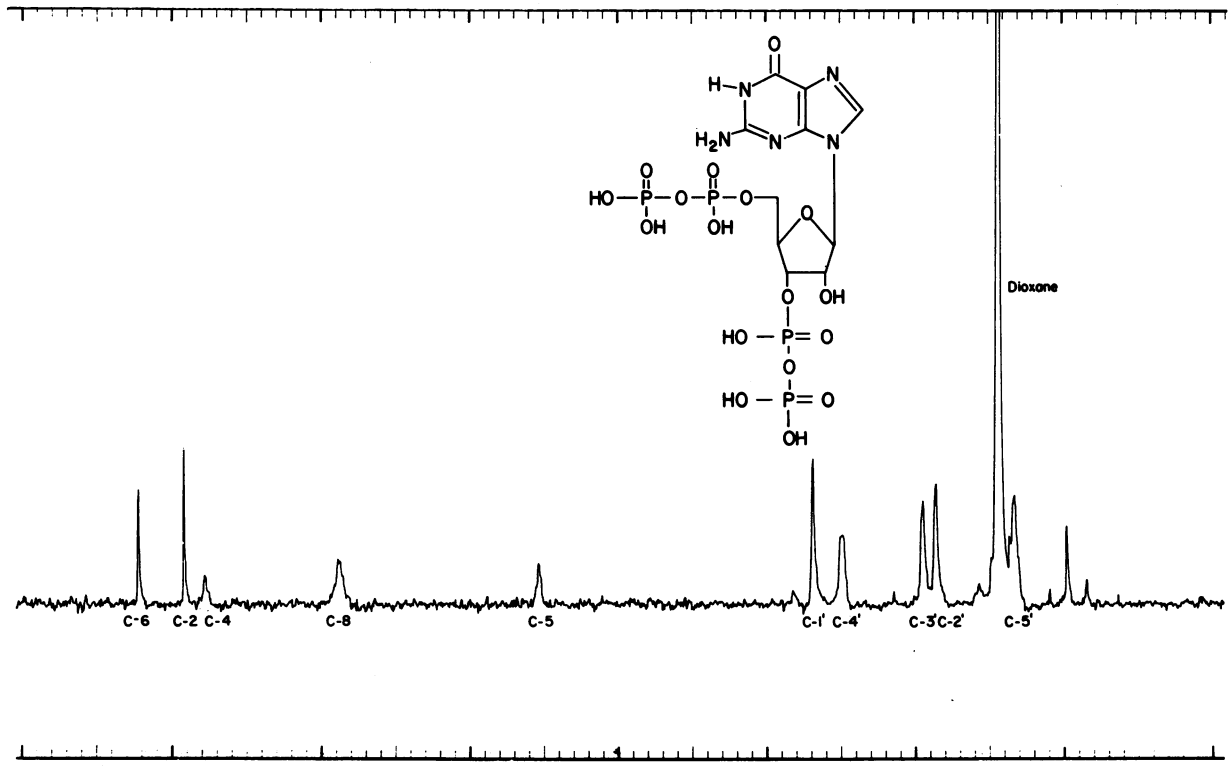

FIG. 1. Natural-abundance ^{13}C NMR spectrum of guanosine 5′-diphosphate, 3′-diphosphate.

and ATP as the pyrophosphate donor (Cashel, M., manuscript in preparation).

In this manner, we have prepared ppGpp and subjected the compound to ^{13}C natural-abundance nuclear magnetic resonance (NMR) spectroscopy in order not only to confirm its structure, but also to localize the positions of ribose attachment of the phosphates in the compound. While this work was in progress, Sy and Lipmann reported localization of the pyrophosphate residues to the ribose 5′ and 3′ positions based on the hydrolytic activities of rye grass 3′ phosphomonoesterase towards guanosine 5′-phosphate, 3′-phosphate, which in turn was produced by hydrolysis of the β-phosphates of ppGpp with zinc-activated pyrophosphatase from yeast (23).

MATERIALS AND METHODS

Preparation and Purity of Guanosine Tetraphosphate. The lithium salt of ppGpp was prepared by *in vitro* enzymatic synthesis by the method of Cashel (Cashel, M., manuscript in preparation). Guanosine tetraphosphate prepared in this way was shown by chemical analysis to contain guanine, ribose, and phosphate in a molar ratio of 1:1:4. Its labilities to acid and base hydrolysis were indistinguishable from guanosine tetraphosphate isolated from bacterial cells. The powder (370 mg dry weight) was dissolved in 1.4 ml of D_2O, and the solution was clarified by passage through a syringe-mounted Millipore filter. A small portion of this clear, colorless, slightly viscous solution (pH about 6.5) was diluted 11,000-fold into neutral aqueous solution. The UV spectrum of this diluted solution was indistinguishable from that of GDP, indicating the absence of detectable adenine nucleotides, and, assuming a molar extinction coefficient at 253 nm of 13,700, yielded a molarity of the concentrated solution of 0.36.

The degree of hydrolysis of the compound was examined by PEI-cellulose thin-layer chromatography by the method of Cashel (6), which separates guanine nucleotides on the basis of their phosphate content. Chromatography of samples containing from 3.5–175 nmol of guanosine tetraphosphate revealed no contaminants despite the fact that as little as 1–2 nmol of nucleotide can be detected by UV absorption.

After completion of the first NMR analysis, during which the sample was maintained at 31° for about 18 hr, chromatographic analysis revealed two hydrolysis products. A minor one migrating in the position of GTP and a more abundant one in the position of GDP. The sum of these hydrolysis products was estimated by visual inspection to account for <10% of the nucleotides.

Before the second analysis, 0.5 ml of D_2O was added to the sample to reduce its viscosity. At the conclusion of this analysis (about 18 hr at 31°) the sample was again analyzed by chromatography. The same two hydrolysis products were the only ones observed and were estimated to comprise <20% of the total nucleotide.

Natural-Abundance ^{13}C NMR Spectra. Proton noise-decoupled ^{13}C NMR spectra were obtained at 25.1 MHz on a Varian XL-100-15 NMR Spectrometer operating in the Fourier transform mode. The free induction decay signals were accumulated in 8000 data points over 60,000 transients with a 30-μsec pulse width and a 1-sec acquisition time. A suitable spectrum was not obtained during the first accumulation due to viscosity effects, so the sample was diluted and analyzed again.

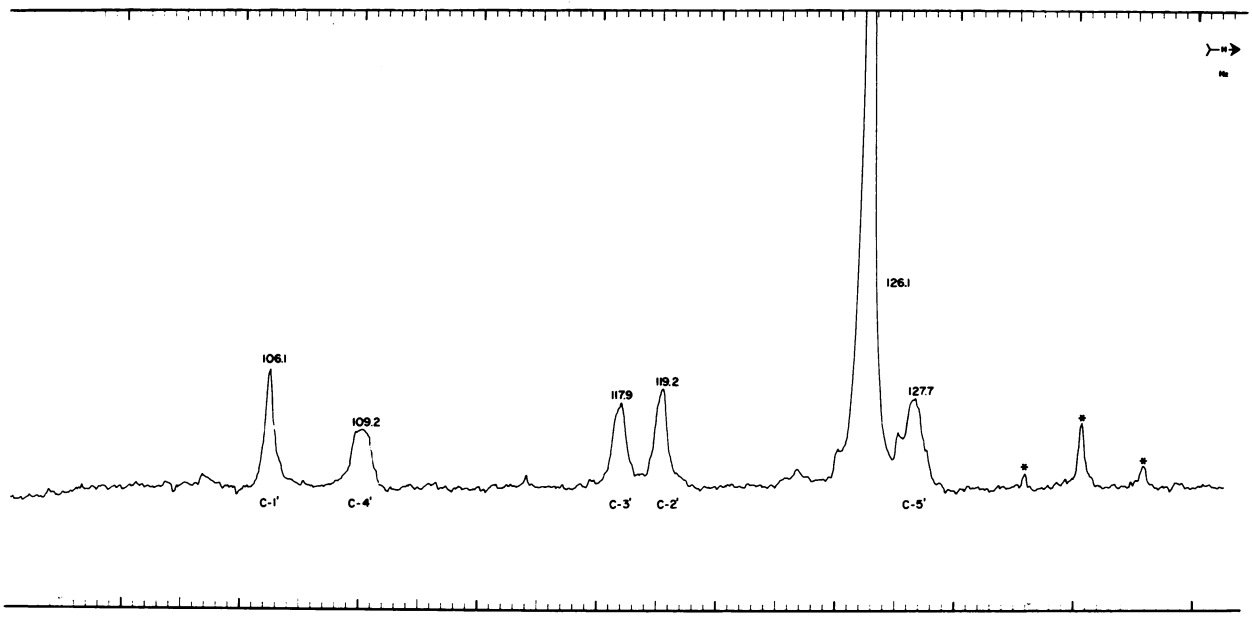

FIG. 2. Natural-abundance ^{13}C NMR spectrum of the ribose carbon region of guanosine 5′-diphosphate, 3′-diphosphate, expanded scale. * Impurities: the resonance at highest field is C-1 of ethanol, and the next lower resonances are the hydroxymethylene and quaternary carbons, respectively, of Tris.

RESULTS

The natural-abundance ^{13}C NMR spectrum of ppGpp (Fig. 1) contains resonances ascribable to 10 carbon atoms. The five resonances at lower field have chemical shifts similar to the ^{13}C resonances of other guanine nucleotides, and the assignments of these resonances is based on the work of Dorman and Roberts (24). The five resonances at higher field have chemical shifts characteristic of the ribose carbon atoms of ribonucleotides, and the assignments of these resonances can be made based on the work of Mantsch and Smith (25) and Kotowycz and Hayamizu (26). These workers observed that phosphorylation of a nucleoside resulted in a downfield shift of the carbon to which the phosphate was directly attached and an upfield shift of the carbons adjacent to that carbon. They also observed ^{13}C–^{31}P coupling to the directly attached carbon as well as to those adjacent to it.

The assignment of the structure of ppGpp as guanosine 5′-diphosphate, 3′-diphosphate is based on interpretation of the chemical shifts and linewidths of the ribose carbon atoms. To aid this interpretation, these data are summarized and compared to other nucleotides in Table 1, and the ribose portion of the ^{13}C NMR spectrum is expanded and presented in Fig. 2.

The C-1′ chemical shift of ppGpp is indicative of the β-configuration for a guanine nucleotide. The slight upfield shift relative to C-1′ of guanosine is also observed for other guanine and adenine nucleotides. More importantly, the small linewidth of C-1′ (8 Hz) indicates that a diphosphate group is not present at C-2′, as the ^{13}C–^{31}P coupling to C-1′ in other 2′-nucleotides is very large (about 9 Hz), which in this spectrum would result in a C-1′ linewidth of about 16 Hz (25, 26). It is possible, however, that a 2′-diphosphate could adopt a conformation such that the phosphorous coupling to C-1′ would be small. But this would result in a downfield shift of C-2′ and an upfield shift of C-3′ in ppGpp relative to guanosine. From the data of Kotowycz and Hayamizu (26), the expected chemical shifts of C-2′ and C-3′ would be calculated to be about 116 and 122 ppm, respectively. The

TABLE 1. *Carbon-13 chemical shifts of the ribose carbons of adenine and guanine nucleotides**

Nucleotide	Ref.	C-1′	C-2′	C-3′	C-4′	C-5′
(Adenosine)	27	103.5	118.0	120.9	105.7	130.0
2′-AMP	26	105.1 (+1.6)†	116.5 (−1.5)	122.1 (+1.2)	107.3 (+1.6)	131.1 (+1.1)
3′-AMP	26	104.2 (+0.7)	118.8 (+0.8)	119.3 (−1.6)	107.4 (+1.7)	131.0 (+1.0)
5′-AMP	26	105.5 (+2.0)	118.1 (+0.1)	122.1 (+1.2)	108.2 (+2.5)	128.7 (−1.3)
ATP	24	105.6 (+2.1)	118.3 (+0.3)	122.4 (+1.5)	108.7 (+3.0)	127.3 (−2.7)
(Guanosine)	27	105.4	117.8	121.1	106.2	130.4
5′-GMP	24	105.5 (+0.1)	118.2 (+0.4)	121.9 (+0.8)	108.4 (+1.8)	128.6 (−1.8)
ppGpp		106.1 (+0.7)	119.2‡ (+1.4)	117.9‡ (−3.2)	109.2 (+3.0)	127.7 (−2.7)

* In parts per million upfield from CS_2.

† Upfield or downfield shift in ppm with respect to the corresponding nucleoside.

‡ Assignments may be reversed; see discussion in *text*.

observed shifts of C-2′ (119.2 ppm) and C-3′ (117.9 ppm) demonstrate that a diphosphate group is not present at C-2′ in ppGpp.

The assignments of C-2′ and C-3′ are based on the magnitudes of the expected downfield shift for C-3′ and the expected upfield shift for C-2′ due to the diphosphate group at C-3′. In adenine nucleotides (Table 1), phosphorylation at a secondary carbon produces a greater downfield shift of that carbon than phosphorylation at a primary carbon. Since the observed downfield shift of C-5′ in ppGpp is 2.7 ppm relative to guanosine, the resonance at 117.9 ppm was assigned to C-3′. This assignment results in a downfield shift for C-3′ of 3.2 ppm relative to guanosine, and an upfield shift of 1.4 ppm for C-2′ relative to guanosine. If these assignments are reversed, a downfield shift of 1.9 ppm is observed for C-3′ and an upfield shift of only 0.1 ppm for C-2′. Regardless of these assignments, only C-3′ is shifted downfield and therefore must contain a diphosphate substituent, but the assignments given more nearly represent the expected shifts of C-2′ and C-3′.

The chemical shifts of C-4′ and C-5′ and the linewidth of C-4′ further substantiate the structure of ppGpp as guanosine 5-′diphosphate, 3′-diphosphate. The large linewidth of C-4′ (19 Hz) is consistent with coupling from the diphosphate substituents at both C-3′ and C-5′, and the upfield shift of C-4′ reflects the fact that it is adjacent to both the C-3′ and C-5′ diphosphate groups.

DISCUSSION

Our concern here is in confirming the structure of ppGpp with the intact compound through the use of ^{13}C natural-abundance NMR spectroscopy. There is a clear indication from these spectra that the pyrophosphate residues are indeed attached to the 5′ and 3′ ribose carbons and that there are no indications of unusual spectral shifts in the guanosine moiety of ppGpp. Thus, these determinations with the intact compound clearly confirm the assignments made by Sy and Lipmann using evidence from enzyme specificity with guanosine 5′-phosphate, 3′-phosphate, the hydrolytic product of ppGpp (23).

There is chromatographic evidence that ppGpp isolated from whole cells is in fact the same compound produced by the *in vitro* reaction. Comigration of these two isolates has been seen in various chromatographic development systems including borate, neutral and acidic lithium chloride, sodium formate, and potassium phosphate at pH 3.4 (ref 7; M. Cashel, manuscript in preparation). In addition, by use of [^{33}P]ppGpp obtained from whole cells mixed with [^{32}P]ppGpp from *in vitro* preparations, it is not possible to distinguish the source of ppGpp from an analysis of the detailed rate of hydrolysis in either acid or alkali (M. Cashel, manuscript in preparation). Similar results have been obtained with pppGpp isolates.

In view of the fact that both GDP and GTP can serve as pyrophosphate acceptors from ATP (7, 23), it seems likely that a structural assignment for pppGpp will be analogous to that of ppGpp. However, NMR spectra on pppGpp have not been obtained.

It is perhaps notable that neither dGTP nor dGDP serve as efficient pyrophosphate acceptor substrates *in vitro* as compared to GDP and GTP (7). Since the deoxyribonucleotides do contain a 3′-hydroxyl group, the ability of the enzyme system to discriminate between these compounds and GDP or GTP is apparently more complex than simply the availability of an appropriate 3′-hydroxyl group.

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