Diadenosine 5',5''''-P1,P4-tetraphosphate and Related Adenylylated Nucleotides in Salmonella typhimurium*

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Salmonella typhimurium LT2 rapidly accumulates high levels of a family of five adenylylated nucleotides following exposure to a bacteriostatic quinone, 6-amino-7-chloro-5,6-dioxoquinoline. These compounds have been analyzed using our recently described two-dimensional thin layer chromatographic method. The five dinucleotides, which cannot be detected in exponentially growing cells, have been identified as diadenosine 5',5''''-P1,P4-tetraphosphate (AppppA), AppppGpp (guanosine 3'-diphosphate-5'-adenosine-5''''-(P1,P4-triphosphate)), AppppG (adenosine 5'-guanosine-5'- (P1,P4-tetraphosphate)), AppppA (diadenosine 5',5''''-P1,P4-triphosphate), AppppA has been previously detected in vitro as an enzymatic product of aminoacyl-tRNA synthetases and in vivo at submicromolar levels in eucaryotic cells. The induced intracellular concentration of AppppA and the other adenylylated nucleotides in S. typhimurium is approximately 100-fold higher than that found in eucaryotic cells. We propose that these dinucleotides are alarmones, regulatory molecules signaling a particular metabolic stress.

In 1966, Zamecnik and his colleagues discovered that AppppA1 was synthesized in an in vitro enzymatic system consisting of ATP, l-lysine, Mg2+, and purified Escherichia coli lysyl-tRNA synthetase (1). This dinucleotide is produced when ATP replaces PPi in the back reaction of amino acid activation and is adenylylated by the enzyme (see Fig. 1) (2–4). In fact, it has been found that many nucloside 5'-di- and 5'-triphosphates can be adenylylated when they substitute for PPi, in this reversible pyrophosphate exchange catalyzed by the synthetase (2–6). A variety of aminoaac-tRNA synthetases from both procaryotic and eucaryotic cells can synthesize AppppA in vitro, although some tRNA synthetases may not have this capability (3, 4, 7, 8). Therefore, it is likely that a wide range of living cells can synthesize AppppA and other adenylylated nucleotides.

Thus far, AppppA has been detected in vivo in lower eucaryotic cells (e.g. Tetrahymena pyriformis (9) and Physarum polycephalum (10)) and in a variety of mammalian cells at very low concentrations of 10–1200 nM (3, 4, 11). Rapaport and Zamecnik have reported a correlation between increased levels of AppppA and cellular proliferative activity and have proposed a function for this dinucleotide as a possible pleiotropic activator of proliferation (11). This hypothesis is supported by findings that AppppA triggers the initiation of in vitro DNA replication in quiescent mammalian cells (12), binds to HeLa cell DNA polymerase α (13, 14), and acts as a primer for DNA synthesis in vitro (15, 16).

We now find that a family of adenylylated nucleotides dramatically accumulate to high levels in a bacterial cell inhibited by a cytostatic quinone, ACDQ. Using our newly developed technology to resolve cellular nucleotides, we have analyzed the novel phosphorylated compounds that are produced, their induced levels, and the kinetics of their accumulation under these conditions. A preliminary account of part of this work has been presented.5

EXPERIMENTAL PROCEDURES

Chemicals—ACDQ was kindly provided by Dr. J. Potter of Farbwerte Bayer, Wuppertal, Federal Republic of Germany. AppA, AppppA, ApppppA, AppppppA, and AppppG were purchased from P-L Biochemicals. Sources of other chemicals have been described previously (17).

Cultivation, Labeling, and Extraction of Cells— Cultures of Salmonella typhimurium strain LT2 were grown, labeled, and extracted as described (17). A radiolabeled culture was grown in 32P, at a specific activity of 500 μCi/μmol (100 μCi/ml of 32P, 0.2 mM KH2PO4). When the density of the culture reached a 4×106 range of 0.5 to 0.7 (approximately 5×109 cells/ml), an initial aliquot was taken. Two minutes after removing this sample, ACDQ (5 μg/ml) was added to the radioactive culture, and aliquots were taken at designated time intervals. When formic acid extracts of labeled cells were resolved using our newly developed technology to resolve cellular nucleotides, we have analyzed the novel phosphorylated compounds that are produced, their induced levels, and the kinetics of their accumulation under these conditions. A preliminary account of part of this work has been presented.5

Thin Layer Chromatography, Autoradiographic Detection, and Quantitation—Ten ml of the crude acid extract were applied as a lane (for one-dimensional systems) or as a spot (for two-dimensional...
systems) on polyethyleneimine cellulose plates and developed as described (17). In co-chromatography experiments, 2-µl aliquots of 5 ml standard solutions were mixed with 10-µl radioactive samples before application to the TLC plate. Locations of standards were determined either by UV absorbance (17) or the phosphate detection method (19). 32P-Labeled compounds were visualized by autoradiography (17). When required, the corresponding spots were cut out from the chromatogram and placed in a vial containing 4.0 ml of Betamax (WestChem Products) scintillation fluid. Radioactivity was determined by scintillation counting in a Packard Tri-Carb 300 scintillation counter.

Purification of Quinone-induced Spots and Other Radiolabeled Nucleotides—A 10-ml culture of S. typhimurium was grown and labeled as described above. Thirty min after the addition of ACDQ, the cells were extracted as above and separated into 1-ml portions. The debris was pelleted by centrifugation in a Beckman microfuge. The supernatant was filtered through an Acrodisc disposable filter assembly (0.2 µm) from Gelman and concentrated in a spinning lyophilizer (Savant Instruments, Inc.) overnight. The concentrate was resuspended in 70 µl of distilled water, spotted on 12 TLC plates, and run in the optimized two-dimensional TLC system. Autoradiograms were used as guides to locate the relevant compounds. After the spots were cut out from the chromatograms, the polyethyleneimine cellulose containing each compound was scraped from the plastic backing. Each phosphorylated compound was eluted in 0.5 ml of 0.25 M NH₄HCO₃ (pH 8) for 30 min at room temperature. The polyethyleneimine cellulose was pelleted by centrifugation in a microfuge for 10 min at 4 °C, the supernatant was removed, and the elution process was repeated. The two supernatant fractions were pooled, concentrated in a spinning lyophilizer overnight, and resuspended in 100 µl of 0.25 M NH₄HCO₃ (pH 8). This procedure gave a high yield of purified compound (approximately 90% recovered).

Chemical and Enzymatic Tests—Tests for the characterization of compounds were performed as previously described (17).

RESULTS

S. typhimurium LT2 Rapidly Accumulates Six Novel Phosphorylated Compounds after Inhibition by ACDQ—ACDQ is a cytostatic quinone known to inhibit the growth of E. coli (20, 21). Its primary mode of action, at low concentrations, appears to be the inhibition of charging of tRNAes by reacting with essential sulfhydryl groups of leucyl-tRNA synthetase, resulting in an active site-directed modification of the enzyme (21-23). Cells exposed to this quinone accumulate ppGpp and ppGpp (at a ratio of 10:1) (20, 22). Nucleotide pool changes induced by ACDQ were investigated using our newly developed technology (17). Fig. 2a shows a schematic diagram of our standard two-dimensional TLC system (17). Fig. 2b is an autoradiogram of a normal 32P-labeled crude extract of S. typhimurium, while an autoradiogram of an extract taken after a 30-min exposure to ACDQ (5 µg/ml) is depicted in Fig. 2c. It is apparent that a number of spots (as indicated by arrows) rapidly appear, or increase in intensity, in the lower portion of the autoradiogram after the addition of ACDQ. Their chromatographic locations indicate that they are compounds with high negative charge. A large number of other metabolic stresses did not cause the appearance of these spots (Refs. 17 and 24 and data not shown).

To see whether other changes occur following exposure to
ACDQ, the acid extracts of *S. typhimurium* before and after addition of ACDQ were separated in a two-dimensional TLC system which gives excellent resolution of nucleotides with low negative charge (data not shown) (17). Aside from minor pool fluctuations, there were no other changes in the two-dimensional profiles other than those occurring in the high negative charge region.

Better resolution of compounds in the lower left corner of the chromatogram was obtained by increasing the salt concentration in the first dimension solvent and lowering the salt concentration in the second dimension solvent. A first dimension solvent of 1.75 M morpholine, 0.1 M boric acid, 1.4 M HCl (pH 8.7) gives excellent resolution of ribo- and deoxyribonucleoside tri- and polyphosphates. A second dimension solvent of 3 M (NH₄)₂SO₄, 2% disodium EDTA (pH 5.5) gives improved resolution of hydrophobic nucleotides with a minimal amount of smearing (17). A schematic diagram of this system is shown in Fig. 3a.

With this optimized two-dimensional system, we can clearly visualize the nucleotide pool changes which occur following exposure to ACDQ. Fig. 3c depicts an autoradiogram of a crude extract 50 min after addition of ACDQ using the optimized TLC system. Compared to an autoradiogram of the normal acid extract (Fig. 3b), there are 11 spots which increase in intensity. Six major spots correspond to phosphorylated compounds which accumulate dramatically and are not detectable (i.e. concentrations less than 1 μM) in exponentially growing cells. Since they have not been detected previously in *S. typhimurium*, we designated these as quinone-induced spots (QS) 1–6. According to the “logic” of the two-dimensional TLC system (17), the relative migration of these compounds in the first dimension indicates that they probably contain 3–5 phosphates. Similarly, their location in the hydrophobic (or left) portion of the chromatogram suggests that they are either dinucleotides or CoA derivatives. Another spot (indicated by the arrow) appears to be a result of smearing during chromatography and was not seen in several similar chromatographic runs. In addition to these new spots, five other spots increase, but they have been seen previously. Two of the spots correspond to the “magic spots” (ppGpp and pppGpp) discovered by Cashel and Gallant (25). From previous work (20,22) these were known to accumulate in ACDQ-inhibited cells. Three other spots correspond to ppG>p (guanosine 5’-diphosphate-2’,3’-cyclic monophosphate, two spots) and pppG>p (guanosine 5’-triphosphate-2’,3’-cyclic monophosphate) (see Fig. 3a). ppG>p and pppG>p levels increase on chromatograms simultaneously with the “magic spots” and appear to be minor artifactual degradation products which are produced when ppGpp and pppGpp, respectively, break down before and during chromatography. Several known

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3 B. Bochner, unpublished data.
nucleotides (succinyl-CoA and the AMP precursors, ZMP and succinyl-ZMP (17)) were observed to decrease 30 min after the addition of ACDQ (Fig. 2c).

Characterization of the Quinone-induced Spots—We have developed a battery of chemical and enzymatic tests which can be used to characterize and analyze nucleotides and nucleotide derivatives (17). Only one chromatogram is required to test the susceptibility of several compounds. In addition, the sensitivity of known compounds to a given test verifies the validity of that test. These tests have elucidated the structure of each of these novel metabolites.

The susceptibilities of the quinone-induced spots to the seven tests are summarized in Table I. It is evident that QS 1, 3, 4, and 5 have identical susceptibilities to the battery of tests. This, in addition to the fact that they all migrate in the same region of the two-dimensional TLC system, indicates that they may be structurally related. Their resistance to bacterial alkaline phosphatase (see Fig. 3d), 5'-nucleotidase, and nuclease P1 indicates that these compounds do not contain external phosphate groups. Therefore, they are likely to be dinucleotides rather than CoA derivatives. Furthermore, their resistance to ribonuclease T2 argues against internal 3'-phosphate linkages. The sensitivity of these dinucleotides to oxidation by periodate and digestion by venom phosphodiesterase, however, suggests these phosphorylated compounds are ribose-containing nucleotides with internal 5'-phosphate linkages.

QS6 differs from these four compounds in being sensitive to digestion by alkaline phosphatase (see Fig. 3d), 5'-nucleotidase, and nuclease P1 indicates that this compound contains one or more external phosphates. Its susceptibility to venom phosphodiesterase and ribonuclease T2 suggests that this compound contains both 5'- and 3'-polynucleotides, at least one of which is probably cleaved by alkaline phosphatase. As with QS 1, 3, 4, and 5, QS2 is oxidized by periodate. This compound resembles ppGpp and ppGGpp in its susceptibility to the tests (17).

QS6 is apparently structurally unrelated to the other quinone-induced spots. While QS 1-5 adsorb to charcoal, confirming that they are nucleotides, QS6 does not. We have found that some nucleotides have a decreased affinity for charcoal; for example, uracil nucleotides adsorb less readily than adenine or guanine nucleotides. In order to determine whether the nonbinding of QS6 to charcoal was due to decreased affinity, we exposed this phosphorylated compound to 2- and 4-fold increased amounts of charcoal (1.3 mg/20 μl and 2.6 mg/20 μl, respectively). In each of these instances, QS6 did not appreciably adsorb to charcoal. This characteristic is confounded by the susceptibility of QS6 to digestion by 5'-nucleotidase, indicating that this compound contains a nucleoside linked to a 5'-monophosphate (17). Based on the specificity of this enzyme, it is likely that QS6 is a nucleotide that for some reason is hindered in adsorbing to charcoal rather than a non-nucleotide that is hydrolyzed by the enzyme. However, it is also possible that QS6 was hydrolyzed by a contaminating enzyme. The susceptibility of QS6 to digestion by alkaline phosphatase (Fig. 3d) further indicates that this compound contains at least one external phosphate. Its resistance to venom phosphodiesterase, ribonuclease T2, and nuclease P1 suggests that QS6 is not a nucleoside linked to 5'- or 3'-polynucleotides nor to a 5'-monophosphate. Its migration in the first dimension indicates that this novel compound has a net negative charge similar to that of a nucleotide containing four external phosphates. Because QS6 is present at a very low intracellular concentration, we have been unable to further clarify its structure. Its characteristics, as established by our battery of tests, do not correspond to any normal cellular metabolite. However, it is unlikely that this phosphorylated compound is structurally related to the family of dinucleotides (QS 1-5).

Identification of QS1 as AppppA—QS1 migrates with ATP in the first dimension indicating that they possess similar net negative charge. This evidence suggests that QS1 is a dinucleotide containing four internal phosphates. Based on the earlier findings that ACDQ inhibits leucyl-tRNA synthetase (21-23) and that AppppA has been found to be produced in vitro by aminoacyl-tRNA synthetases (1-8), we suspected that QS1 might be AppppA. In fact, we found that QS1 comigrated precisely with a standard of AppppA.

To further verify its identity, QS1 was purified and digested by venom phosphodiesterase. The digestion was followed by chromatographic analysis with two one-dimensional TLC systems. The two systems resolve nucleotides based upon different principles of separation (i.e. negative charge of their phosphate groups and their content of nucleobases, respectively) (17), in order to avoid the possible problem of two compounds migrating together in one system. Autoradiograms depicting this digestion are shown in Fig. 4, a and b. QS1 before digestion was run in lane 4. 5'-ATP and 5'-AMP were the products of a partial venom phosphodiesterase hydrolysis of QS1 (lane 5). As the digestion was followed to completion (lane 6), the ATP generated from the first cleavage was further dephosphorylated to 5'-AMP and PP, in a final 32P ratio of 1:0.85, respectively. The venom phosphodiesterase digestion products of QS1 were verified by comparing their migration with that of a parallel digestion of purified 32P-labeled ATP. Upon addition of venom phosphodiesterase, ATP was cleaved to 5'-AMP and PP, (Fig. 4, a and b, lanes 2 and 3) in a final 32P ratio of 1:2.0, respectively. Our experiments show that the cleavage of AppppA by venom phosphodiesterase to give ATP and AMP is relatively fast compared to the cleavage of ATP. This phenomenon has been reported previously by Randerath et al., who proposed that AppppA may be a better substrate than ATP for venom phosphodiesterase (2).

It is unlikely that QS1 has an undetected modification on either adenosine. The complete venom phosphodiesterase digestion products of QS1 were resolved in a two-dimensional

| QS   | Proposed structure | Comigration with standard | Adsortion to charcoal | Oxidation by periodate | Susceptibility to digestion by: | Ribonuclease T2 |
|------|--------------------|---------------------------|-----------------------|-----------------------|--------------------------------|----------------|
| 1    | AppppA             | +                         | S                     | S                     | R                              | R              |
| 2    | AppppG             | NA                        | S                     | S                     | R                              | R              |
| 3    | AppppG             | NA                        | S                     | S                     | S                              | R              |
| 4    | AppppG             | +                         | S                     | ND                    | S                              | S              |
| 5    | AppppA             | +                         | S                     | S                     | R                              | S              |
| 6    | AppppA             | +                         | R                     | ND                    | S                              | ND             |

*Explanation of symbols: +, precise comigration with a commercial standard; NA, no commercial standard was available; S, susceptible to the stated condition; R, resistant to the stated condition; ND, not determined.
Fig. 4. One-dimensional resolution of venom phosphodiesterase digestion of QS1. 1.75 M Morpholine, 1.4 M HCl (pH 8.2) was used to separate on the basis of negative charge (a), and 3 M (NH₄)₂SO₄, 2% disodium EDTA (pH 5.5) was used to separate based on the content of nucleobases (b). §P-labeled ATP was run before (lane 1), 40 min (lane 2), and 16 h (lane 3) after addition of snake venom phosphodiesterase. Likewise, §P-labeled QS1 was run before (lane 4), 40 min (lane 5), and 16 h (lane 6) after addition of venom phosphodiesterase. The digestions were performed in 0.25 M NH₄HCO₃ (pH 8) at 37°C.

TLC system which optimally resolves modified mononucleotides (17). The AMP and PP₁ generated from the digestion conformed with standards of 5′-AMP and PP₁, respectively. Any modification of the adenosine moiety or a different linkage of the phosphate to the nucleoside would have resulted in altered migration in this system. Thus, the structure of QS1 has been established as AppppA.

Identification of QS2 as AppppGpp—AppppGpp and AppppGpp can be made in vitro from ppGpp and pppGpp, respectively (6). Therefore, we hypothesized that QS2 might be one of these based on its chromatographic location and susceptibility to ribonuclease T2. QS2 was purified and analyzed, analogous to the method used for QS1. Venom phosphodiesterase cleaves the α,β-pyrophosphate linkage of nucleoside 5′-polynucleotides, and at pH 8.4, it requires a free 3′-hydroxyl group (26). When QS2 was hydrolyzed by venom phosphodiesterase at pH 8.4, 5′-AMP and ppGpp (at a §P ratio of 1:3.95, respectively) were the only digestion products (Fig. 5, a and b, lane 2). The identity of the AMP band was verified by comigration with a standard. In Fig. 5, a and b, lanes 3 and 4 depict the migration of radiolabeled standards of ppGpp andппpGpp, respectively. As in the optimized two-dimensional system, ppG>p is present with ppGpp. The lower band of Fig. 5a, lane 2, clearly migrates with the standard of ppGpp and not ppGpp in the solvent system which separates on the basis of negative charge. Consequently, the identity of QS2 was established as an adenylylated form of ppGpp; however, this dinucleotide could be linked A'ppp²G²pp or A'ppp³G²pp, since both structures yield AMP and ppGpp as venom phosphodiesterase digestion products and show identical susceptibilities to the battery of tests.

To determine the phosphate linkage of this adenylylated nucleotide, we subjected purified QS2 to a mixed enzymatic digestion with ribonuclease T2 and nuclease P1 at pH 6.5. Digestion by this enzyme mixture will distinguish the two phosphate linkages.

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\begin{align*}
\text{A'}^2\text{ppp}^2\text{G}^2\text{pp} & \xrightarrow{\text{ribonuclease T2}} \text{A'}^2\text{ppp}^3\text{G}^2\text{pp} \\
\text{A'}^2\text{ppp}^3\text{G}^2\text{pp} + \text{P} & \xrightarrow{\text{nuclease P1}} \text{ADP} + \text{ppG}^3\text{pp} \\
\text{ADP} + \text{ppG}^3\text{pp} + 2\text{P} & \xrightarrow{} \text{ADP} + \text{GDP} + \text{P}
\end{align*}
\]

When this experiment was performed, QS2 was found (by comigration with standards) to give AppppG and P, in an approximate molar ratio of 1:2, respectively. Thus, the structure is A'ppp²G²pp. The linkage of this dinucleotide is consistent with the finding that AppppGpp is synthesized from ppGpp and aminoacyl-AMP in vitro (6).

Identification of the Other Quinone-induced Spots—QS3, 4, and 5 are induced to very low intracellular concentrations (8–20 μM). Therefore, they have been provisionally identified (without purification) as AppppG, AppppG, and AppppA, respectively.

The identity of QS4 as AppppG is supported by alkaline phosphatase digestion of the acid extract of ACDQ-treated cells (Fig. 3d), in which QS2 (AppppGpp) disappeared with a concomitant increased appearance of QS4 (AppppG). Commercial standards of AppppG and AppppA were available, and they comigrated exactly with QS 4 and 5, respectively. A preliminary purification of QS3 has indicated that AMP, ATP, GTP, and GMP are the partial venom phosphodiesterase digestion products of this dinucleotide, supporting its identity as AppppG; however, no commercial standard of AppppG was available for comparison. Alternative structures for QS 4 and 5 are possible; for example, AppppG and AppppA would show similar susceptibilities to the battery of tests and could be expected to run in the same region as QS 4 and 5, respectively, since they might have a decreased affinity for borate. However, it is unlikely that these dinucleotides would comigrate exactly with standards of AppppG and AppppA, respectively. Commercial standards of AppppG and AppppA were not available.

It is interesting to note that QS 1–5 preserve a relative order of migration in accordance with the empirical logic of the two-dimensional TLC system (Fig. 3c). For example, ATP, GTP, and ppGpp migrate along a diagonal line in this system; in a similar manner, AppppA (QS1), AppppG (QS3), and AppppGpp (QS2) also form a line parallel to, but shifted to the left of, the ATP-ppGpp line. In addition, the relative positions of AppppA (QS5) and AppppG (QS4) to AppppA (QS1) and AppppG (QS8) are parallel to the positions of the unadenylated forms, i.e. ADP and GDP to ATP and GTP, in like fashion. Consequently, the identities of QS 1–5 are consistent with their chromatographic locations.

We have located where standards of AppppA, AppppppA, and AppppppA migrate in the optimized two-dimensional system (data not shown). There are no spots present in crude extracts of ACDQ-treated cells which comigrate with these dinucleotides. It is also known that adenylylated pyrimidine nucleoside 5′-polynucleotides can be synthesized in the in vitro back
Fig. 5. One-dimensional resolution of venom phosphodiesterase digestion of QS2. 0.5 M Triethanolamine, 1.0 M LiCl, 0.2 M HCl (pH 8.0) was used to separate on the basis of negative charge (a), and 3 M (NH₄)₂SO₄, 2% disodium EDTA (pH 5.5) was used to separate based on the content of nucleobases (b). ³²P-Labeled QS2 was run before (lane 1) and after (lane 2) digestion by snake venom phosphodiesterase. The 16-h digestion was performed in 0.25 M NH₄HCO₃ (pH 8.4) at 37 °C. ³²P-Labeled standards of ppGpp and pppGpp were run in lanes 3 and 4, respectively. The chromatographic location of AMP was determined by migration of a standard.

Fig. 6. Kinetics of nucleotide pool changes after addition of ACDQ (5 µg/ml) to S. typhimurium. ACDQ was added at time 0. a, pool changes of ATP (●), ppGpp (○), ppGpp (●), CTP (●), GDP (●), and ADP (●). b, induced accumulation of AppppA (●), ApppGpp (○), AppppG (●), ApppG (●), and AppppA (●).

The reaction of lysyl-tRNA synthetase (3, 4, 27). These would be expected to run near ATP and could be obscured. In Fig. 3d, we do see one alkaline phosphatase-resistant spot near ATP which could correspond to one of these.

Quantitation of Quinone-induced Spots and Related Nucleotides—Fig. 6a depicts the kinetics of ATP, ppGpp, ppGpp, and other nucleotide pools in cells exposed to ACDQ (5 µg/ml). These changes are comparable to those reported previously (20). Whereas ATP increases slightly from 3.0 mM (the basal level in normal cells (17)) to 3.6 mM, the ppGpp concentration increases dramatically shortly after addition of the quinone. Using the initial cellular concentration of ATP to normalize the concentrations of other phosphorylated compounds, we found that ppGpp increases 79-fold to a level of 102 µM while pppGpp reaches a significantly smaller peak concentration of 164 µM. The other nucleotides undergo less dramatic changes.

The induction of QS 1–5 following exposure to ACDQ is shown in Fig. 6b, which can be compared to the levels of the unadenylylated forms in Fig. 6a. The two major quinone-induced spots, AppppA and ApppGpp, peak 30 and 20 min, respectively, after the addition of ACDQ. AppppA increases from undetectable levels (<1 µM) to a peak concentration of 102 µM while ApppGpp accumulates from undetectable levels to 86 µM, resulting in at least an 80-fold increase in the levels of these dinucleotides. The other minor quinone-induced spots increase less dramatically and attain levels of 8–20 µM.

The phosphate concentration of QS6 increases to a maximum concentration of 119 µM 30 min after the cell is exposed to ACDQ. Since the identity of QS6 has not been determined, we were unable to convert these values to the concentrations of this compound. If we assume that this novel phosphorylated
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compound contains 5 phosphates (based on its chromatographic location), we can estimate an approximate induced intracellular concentration of 24 μM.

**DISCUSSION**

Using our recently developed method for monitoring the in vivo metabolism of cellular nucleotides (17), we have been attempting to systematically identify new nucleotide signal molecules (24). As part of this study, we now find that the bacterium *Salmonella typhimurium* accumulates a family of adenylylated nucleotides at intracellular concentrations of up to 100 μM following exposure to ACDQ but not under a variety of other metabolic stresses (Refs. 17 and 24 and data not shown). Characterization of these compounds by investigating their susceptibility to our battery of chemical and enzymatic tests and further studies on the purified compounds have established the structures of the two major nucleotides as AppppA and AppppGpp. Preliminary evidence indicates that the other compounds are AppppG, ApppG, and ApppA.

Adenylylated nucleotides can be synthesized in the back reaction of aminoacyl-tRNA synthetases in vitro (2-6, 27). AppppA, AppppGpp, AppppG, and ApppA could, therefore, be produced from ATP, ppGpp, GTP, GDP, and ADP, respectively (see Fig. 1). AppppG has been synthesized in vitro in a similar manner from ppGpp (6), and it is likely that cells do have the capability to synthesize this dinucleotide. As can be seen from Fig. 6a, however, cells exposed to ACDQ have “magic spot” pools similar to those of spoT mutants (22, 28), that is, the level of ppGpp is much higher than that of ppGpp. With the concentration of ppGpp attained a level of only 160 μM it is possible that there is not a high enough concentration of this nucleotide from which the adenylylated form (AppppGpp) could be derived. This explanation, however, is confounded by the observation that AppppG is produced in vivo even though the level of GDP, the unadenylylated form, never exceeds 100 μM under these conditions. The specificity of nucleotide adenylylation in vivo requires further elucidation.

The finding that AppppA and other adenylylated nucleotides accumulate to high levels in a bacterial cell raises the question of their function. In eucaryotic cells, AppppA has been postulated to be a signal for cell proliferation (11). Our results suggest a different function in bacteria because these dinucleotides accumulate under a particular condition where the growth of cells is severely inhibited. We propose, instead, that AppppA and the related adenylylated nucleotides are alarmones (24, 29, 30), signal molecules, such as ppGpp (29, 31), that are produced under specific metabolic stresses. This is supported by the finding that the enzymatic synthesis of AppppA by *E. coli* phenylalanine-tRNA synthetase is decreased upon the addition of phenylalanine-specific tRNA (7). However, if the cell is experiencing a shortage of tRNA, this would tend to increase the aminoacyl-adenylate pool and could promote the pyrophosphate exchange back reaction resulting in production of AppppA and the other adenylylated nucleotides.

Another possible hypothesis is that these adenylylated nucleotides signal oxidative damage to the cell. Quinones, such as ACDQ, are strong oxidizing agents (33) as they cause redox cycling (34). Leucyl-tRNA synthetase, an enzyme hyposensitive to oxidation (23, 35, 36), is the most susceptible target of ACDQ (21-23). In fact, it has been demonstrated that ACDQ not only inhibits the tRNA charging reaction but also decreases the binding affinity of leucyl-tRNA synthetase for PP (22) as it reacts with a pair of sulfhydryl groups located near the active site of the enzyme (23). Consequently, this enzyme could have a higher affinity for nucleoside 5’-phosphates than for PP, if the back reaction were to occur. The accumulation of these dinucleotides in *S. typhimurium* and *E. coli* under a variety of other oxidative stresses, but not under other metabolic stresses, supports this latter possibility.

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