A novel nucleotide has been detected in _Escherichia coli_ subjected to the stringent response. However, this nucleotide does not accumulate in _relA_ cells subjected to heat shock, in which guanosine 5'-diphosphate-3'-diphosphate does accumulate but stable RNA synthesis is not restricted. The intracellular level of this new nucleotide thus correlates well with control of stable RNA synthesis. Chemical and enzymatic analysis shows that the new nucleotide is guanosine 5'-diphosphate-3'-monophosphate. It is suggested that this nucleotide may play a role in stringent control of stable RNA synthesis.

When the aminoacylation of any tRNA species is restricted in _Escherichia coli_, there ensues a major readjustment of the pattern of transcriptional and metabolic activity, including a restriction of stable RNA synthesis, termed stringent control (reviewed in Refs. 1 to 3). This adjustment requires a functional _relA_ gene product, which has been identified as an enzyme which produces the two regulatory nucleotides, ppGpp and ppGpp (reviewed in Ref. 4). Stringent control is unimpaired in _spoT_ mutants, which accumulate only ppGpp (5, 6). It follows that the ability to generate a high level of ppGpp is necessary for stringent control.

The nucleotide has proven to be a pleiotropic effector of a variety of metabolic steps and transcriptional processes in _vitro_ (reviewed in Refs. 1 to 3). Nonetheless, its reported effects on tRNA and tRNA synthesis in _vitro_ and in permeabilized cells have not, on the whole, seemed sufficient to account in full for stringent control of the formation of these RNA species in real life (reviewed in Ref. 7). Under various growth regimes, high levels of ppGpp are generally correlated with restricted synthesis of tRNA and tRNA. But special cases have been observed where this correlation breaks down (7-9). In particular, we recently found that certain strains accumulate a high level of ppGpp upon heat shock but do not at the same time restrict RNA and tRNA formation (7). (The interpretation of these results has recently been challenged by Challoner-Larson and Yamazaki (10). However, their objections are obviated by the fact that the original results depended on the comparison of the behavior of _isogenic relA_ and _relA_ cells following temperature upshift. Challoner-Larson and Yamazaki (10) reported no such comparison in their work, and we feel that such a comparison is essential.) It follows that a high level of ppGpp may be necessary, but it is not sufficient, for stringent control of stable RNA synthesis.

To explain this puzzle, we speculated that some derivative of ppGpp, formed during the stringent response but not upon heat shock, might be an essential element of the stringent control system (7). In this article, we describe the identification and characterization of a new member of the MS nucleotide family which might fill the bill.

**MATERIALS AND METHODS**

_Bacterial Strains—_The strains employed were CP78 (R+, arg-, his-, thr-, leu-, _relA_); CP79, an _isogenic relA_-descendant of CP78; NF161 (met-, arg-, _relA_); and CP78GA2, an _isogenic guaA_-mutant (defective in xanthosine-5'-monophosphate amidotransferase) derived from CP78. CP78, CP79, and NF161 are available through the _E. coli_ stock culture collection, care of Dr. Barbara Bachmann, Department of Human Genetics, Yale University Medical School.

_Media and Culture Conditions—_Tris/glucose minimal medium supplemented with appropriate required amino acids and KH₂PO₄, at 0.5 mm was used throughout and has been described before (7). Growth of cells was under forced aeration at 37°C and monitored turbidimetrically at 720 nm in a Beckman DB spectrophotometer; an absorbance of 1.0 corresponds to about 10⁷ cells or 200 µg of protein/ml.

_Chemicals and Supplies—_³²P was purchased from New England Nuclear as orthophosphoric acid, carrier-free. Common nucleotides and enzymes were obtained from Sigma. Unusual nucleotides were from either ICN Pharmaceutical or P-L Biochemicals. PEI-cellulose sheets were obtained from both Brintmann (cellulose-polyethyleneimine Polygram Cell 300 PEI) and Merck (PEI-cellulose F). Cellulose sheets were purchased from Eastman-Kodak Co. (Eastman Chromatographic-cellulose with fluorescent indicator).

_Labeling and Extraction of Nucleotides—_Small volumes of growing cultures were labeled as indicated, and extracted in 0.1 M formic acid. After neutralization with Tris buffer, the cell pellet was centrifuged down in a Beckman microfuge, and the supernatant, containing some cases, nucleotides were adsorbed to a small Norit column, as described in the legend to Fig. 2. After preparative thin layer chromatography, MS₃ was eluted with 2 µ l triethylammonium bicarbonate, followed by lyophilization.

**RESULTS**

_Detection of MS₃—_Fig. 1 shows radioautograms produced by a certain group of _³²P-labeled nucleotides, resolved by a_}

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† The abbreviations used are: pnpGpp, guanosine 5'-triphosphate-3'-diphosphate; ppGpp, guanosine 5'-diphosphate-3'-diphosphate; ppGp, guanosine 5'-diphosphate-3'-monophosphate; PEI-cellulose; polyethyleneimine-cellulose.

‡ We use this abbreviation by analogy to MS1 and MS2, the original designations of ppGpp and pnpGpp before these compounds were characterized. We report the chemical characterization of MS3 below, and will call it by its proper name once we have completed the description of the structural analysis.
microliters of cell-free extract labeled at 0.5 mCi/ml were applied to a thin layer chromatography on PEI-cellulose (Brinkmann). Twenty microliters of cell-free extract labeled at 0.5 mCi/ml were applied together with 0.1 nmol of each of the markers indicated. First dimension, 4 M HCOOH + 1 M LiCl; second dimension, 1.5 M KH₂PO₄. The figure shows autoradiograms of these chromatograms after 3-day exposure; the positions of marker GTP, ATP, and adenosine tetraphosphate, visualized by ultraviolet absorption, are circled in solid lines. Conditions were: Panel A, strain CP78 (rel') in exponential growth; Panel B, strain CP78 9 min after adding 500 µg/ml of serine hydroxamate (a dotted circle just above the GTP spot); Panel C, strain CP78 (relA) after serine hydroxamate; Panel D, CP78, 6 min after shift from room temperature to 40°C.

new method. In Panel A, the nucleotides were those found in a relA' strain growing exponentially; in Panel B, those found in an isogenic relA mutant also subjected to serine hydroxamate inhibition. It can be seen that an increased level of the regulatory nucleotides ppGp and ppGpp is specific to the conditions of Panel B. Another compound, just above GTP in Panel B, shares this specificity as well. We call this compound MS3. The nucleotide predicted by our hypothesis should accumulate during the stringent response but not upon heat shock. Panel D, in which the cells were the relA' strain subjected to heat shock, shows that the level of MS3 behaves as predicted.

MS3 has not been detected before because it co-migrates with GTP in most commonly employed separation systems. In the system of Fig. 1, MS3 is just resolved from GTP and co-migrates with its two isomers, ppGp and ppGpp. At this point, a serious possibility of artifact needs to be considered. The first dimension solvent of the separation system is strongly acidic, and the 3'-pyrophosphate group of ppGpp is somewhat acid-labile. Thus, some labeled ppGp could be generated from labeled ppGpp during chromatography in the first dimension. We have therefore taken some pains to determine how much, if any, of the material we term MS3 is of authentic biological origin.

First, we have simply reversed the order in which the two dimensions of the separation are developed. The second dimension solvent, 1.5 M KH₂PO₄, at pH 3.4, is only weakly acidic; using authentic ppGp, we find 0.5% or less hydrolysis to ppGp during chromatography in this solvent. Since the R₂ of ppGp is much lower than that of ppGp in the KH₂PO₄ solvent, any ppGp generated from ppGpp during subsequent development at right angles in the acidic solvent will not co-migrate with ppGp in the two-dimensional pattern. Thus, we would expect material co-migrating with ppGp in both dimensions to contain artifactually generated ppGp amounting to a maximum of 0.5% the quantity of ppGpp. On the contrary, the labeled MS3 detected by this procedure (Fig. 2) amounts to nearly 3% the quantity of ppGpp. The bulk of this material must therefore be of authentic biological origin.

We should warn the reader that the method illustrated in Fig. 1 does risk considerable contamination of MS3 by spurious ppGp generated by hydrolysis of ppGpp during the first, acidic dimension. The quantity will vary with the length of time the first dimension takes to run, which varies unpredictably from one batch of PEI-cellulose plates to the next. Indeed, with unusually slow running plates we have seen clear evidence of this chromatographic artifact, in the form of a trail of radioactivity running down from MS3 through the GTP spot to the level of ppGpp in the first dimension. Thus, the reversed two-dimensional procedure illustrated in Fig. 2 is the method of choice for detecting authentic MS3 and is the method we used in the [³¹C]guanosine labeling studies described below.

In order to obtain sufficient MS3 for chemical characterization, we have employed the following three-step procedure. First, the nucleotide fraction was purified and concentrated by adsorption to activated charcoal, followed by desorption in 50% ethanol containing 2.5% NH₄OH. The material was then subjected to column chromatography on DEAE-Sephadex in a neutral pH NaCl gradient containing urea by the method shown in Fig. 3. The small peak (Fraction 26) following marker GTP proved to consist mainly of MS3 (Fig. 3A), which was subsequently isolated by preparative thin layer chromatography (Fig. 3B). Here again, we have assessed the extent of artificial hydrolysis of ppGpp to ppGp during work-up, in particular the brief exposure to 2.8% NH₄OH during desorption from Norit. When authentic ppGpp was carried through the latter procedure, we again found less than 0.5% hydrolysis to ppGp. Thus, MS3 isolated in this manner should also consist mainly of authentic biological material.

Chemical Characterization of MS3—MS3 is absorbed by activated charcoal and labeled by [³¹C]guanosine (see below), indicating that it is a purine nucleotide. It is insensitive to periodate oxidation and fails to complex borate ion, indicating

![Image 1](http://www.jbc.org/)  
**Fig. 1.** Thin layer chromatography of [³¹P]labeled MS3. [³¹P]-labeled nucleotides were extracted from cells as described under “Materials and Methods” under four different conditions and resolved by thin layer chromatography on PEI-cellulose (Brinkmann). Twenty microliters of cell-free extract labeled at 0.5 mCi/ml were applied together with 0.1 nmol of each of the markers indicated. First dimension, 4 M HCOOH + 1 M LiCl; second dimension, 1.5 M KH₂PO₄. The figure shows autoradiograms of these chromatograms after 3-day exposure; the positions of marker GTP, ATP, and adenosine tetraphosphate, visualized by ultraviolet absorption, are circled in solid lines. Conditions were: Panel A, strain CP78 (rel') in exponential growth; Panel B, strain CP78 9 min after adding 500 µg/ml of serine hydroxamate (a dotted circle just above the GTP spot); Panel C, strain CP78 (relA) after serine hydroxamate; Panel D, CP78, 6 min after shift from room temperature to 40°C.

![Image 2](http://www.jbc.org/)  
**Fig. 2.** Resolution of MS3 by reversed two-dimensional thin layer chromatography. Aliquots of an exponential, [³¹P]labeled culture of NF161 were submitted to the stringent response by addition of 800 µg/ml of valine (A) or 500 µg/ml of serine hydroxamate (B). Nucleotides were extracted from A after 18 min and from B after 7 min, and resolved with solvents reversed as described in the text. The radioautogram of Culture A is shown at left, that of Culture B at right. (No MS3 was detected in the control, uninhibited culture.) The ratio of MS3 to MS1 was 2.75% in A and 2.64% in B. In two other experiments with the same strain subjected to valine inhibition, the MS3/MS1 ratio was 2.62 and 2.87%, giving an overall average of 2.72%. Since the amount of ppGp generated artifically by hydrolysis of ppGpp during chromatography is less than 0.5% (see text), more than 90% of the MS3 detected in these experiments must have originated in the cells.
the presence of a substituent on position 2' or 3' (11). To identify the nucleoside residue, we prepared MS3 from cells labeled with [14C]guanosine, stripped off the phosphates by alkaline phosphatase treatment, and submitted the product to two-dimensional thin layer chromatography along with appropriate markers. Fig. 4 shows that the product co-migrated exactly with guanosine. The separation system employed resolves guanosine from all known purine nucleosides, including, in particular, 2'- and 3'-modified guanosine derivatives as shown. We conclude that the nucleoside residue of MS3 is unmodified guanosine. Since alkaline phosphatase hydrolysis converts MS3 to guanosine, it follows that the substituent on position 2' or 3' is one or more of the phosphate groups.

After equilibrium labeling with [14C]guanosine and [32P]-Pi, the ratio of 14C to 32P found in MS3 was 97% that found in GTP and 71% that found in ppGpp, indicating that the compound contains three phosphate groups/guanosine residue. Accordingly, the chromatographic behavior of MS3 was compared with that of authentic ppGpp and pGpp. Both compounds co-migrated with MS3 in the two-dimensional separation of Fig. 1 and in several other systems. Moreover, nitrous acid treatment of MS3 converted it to a product which co-migrated in a two-dimensional separation with the nitrous acid product of ppGpp, presumably ppXp (data not shown). All of the foregoing results indicate that MS3 is an isomer of GTP.
Guanosine 5'-Diphosphate-3'-monophosphate in Escherichia coli

3-P dehydrogenase and 3-P-glycerate phosphokinase: hydrolysis, indicating that it does not have a diphosphate between inorganic phosphate and ATP catalyzed by glyceraldehyde-3-P dehydrogenase and 3-P-glycerate phosphokinase:

\[
glyceraldehyde-3-P + NAD \rightarrow 1,3-PP-glycerate + NADH
\]

1,3-PP-glycerate + ADP \( \rightarrow \) 3-P-glycerate + ATP

ppG, ppGpp MS3, and ppGpp were tested for their ability to serve as an acceptor in such a reaction. Ten-microliter reaction mixtures contain the following: Tris-HCl buffer at pH 7.4 (0.3 μmol), glyceraldehyde-3-P (10 μmol), MgSO4 (0.05 μmol), KCl (0.5 μmol), K2HPO4 (1 μmol), NAD (1 μmol), \([\gamma^32P]phosphoric acid (0.2 μCi), glyceraldehyde-3-P dehydrogenase and 3-P-glycerate phosphokinase (Sigma) at 0.1 and 0.3 unit, respectively. The reaction mixtures were incubated at 37°C for 60 min and followed by two-dimensional thin layer chromatography. First dimension, 2 M HCOOH + 1.5 M LiCl; second dimension, 1.5 M KH2PO4. Panel A, ppG as acceptor; Panel B, ppGpp as acceptor; Panel C, purified MS3 as acceptor (the expected radioactive product is ppGpp which migrates close to the position of ppGpp); and Panel D, ppGpp as acceptor (note that there is no radioactive compound close to ppGpp). Circles represent the positions of marker nucleotides. The chromatograms were exposed to Kodak autoradiography films for 16 h.

Location of the Phosphate Groups in MS3—The 3'-diphosphate groups of such nucleotides as ppGpp and ppGpp are sensitive to alkaline hydrolysis, whereas the 5'-diphosphate residue of ppGpp is not. MS3 is not sensitive to alkaline hydrolysis, indicating that it does not have a diphosphate group on position 3' or 2' (12). A complementary test is provided by the Glynn-Chappell reaction (13, 14), which phosphorylates guanosine nucleotides bearing a diphosphate group in position 5'. Both MS3 and ppGpp serve as acceptors in this reaction, whereas ppGpp does not (Fig. 5). We conclude that MS3 is a guanosine 5'-diphosphate with another phosphate on either position 2' or 3'.

To determine the location of the third phosphate group, we hydrolyzed MS3 with snake venom phosphodiesterase and submitted the diphosphate product to two tests. First, the product co-migrated with authentic ppGp(3') in a separation system which resolves this compound from ppGp(2') (Fig. 6, Panel A). Second, the diphosphate product was hydrolyzed virtually completely by ryegrass 3'-nucleotidase (15, 16) (Fig. 6, Panel B), which was shown in parallel incubations to be wholly inactive against ppGp(2'). These tests indicate that the third phosphate group in MS3 is esterified to position 3', and complete the structural proof that MS3 is identical to ppGp.

We have performed one further enzymatic test of this identity. Snake venom phosphodiesterase cleaves the 5' α-β bond of different nucleotides at characteristic rates and works particularly slowly on nucleotides bearing a 3' substituent (17-19). Slow hydrolysis of MS3 and authentic ppGp by this enzyme proceeded with identical kinetics (Fig. 7).

Kinetics of MS3 Labeling in Vivo—Fig. 8 shows the kinetics of [14C]guanosine entry into the steady state pools of GTP, ppGpp, and MS3 (ppGp). The lag between the labeling of GTP and ppGpp, as pointed out by Fiil et al. (20, 21) is
This report brings the number of these nucleotides identified in *E. coli* to three. It is by no means impossible that other such compounds remain to be discovered in *E. coli*, especially in view of the existence of related nucleotides in *Actinomycetes* (22, 23) and *Bacillus subtilis* (24).

This increasingly numerous tribe of 3'-phosphorylated nucleotides, and their putative regulatory functions, presents itself as an intracellular, prokaryotic counterpart to the families of chemically related hormones of higher organisms, each with particular although sometimes overlapping functions in a network of specificities. (We are indebted for this analogy to the late Gordon Tomkins (25).) We believe that the burgeoning literature on control effects of the most abundant of these compounds, ppGpp, will prove to be only the first installment of a much more elaborate story.

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Note Added in Proof—Lagosky and Chang (26) have recently reported in detail on the production of ppGp by acid hydrolysis of msGms, and they have also developed a lysozyme-deoxycholate method for extracting nucleotides from cells without this artifact; using this method, we are happy to note, they find a biological level of ppGp virtually identical to the one we have observed.

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