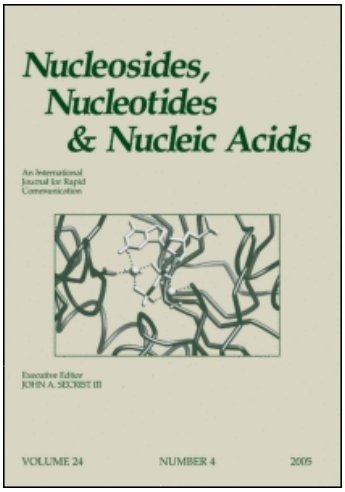


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# Induction of Bacterial Differentiation by Adenine- and Adenosine-Analogs and Inhibitors of Nucleic Acid Synthesis

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# INDUCTION OF BACTERIAL DIFFERENTIATION BY ADENINE- AND ADENOSINE-ANALOGS AND INHIBITORS OF NUCLEIC ACID SYNTHESIS

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## ABSTRACT

Several adenine- or adenosine-analogs, which inhibited growth and decreased the intracellular GTP pool, induced sporulation of Bacillus subtilis. The inducers were added to cultures growing in a medium containing excess ammonium ions, glucose, and phosphate in which cells normally cannot differentiate. They included compounds that are modified in the ribose unit (decoyinine, psicofuranine, cordycepin) or are substituted within the purine ring or at the 6-N position of adenosine (6-methylaminopurine, zeatin, 6-anilinopurine, formycin). Their effects on the cellular concentration of nucleotides were also measured. All sporulation inducers except formycin-A caused a decrease of GMP, GDP and GTP, some by inhibiting IMP dehydrogenase and others by inhibiting GMP synthetase. In contrast, formycin-A caused an increase of GMP while GDP and GTP decreased. Therefore, the compound (signal) controlling sporulation is GDP or GTP but not GMP. Antibiotics inhibiting growth by direct inhibition of nucleic acid synthesis did not induce sporulation.

## INTRODUCTION

Bacillus subtilis normally sporulates when carbon, nitrogen, or phosphate sources are growth limiting. Several studies from this laboratory[1-6] have shown that sporulation can be initiated even in an excess of all these nutrients if the synthesis of purine nucleotides, in particular guanine nucleotides, is reduced but not abolished. This was demonstrated with purine requiring mutants,[2,3] purine analogs, and other inhibitors of purine nucleotide synthesis.[4,5] The most effective compound was decoyinine, an adenosine analog that specifically inhibits GMP synthetase.[5] Measurements of nucleotide pools have shown that a decrease in the intracellular concentration of the guanine nucleotide pools is not only sufficient for the initiation of sporulation but is





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observed under all conditions under which sporulation has so far been found.[1,6]

To determine which nucleotides control sporulation, we investigate in this paper which adenine- and adenosine-analogs induce sporulation. We show that this induction is correlated with a decrease in the cellular pools of GTP and GDP but not GMP. We also show that compounds which directly inhibit nucleic acid synthesis do not induce sporulation.

## MATERIALS AND METHODS

Growth conditions. Bacillus subtilis strain 60015 = metC7 trpC2 was used. Synthetic medium contained 20 mM ammonium sulfate; 5 mM potassium phosphate buffer, pH 7.0; 100 mM morpholinopropanesulfonic acid (MOPS), pH adjusted to 7.0 by KOH; 2 mM $MgCl_2$; 0.7 mM $CaCl_2$; 50 μM $MnCl_2$; 1 μM $ZnCl_2$; 5 μM $FeCl_3$; 50 μg/ml L-tryptophan; 20 μg/ml L-methionine; 2 μM thiamine; 20 mM glutamic acid, adjusted to pH 7.0 by KOH; and 55 mM D-glucose.

The bacteria were streaked onto tryptose blood agar base (Difco) plates and grown for 6 to 8 h at 37°C. The cells were inoculated into synthetic medium at an absorbancy at 600 nm ($OD_{600}$) of about $5 \times 10^{-4}$. These cultures were shaken overnight at 200 strokes per minute in a 37°C waterbath until the $OD_{600}$ reached 0.5. At this time (called $t_o$) the drug, usually dissolved in 0.1 N KOH, was added. Control flasks received the solvent (0.1 N KOH) alone. Growth was followed by the change of $OD_{600}$. The viable cell titer (V) was measured by diluting the cultures in 0.1 M potassium phosphate buffer, pH 6.5, plus 1 mM $MgCl_2$ and plating on tryptose blood agar base plates. The heat resistant spore titer (S) was usually determined 10 h after addition of the drug ($t_{10}$) by heating the dilution tubes for 20 min at 75°C and plating.

Cordycepin, 9-β-D-arabinofuranosyladenine (Ara-A), 6-methylaminopurine, zeatin, 6-anilinopurine, 6-benzyladenine, tubercidin, 6-isopentenyladenine, kinetin, tyrothricin, and emetin were purchased from Sigma Chemicals, St. Louis, MO. Decoyinine, psicofuranine, nogalamycin, and lomofungin were gifts from Dr. Grady, Upjohn Co., Kalamazoo, MI. Formycin-A, formycin-B, and co-formycin were gifts from Dr. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan, and the Developmental Therapeutic Program and Chemotherapy, National Cancer Institute (Dr. Douros). Echinomycin was a gift from Dr. Scott, Hoffman LaRoche, Nutley, NJ; hedamycin from Dr. Bradner, Bristol Labs, Syracuse,

NY; and lipiarmycin from Dr. Lancini, Gruppo Lepetit, Milan, Italy.

Determination of the nucleotide pools: To label the nucleotide pools completely, $K_2H^{32}PO_4$ (specific activity of 50 μCi/umole $P_i$) was added to a growing culture when the $OD_{600}$ was about 0.05. When the $OD_{600}$ was 0.5, the drugs were added. At appropriate times thereafter 5 ml of culture were filtered through a Millipore filter (0.45 μm), and the filter (containing the cells) was immediately laid upside down onto 0.3 ml formic acid (0.5 M) kept chilled in a Petri dish. After 15 to 30 min the filters were rinsed with the formic acid on the Petri dish, and the suspension was collected and centrifuged in a microfuge for 2 min. Five μl samples of the supernatant were applied to washed,[6] 0.1 mm thick PEI-cellulose plates (Polygram CEL 300 polyethylenimine, Macherey Nagel Co., Duren, Germany). Appropriate standards (2 to 10 μg) were also applied for UV monitoring of the spots. Chromatograms were developed in one dimension, electrophoresed in the second dimension, and the radioactivity of the spots was counted, all as described before.[6] The cellular concentrations of nucleotides were expressed as pmoles/$OD_{600}$, where 1 $OD_{600}$ unit is the amount of cells which would give an $OD_{600}$ of 1 if they were contained in 1 ml. For a culture grown to $OD_{600}$ of 1, 1 $OD_{600}$ unit corresponds to about 2.6 x $10^8$ cells/ml, a volume of about 1 μl, 0.5 mg dry weight, and 0.25 mg protein. As these relationships vary with the $OD_{600}$ of the culture and the culture medium, we have expressed our data in terms of the actually measured $OD_{600}$ rather than a calculated mg dry weight value or as a molar concentration. The phosphate concentration was measured in each culture according to Ames and Dubin,[7] and the cpm/pmol of $^{32}P_i$ was determined.

## RESULTS

Adenosine analogs altered in the ribose moiety. Adenosine analogs differing in their ribose moiety induced sporulation when they were added at partially inhibitory concentrations to cultures of B. subtilis growing exponentially in synthetic medium (Table 1). Psicofuranine, which - like decoyinine - inhibits GMP synthetase,[8] was required in 4 to 5 times higher concentration (8 mM) than decoyinine to produce the same degree of growth inhibition and sporulation (Fig. 1). Whereas cordycepin inhibited growth at about the same concentration as decoyinine, it was less effective in inducing sporulation (Fig. 2). Arabinosyladenine (Ara-A), which has arabinose in the place of ribose, inhibited growth at

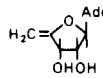
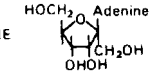
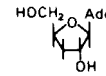
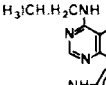
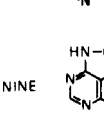
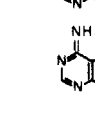
| DRUG | CHEMICAL STRUCTURE | CONCENTRATIONS CAUSING: OPTIMAL SPORULATION | CONCENTRATIONS CAUSING: 50% GROWTH INHIBITION | OPTIMUM SPORE TITER IN MILLIONS/ml | INTRACELLULAR GTP PERCENT OF CONTROL |
|---|---|---|---|---|---|
| NONE | | | | 0.5 – 1.0 | 100 |
| DECOYININE |  | 2mM | 2mM | 100 | 30 |
| PSICOFURANINE |  | 8.4mM | 8.4mM | 30 | 50 |
| CORDYCEPIN |  | 2mM | 2mM | 40 | 60 |
| Ara-A |  | .9mM | .9mM | 1 | 110 |
| 6-METHYL AMINO-PURINE |  | 8mM | 8mM | 200 | 40 |
| ZEATIN |  | 3.2mM | 3.2mM | 200 | 55 |
| 6-ANILINO PURINE |  | 2.4mM | 4mM | 100 | 52 |
| 6-ISOPENTENYL-ADENINE |  | 2.95mM | 4.2mM | 20 | 70 |
| KINETIN |  | 0.95mM | 4mM | 10 | 110 |
| 6-BENZYL ADENINE |  | 1.3mM | 3mM | 5 | 100 |
| FORMYCIN-A |  | 1.9mM | 4mM | 20 | 73 |
| TUBERCIDIN |  | 5mM | >10mM | 0.8 | 120 |

TABLE 1

Adenine and adenosine analogs and their effect on growth, sporulation induction and intracellular GTP pools. The concentration of the drug causing 50% growth inhibition (after 3 h) was determined by interpolation of the results at different concentrations. The drug concentration causing optimal sporulation and the average spore titers are stated. For GTP measurements, cells were grown in synthetic medium containing 25 $\mu C_i$/ml $^{32}P_i$ from $OD_{600}$=0.05 to 0.5, at which time the compound was added. The amount of GTP per $OD_{600}$ unit was determined just before inhibitor addition (=control) and 60 min later. The initial concentration of GTP in this experiment was 154 pmol/$OD_{600}$. In 16 independent experiments in which cells were grown in the same medium and to the same $OD_{600}$, an average of 171.5 pmol/$OD_{600}$ with a standard deviation of 61.1 was calculated. Ara-A = arabinosyladenine.

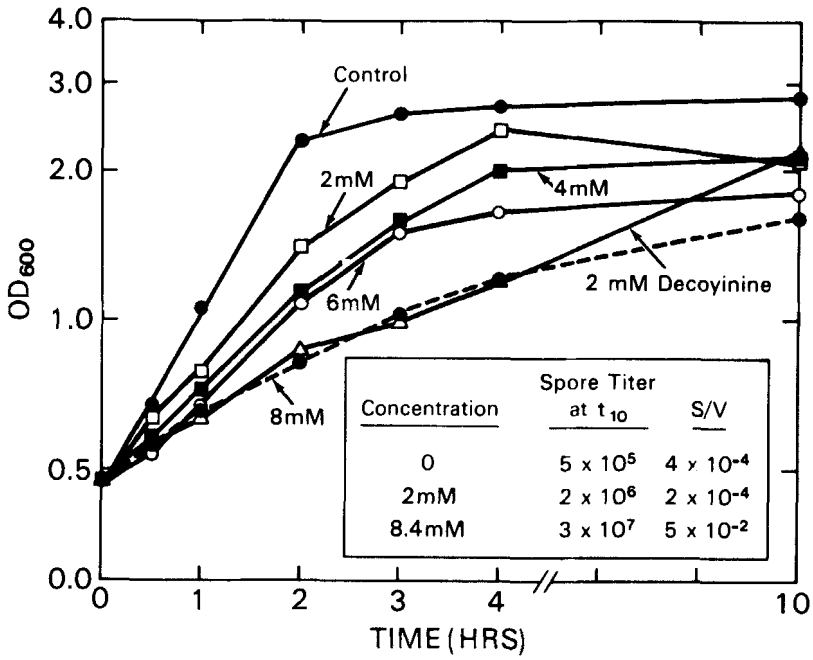



FIG. 1 Growth inhibition and sporulation induction at different concentrations of psicofuranine. S/V = heat resistant spores per viable cell. The growth inhibition by 2 mM decoyinine is shown for comparison.



lower concentrations than the other adenosine analogs and caused cell lysis after 2-3 h; it did not induce sporulation (Table 1).

The intracellular concentration of GTP decreased within 60 min after addition of 2 mM of the above compounds, with the exception of Ara-A which caused an increase (Fig. 3). Specifically, the reduction was 60% with decoyinine, 50% with cordycepin, and 37% with psicofuranine. Both psicofuranine and decoyinine caused a several fold increase of XMP obviously because they inhibit GMP synthetase. Cordycepin did not cause an accumulation of either IMP or XMP. Ara-A caused an increase in the concentration of all 4 nucleoside triphosphates (Fig. 3).

Adenine derivatives with 6-N substitutions. 6-N substituted adenines inhibited growth and induced sporulation to differing degrees (Table 1). The most effective inducers (100- to 200-fold) were 6-methylaminopurine and zeatin, less effective were 6-isopentenyladenine and 6-anilinopurine, while 6-benzyladenine and kinetin were least effective (Fig. 4). The pool levels of nucleotides were examined for

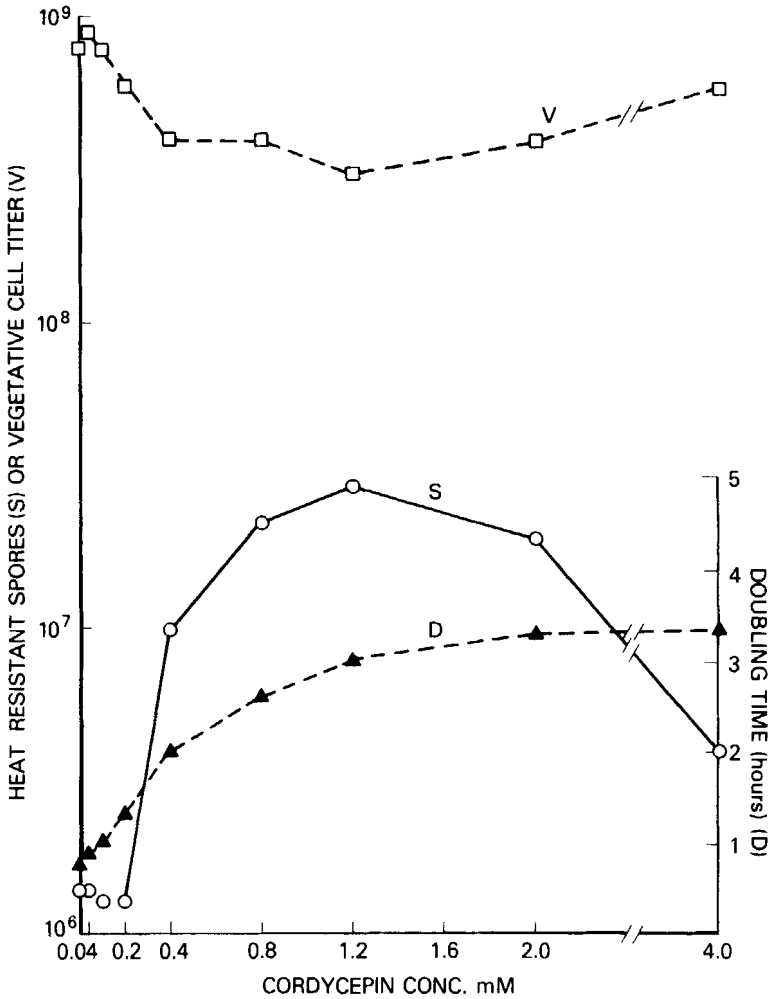



FIG. 2 Effect of cordycepin on growth and sporulation. V = viable cell titer; S = spore titer; D = doubling time, in h.

those concentrations of 6-methylaminopurine (Fig. 5) and zeatin (Fig. 6) which induced the highest spore titers. With either inhibitor, the intracellular concentrations of all 3 guanine nucleotides and of UTP decreased rapidly while the concentration of ATP decreased more slowly or remained constant and that of CTP increased; the concentration of IMP increased more than 2-fold without any increase in XMP, indicating an inhibition of IMP dehydrogenase.

Adenosine analogs altered in the purine moiety. Formycin-A, formycin-B, and tubercidin were among the compounds used that are altered in

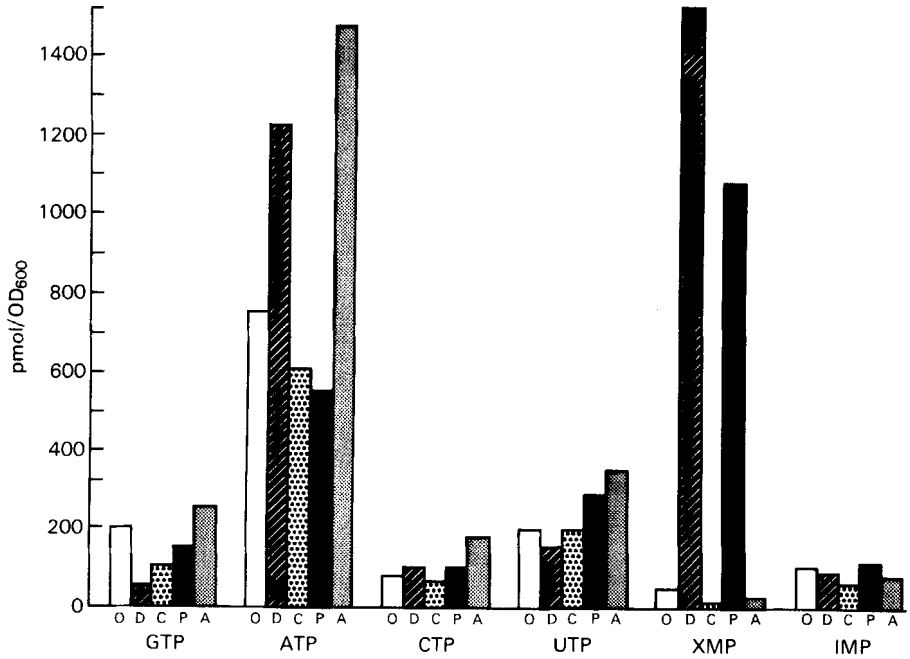



FIG. 3 Effect of adenosine analogs on the intracellular concentration of nucleoside triphosphates and some pertinent monophosphates. Two mM decoyinine (D), cordycepin (C), psicofuranine (P), and arabinosyladenine (A), were added to growing cultures ($OD_{600}$=0.5); the nucleotide pools were analyzed 1 h later. Control (O) cultures received no drug.

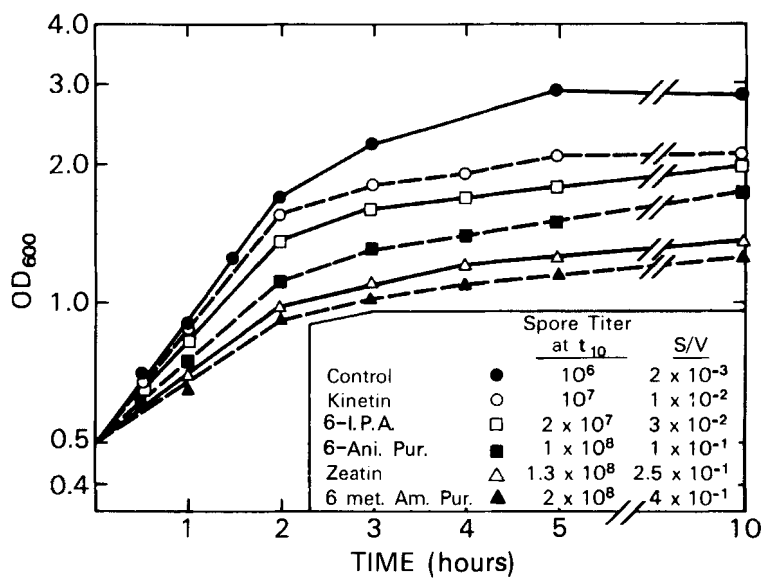



FIG. 4 Effect of various adenine analogs on growth and sporulation.

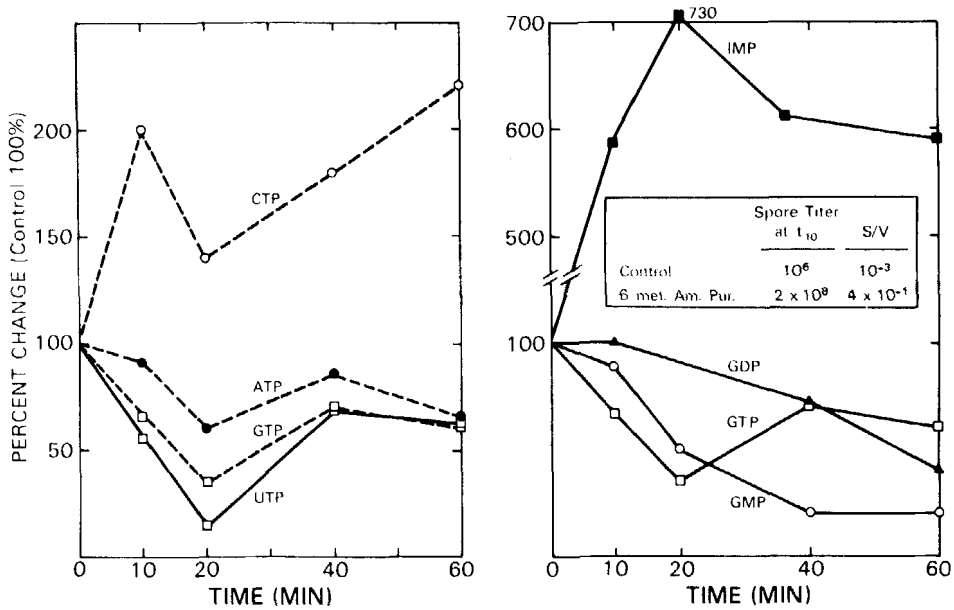



FIG. 5 Changes in the intracellular nucleotide pools caused by addition of 6-methylaminopurine (6 mM); initial values (time 0) were (in pmol/$OD_{600}$): ATP=1040, CTP=89, GMP=81, GDP=42, GTP=219, IMP=128, UTP=280.

the purine nucleus. Formycin-A has a carbon in the place of a nitrogen in the 9-position and tubercidin has the same in the 7-position of the purine ring; both have a nitrogen in the 8-position. Formycin-B is the 6-deaminated product of formycin-A. At concentrations of 1-2 mM, formycin-A and formycin-B inhibited growth moderately and increased the spore titer to $2 \times 10^7$/ml. Increasing the inhibitor concentration to 4 mM produced additional growth inhibition without any further increase in the spore titer. The addition of co-formycin, an inhibitor of adenosine deaminase in mammalian cells,[8,9] together with formycin-A did not potentiate the formycin action. Co-formycin alone (0.5 mM) had no effect on growth and sporulation. Tubercidin slightly (20%) inhibited growth only at high concentrations (5 mM) and did not induce sporulation. Formycin-A (3.5 mM) caused a decrease in the concentrations of GDP and GTP as well as several other nucleotides (Fig. 7), but it caused an increase in the concentration of GMP.

Inhibitors of nucleic acid synthesis. The effects of the following compounds are summarized without showing the experimental data. Tyro-

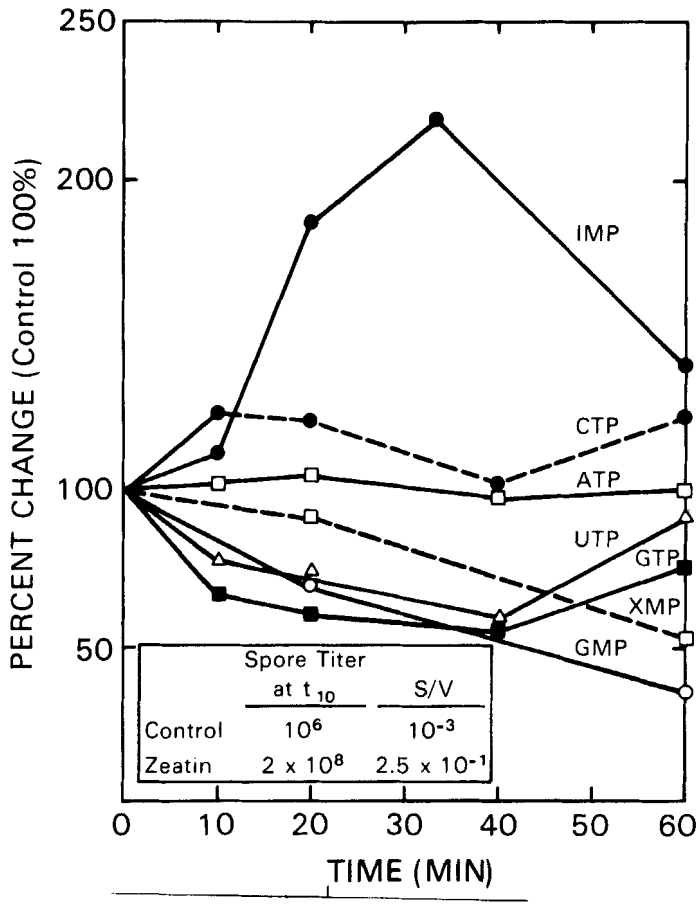



FIG. 6 Changes in the intracellular nucleotide pools caused by the addition of zeatin (3 mM); initial values (in pmol/$OD_{600}$): ATP=840, CTP=85, GMP=52, GTP=167, XMP=16, IMP=95, UTP=208. The curve for GDP is almost the same as that for GMP and was not drawn to avoid crowding; the initial value for GDP was 25.

thricin complex[10-12] (a peptide antibiotic elaborated by B. brevis), which is known to inhibit RNA synthesis,[10] inhibited growth to 50% at a concentration of 0.1 μg/ml and caused complete cessation of growth at 1 μg/ml. However, the cells slowly resumed growth 4-5 h later. At higher concentrations (5 μg/ml) the cells lysed after an initial lag of about an hour. At all tyrothricin concentrations the spore titers at $t_{10}$ and $t_{20}$ were much lower than in the controls. Similarly, echinomycin, another inhibitor of RNA synthesis,[13] inhibited growth progressively between 0.1 and 1.0 μg/ml and caused lysis of the cells at higher concentrations. Again the spore titers were lower than in the controls.

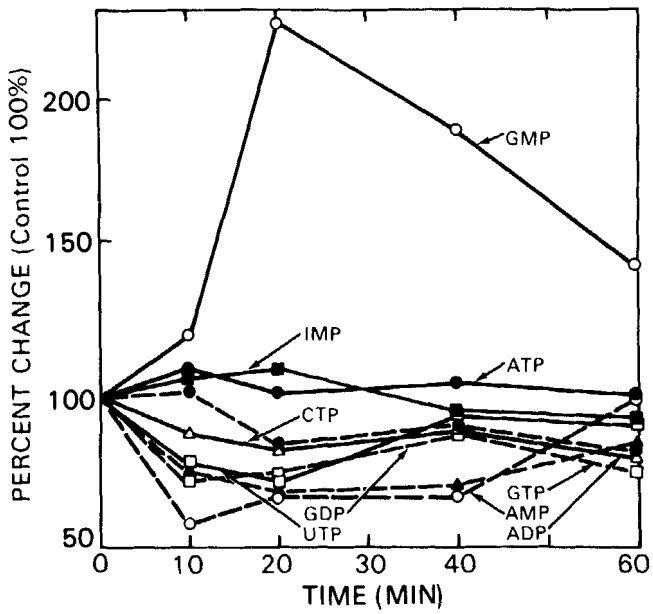



FIG. 7 Changes in the intracellular nucleotide pools caused by the addition of formycin-A (3.5 mM); initial values (in pmol/$OD_{600}$): AMP=171, ADP=94, ATP=740, CTP=85, GTP=137, GDP=52, GMP=88, IMP=136, UTP=188.

Lomofungin, which inhibits RNA and DNA synthesis in yeast,[14-17] inhibited growth and reduced the spore titer (at $t_{10}$) at concentrations of 5 to 40 μg/ml (50% inhibition at 40 μg/ml). Emetin, an alkaloidal eukaryotic ribosomal RNA inhibitor,[18,19] affected neither growth nor sporulation even when used at a concentration of 1 mg/ml.

Nogalamycin[20,21] and hedamycin[22] are known to inhibit RNA synthesis (by binding to DNA) in mammalian cell cultures as well as in many microorganisms; they increasingly inhibited growth of B. subtilis at concentrations of 0.1 to 10 μg/ml. At higher concentrations, growth ceased for about an hour, after which the cells lysed. The spore titer of the surviving cells was very low ($10^3$/ml).

Lipiarmycin, which binds to RNA polymerase,[23-26] inhibited growth transiently at concentrations up to 2 μg/ml and completely (leading to eventual lysis) at higher concentrations; no increase in the spore titer (at $t_{10}$) was observed in any of these cultures.

## DISCUSSION

Several adenosine analogs examined here decreased the intracellular GTP (and GDP) pools and induced sporulation at a drug concentration at

which growth was partially inhibited. The concentration causing a certain degree of growth inhibition and the concentration optimal for inducing sporulation depended on the analog used, reflecting differences in cellular transport, metabolism, and mode of action.

Of the four adenosine analogs whose pentafuranose moiety differed from D-ribose, three (decoyinine, cordycepin, and psicofuranine) induced sporulation and decreased the intracellular GTP pools. The fourth, Ara-A, a known antiviral agent, which is incorporated into DNA and thus blocks its synthesis,[27,28] neither decreased GTP nor induced sporulation; similar to many other inhibitors of DNA synthesis, it caused cell lysis. Both psicofuranine and decoyinine inhibit GMP synthetase in mammalian and microbial systems;[8,29,30] this effect was also demonstrated by the increase of intracellular XMP following the addition of either inhibitor to growing cultures of B. subtilis (Fig. 3).[6] Five times more psicofuranine was needed to match the inhibition of decoyinine. Studies by Rottman et al.[31-33] in B. subtilis have shown that the 5'-phosphate of cordycepin inhibits some early step(s) of de novo purine biosynthesis; the authors also demonstrated that purified phosphoribosylpyrophosphate amide transferase (E.C.2.4.2.14) is inhibited by cordycepin phosphate. In other cell types, cordycepin inhibits the incorporation of phosphate into nucleic acids,[34] inhibits the formation of D-5-phosphoribosylpyrophosphate from D-ribose-5-phosphate,[35] and causes chain termination during RNA synthesis as was shown in vitro.[36] The induction of sporulation by cordycepin probably resulted from the observed decrease of guanine nucleotides, which is known to induce sporulation;[1,2,6] cordycepin is less effective than decoyinine, presumably because its effects on RNA synthesis prevent the timely synthesis of mRNA needed for spore development.

Several 6-N substituted adenosine analogs are known cytokinins (zeatin, kinetin, 6-benzyladenine) or are structurally closely related to them (6-methylaminopurine, 6-anilinopurine, 6-isopentenyladenine). Most of these compounds inhibited the growth of B. subtilis at concentrations of 1-6 mM. However, they differed in their spore inducing potency (Fig. 4). 6-Methylaminopurine, zeatin, and 6-anilinopurine were most effective; 6-isopentenyladenine and kinetin were less effective; and 6-benzyladenine was least effective. The most effective spore inducers also reduced the intracellular GTP pool significantly. The difference in the degree of sporulation induction between zeatin and isopentenyladenine (which are chemically closely related) is not under-

stood. As 6-benzyladenine is one of the most potent cytokinins but was rather ineffective as an inducer of sporulation, there may be no correlation between the mechanisms of sporulation induction and the cytokinin effect in plants. However, differences in transport or metabolism could also account for the different responses. Interestingly, several 2,6-substituted purine analogs induce differentiation in mammalian cells without being incorporated into nucleic acids.[27]

The third group of adenosine analogs used here has exchanged carbon and nitrogen atoms in the imidazole ring (see Table 1). Formycin-A can replace adenosine in a number of reactions.[8,9] At a concentration of 3.5 mM, it inhibited growth of B. subtilis by 45% and caused a 20-fold increase in the spore titer (at $t_{10}$). Our nucleotide measurements indicated that one effect of formycin-A or its nucleotide was to inhibit the further phosphorylation of GMP to GDP (and GTP) because the concentrations of GDP and GTP decreased while the concentration of GMP increased (to 2-fold) (Fig. 7). In mammalian cells, formycin-A is deaminated to formycin-B,[37] which inhibits poly-(ADP-Rib) polymerase.[36] In B. subtilis formycin-B had the same effect as formycin-A. Tubercidin is a potent inhibitor of mammalian mRNA synthesis and is incorporated preferentially into poly-A (+ Strand) RNA.[36] It has antibiotic activity only in some microorganisms, probably by interfering with their glucose utilization.[9] In B. subtilis it affected neither growth nor sporulation.

Other antibiotics which inhibit growth by directly interfering with RNA synthesis (without decreasing the GTP concentration) did not induce sporulation. They include the peptide antibiotics tyrothricin and echinomycin;[12,13] lipiarmycin and lomofungin which inhibit RNA polymerase;[15,23] and hedamycin[22] which inhibits growth by binding to DNA. The inability of other inhibitors of RNA synthesis: actinomycin-D, chromomycin-A3, daunomycin, rifampin, streptolydigin, and streptovaricin, to induce sporulation (at any inhibitory concentration) has been reported before.[4] Emetin,[18,19] an alkaloidal inhibitor of eukaryotic ribosomal RNA synthesis, affected neither growth nor sporulation. We do not know whether this compound is taken up by B. subtilis.

In most (or all) biological systems, the commitment of cells to undergo functional and structural differentiation is preceded by a decrease or an arrest in cell proliferation. This differentiation probably started (at least in microorganisms) as an evolutionary adapta-

tion to adverse growth conditions (limitation of nutrients).[1] Early in evolution, depletion of an already used metabolite such as GTP, which contains the key elements (C, N, O), may have regulated the transition from vegetative growth to differentiation. Later evolution, requiring different cells in a multicellular organism to develop differently, introduced additional signals such as cyclic AMP [which in Bacilli is undetectable during growth and sporulation (concentration less than 1 nM)],[38,39] hormones, etc. The evidence presented earlier[2-6] and here indicates that a decrease of GTP (or GDP) is used as a signal to initiate sporulation of B. subtilis. If one produces a specific decrease of guanine nucleotides, sporulation occurs and, moreover, GTP decreases under all conditions under which massive sporulation is observed, whereas all other nucleotides increase under some and decrease under other conditions. This paper shows for the first time that GMP cannot be a sporulation signal because its concentration decreased under most sporulation conditions but increased during sporulation induction by formycin-A. It is also worth mentioning that the signal is not ppGpp or pppGpp, which are made during the stringent response to amino acid starvation, because other studies in our laboratory have shown that the sporulation observed under such conditions actually results from the decrease of GTP, which is also a consequence of the stringent response; relaxed (relA) mutants, unable to produce the stringent response, no longer sporulate during partial amino acid deprivation but can still sporulate when their GTP concentration is decreased otherwise.[40-42] Numerous unusual nucleotides have been observed by us and by others in thin-layer chromatography or electrophoresis of $^{32}$P-labeled compounds.[1] However, most of these compounds are observed only under special (not all) sporulation conditions. We cannot rule out the possibility that some compound, made in minute quantity, might ultimately control the onset of sporulation. But no convincing evidence for the role of such a compound has yet been provided.[1]

GTP is a precursor of folate and its derivatives which are needed for formylation and certain methylation reactions. Bochner and Ames found in Salmonella typhimurium that the decrease of GTP leads to a surprisingly rapid accumulation of 5-amino 4-imidazole carboxamide riboside 5'-monophosphate (=AICAR or ZMP), 4-N-succino-ZMP, and the triphosphate ZTP.[43] ZMP is synthesized in the purine path and is a side

product of the histidine path. One would therefore expect that the accumulation of ZMP and its derivatives is prevented in purine auxotrophs blocked before ZMP if they are grown in the presence of histidine which inhibits de novo synthesis of histidine by feedback inhibition of the first enzyme in the histidine pathway. In fact, the above mentioned S. typhimurium studies show that under such conditions the sensitivity of cells to folate antimetabolites is eliminated.[43] We have not measured the accumulation of these compounds under conditions causing GTP deprivation in B. subtilis. Although they may be used by the cell to recognize folate deficiency,[43] earlier results from our laboratory make it very unlikely that the increase of any one of these compounds is necessary for the initiation of sporulation: 1) Purine auxotrophs of B. subtilis blocked at different steps before or after ZMP sporulate at a frequency that depends on their leakiness rather than on the position of the block in the purine path.[2] 2) This is even true for his pur mutants (with both blocks before ZMP) for which sporulation was measured in the presence of histidine (50 µg/ml), i.e., under conditions that should enable no ZMP accumulation.[2] 3) Non-leaky pur auxotrophs blocked before ZMP sporulate best if the medium contains an intermediate concentration of 5-amino 4-imidazole carboxamide (AICA) as an extracellular precursor of ZMP.[2] This amount of AICA is not able to supply purines at the normal rate because cells grow in it only very slowly. High concentrations of AICA enable growth at a normal rate and do not cause sporulation. Thus, it is the limitation of purine precursors, including ZMP, and of the derived nucleotides which causes sporulation and not the accumulation of ZMP.

One could also argue that GTP deprivation and the possibly resulting deficiency in 5-methyl tetrahydrofolate might cause a deficiency of methionine and derived compounds and that this deficiency could be responsible for sporulation. However, the following results argue against this possibility: 1) 1% casein hydrolysate (which contains methionine) or 1 mM L-methionine in combination with other amino acids do not prevent sporulation induction by decoyinine.[5,6] 2) The deprivation of methionine does not initiate sporulation of a met rel mutant[41] (in a met rel$^+$ mutant methionine deprivation does initiate sporulation but that effect results from the stringent response to any amino acid deficiency).

We do not know how GTP exerts its control on sporulation. It is known to be a specific substrate for only a few enzymes of B. subtilis, i.e., the ppGpp and pppGpp synthesizing enzymes, the various factors used in protein synthesis, and RNA polymerase. The synthesis of ppGpp is not essential for sporulation, and the enzymes used in protein synthesis apparently all have $k_m$ values of a few μM, whereas a decrease in the intracellular GTP concentration from about 300 to 50 mM GTP sufficed to initiate sporulation [one $OD_{600}$ unit occupies a volume of about 1 μl]. The GTP decrease does not affect the elongation of RNA synthesis but it does decrease the initiation of new RNA chains, at least with respect to ribosomal RNA (Vasantha and Hansen, in preparation). Therefore, the GTP deprivation might initiate sporulation by affecting the relative frequency at which different RNA molecules are synthesized. But it could also inhibit or activate an enzyme such as one of the numerous enzymes using ATP or other purine derivatives (e.g. a protein kinase), it could interfere with the mechanism of cell septation (because sporulation starts by the formation of an asymmetric septum), or it could act directly at the genetic level, e.g. as a corepressor.

The differentiation of certain other cell types apparently is also controlled by nucleotides. For example, myxospore formation of Myxococcus xanthus is accompanied by accumulation of ppGpp and pppGpp.[44] In the yeast Saccharomyces cervisiae, sporulation can be initiated by the deprivation of GTP (E.B. Freese, Olempska-Beer, Hartig, and Freese; in preparation). In myxomycetes, formycin-B interferes with the normal process of fruiting body formation.[45] In plants, the ratio of auxins to cytokinins (adenosine analogs) determines whether the callus cells differentiate into a root or a shoot.[46,47] Erythroid differentiation of erythroleukemic cells can be induced by purines and purine analogs.[27]

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