A Guanosine 3'':5'-Monophosphate-sensitive Nuclease from Bacillus brevis*

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SUMMARY

In toluene-treated cells of Bacillus brevis, newly synthesized RNA is rapidly degraded in a reaction that is inhibited by cyclic guanosine 3'':5'-monophosphate (cGMP) and by 1,1O-phenanthroline. This appears to be due to a ribonuclease found in cell-free extracts of B. brevis which is inhibited by cGMP and related compounds as well as by 1,1O-phenanthroline.

The cGMP-sensitive nuclease hydrolyzes synthetic polynucleotides, yielding nucleoside 5'-monophosphates as the sole products, even during the early stages of hydrolysis. Synthetic polynucleotides terminated by a 3'-phosphate are resistant to hydrolysis, while with 3'-hydroxyl-terminated substrates the release of the 3'-terminal nucleotide occurs much more rapidly than the over-all hydrolysis of the polymer. The enzyme is therefore an exonuclease that degrades polynucleotides from the 3' end to produce 5'-mononucleotides. It also acts on denatured but not on native DNA. Activity is greatest in the presence of Mn2+ and is not affected by the presence of monovalent cations. 1,1O-Phenanthroline. This appears to be due to a ribonuclease found in cell-free extracts of B. brevis which is inhibited by cGMP and related compounds as well as by 1,1O-phenanthroline.

The inhibition of the enzyme by cGMP is noncompetitive, and cGMP itself is not hydrolyzed. The sensitivity of the nuclease to inhibition depends strikingly on the nature of the substrate and is lost when the enzyme is assayed at high pH. These observations suggest that cGMP inhibits the nuclease by combining with an allosteric site on the enzyme.

Although cGMP was found to be the most effective inhibitor, other nucleoside 3'':5'-monophosphates and derivatives of 5'-GMP can also inhibit the nuclease. Since measurements of cGMP in B. brevis have not revealed detectable amounts (less than 5 × 10−8 M), the substance that modulates the activity of the nuclease under physiological conditions remains to be identified.

In the course of studies on RNA synthesis in cells of Bacillus brevis made permeable to small molecules by treatment with toluene, we observed a striking stimulation by cyclic guanosine 3'':5'-monophosphate, especially in cells derived from sporulating cultures (1). Further analysis of this phenomenon revealed that the primary effect of the cyclic nucleotide was not on the rate of RNA synthesis but rather on the rate of RNA degradation. In this paper, we describe some of the properties of a nuclease from B. brevis whose activity is inhibited by cGMP 1.

EXPERIMENTAL PROCEDURE

Materials—Chemicals were obtained from the following sources: cGMP, cAMP, and guanosine 3'':5'-bisphosphate from Boehringer; cUMP, cCMP, cyclic guanosine 2'':3'-monophosphate, 5'-AMP, 5'-UMP, and 5'-CMP from Sigma; GDP, ADP, UMP, CDP, GTP, ATP, UTP, CTP, [2-14C]UDP (41.6 Ci per mol), [5-3H]UTP (17.5 Ci per mmol), [5-3H]UTP (15 Ci per mmol), and ammonium sulfate (enzyme grade) from Schwarz-Mann; [8-3H]GTP (53.6 Ci per mol) and [G-3H]GMP (3.6 Ci per mmol) from New England Nuclear; polynucleotide phosphorylase (Micrococcus luteus, type 1) from P-L Biochemicals; alkaline phosphatase (Escherichia coli, chromatographically purified), β-galactosidase (E. coli), DNase I (bovine pancreas, electrophoretically purified), and ribonuclease A (bovine pancreas, crystalline) from Worthington; 111I-labeled 2'-O-succinyl cGMP tyrosine methyl ester, 32P-labeled 2'-O-succinyl cAMP tyrosine methyl ester, cGMP antisera (rabbit), cAMP antisera (rabbit), and anti-rabbit immunoglobulin (sheep) from Collaborative Research, Waltham, Mass.; p-toluene sulfonil fluoride from Aldrich; polyethylenimine-impregnated cellulose sheets from Brinkmann; and DEAE-cellulose (Cellex D), ECTEOLA (epichlorohydryl triethanolamine)-cellulose (Cellex E) and Agarose gel (Bio-Gel A-0.5m) from Bio-Rad. Glyceraldehyde kinase was purified from E. coli by the method of Thorner and Paulus (2). DNA cellulose was prepared from denatured salmon sperm DNA as described by Alberts et al. (3). Guanosine 3'':5'-diphosphate-5'-diphosphate was a gift of Dr. Ricardo Block and streptolysin was donated by Dr. G. B. Whitfield of the Upjohn Company. 32P-labeled T7 phage DNA (0 Ci per mol) was the gift of Mr. D. M. Livingston. It was denatured by heating for 5 min at 100° in 10 mM Tris, pH 7.4, 10 mM NaCl, and 1 mM EDTA at a concentration of 0.1 μmol per ml, followed by rapid cooling at 0°.

Synthetic Polynucleotides—Radioactive polynucleotides were prepared from nucleoside diphosphates with polynucleotide phosphorylase from M. luteus (4) and purified by the method of Chamberlin (5). The following polymers were prepared: poly(A) from [32P]ADP (0.1 Ci per mol); poly(A,C) from equimolar amounts of [14C]ADP (0.2 Ci per mol) and unlabeled CDP; and poly(U) from [32P]UDP (0.1 Ci per mol) or from [3H]UDP (0.2 Ci per mol). The yields of radioactive polynucleotides were 40 to 50%.

1 The abbreviations used are: cGMP, cAMP, cUMP, cCMP, and eIMP, the cyclic 3'':5'-monophosphates of guanosine, adenosine, uridine, cytidine, and inosine, respectively.

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(Ap)U₉ was prepared by the digestion of poly(A, U) with pancreatic ribonuclease. The latter polymer was synthesized from 100 μmol of [8-3H]ADP (10 μCi) and 3 μmol of [5-3H]UDP (30 μCi) with polyuridylic acid polynucleosidase (4). The product was incubated for 16 hours at 37°C with 0.15 mg of pancreatic ribonuclease H as UMP and then for 2.5 hours at 25°C with 0.1 M HCl in order to hydrolyze cyclic phosphate esters. The yield of polynucleotide, based on the recoveries of 3H and 8°C, was 47% and 23%, respectively, indicating that the product had a molar ratio of adenine to uracil of 7. After alkaline hydrolysis (0.3 n KOH at 37°C for 16 hours) and thin layer chromatography on cellulose impregnated with polyethyleneimine, 94% of 8°C was recovered as AMP and 97% of 3H as UMP. On the other hand, the polynucleotide was first treated with bacterial alkaline phosphatase (1.3 μmol of polynucleotide and 0.3 unit of alkaline phosphatase in 0.2 ml of 0.5 M Tris hydrochloride, pH 8.0, at 25°C for 30 min) and then subjected to alkaline hydrolysis and thin layer chromatography, 95% of 8°C was associated with AMP and none with adenosine, while 94% of 3H was found in uridine and only 3% in UMP. These analyses showed that in the original polymer, at least 95% of the uridine residues were at the 3' end and terminated by a 2'- or 3'-phosphate while no adenosine residues were 3'-terminal, consistent with the structure (Ap)₉U₉.

The concentration of polynucleotides is always expressed in terms of their phosphate content.

Enzyme Assay—Ribonuclease activity was measured by the conversion of polynucleotides to radioactive products. Unless otherwise stated, the conditions of the assay were as follows: 89 μmol of [3H]poly(U) were incubated with 0.1 n Tris, pH 7.1, 10 mM MgCl₂, 2 mM MnCl₂, 10 mM 2-mercaptoethanol, and appropriate amounts of enzyme in a final volume of 0.1 ml for 15 min at 37°C. The reaction was terminated by the addition of 1.5 ml of cold ethanol and 0.4 ml of RNA (1 mg per ml) in 10 mM Tris. At the appropriate time, the mixtures were acidified to pH 1.60 with 2N HCl and the precipitate was centrifuged at 4°C to remove the sample (1 ml) of the supernatant solution was transferred to a vial, and Bray's scintillation fluid (6) (10 ml) was added for counting in a Packard Tri-Carb liquid scintillation spectrometer. The amount of alcohol-soluble product formed was a linear function of time and enzyme concentration as long as not more than 95% of the substrate was hydrolyzed. A unit of ribonuclease activity was defined as the amount of enzyme that produced 1 nmol of alcohol-soluble product per min from poly(U) under the conditions described.

Bacterial Growth—Bacillus brevis ATCC 8185 was grown in the medium of Hansen et al. (7) at 37°C on a rotary shaker or in a New Brunswick Fermentor with vigorous aeration. Growth was monitored with a Klett-Summerson photoelectric calorimeter using the No. 42 filter. The transition from exponential growth was characterized by a sharp increase in absorbance at 685 nm, followed by a period of more gradual increase, with a change in slope of 180 Klett units, washed with a buffer containing 10 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 mM 3,5-DTA, 0.1 mM KCl, and 1 mM p-toluene sulfonyl fluoride, and stored at -20°C.

Cell-free extracts of B. brevis were made permeable to small molecules by treatment with toluene, which can incorporate [3H]UTP into RNA in a reaction that depended on all four nucleoside triphosphates, and that was inhibited by rifampicin and streptolydigin (1). When further RNA synthesis was prevented by the addition of streptolydigin, the radioactive product was found to be rapidly degraded. As shown in Fig. 1, the half-life of newly synthesized RNA was 10 min. The addition of cGMP reduced the rate of RNA degradation to one-half, while cAMP had a smaller effect. 1,10-Phenanthroline (1.0 mM) completely inhibited RNA degradation.

Cell-free extracts of B. brevis mediated the rapid hydrolysis of synthetic polynucleotides. Fig. 2 shows the effect of cGMP on the hydrolysis of [3H]poly(U) in the presence of Mn²⁺ and Mg²⁺ at pH 7.1. The reaction was inhibited by low concentrations of cGMP, with 89% inhibition at 1 mM. This indicated that most of the ribonuclease activity measured under these conditions was due to an enzyme which was sensitive to the cyclic nucleotide.

Enzyme Purification—All operations were carried out at 4°C. Thawed cells (10 g) of B. brevis were suspended in 20 ml of Buffer A (10 mM Tris, pH 7.1, 10 mM 2-mercaptoethanol, 2 mM MnCl₂, and 20% ethylene glycol) supplemented with 1 mM NH₄Cl, 1 mM p-toluene sulfonyl fluoride, and 0.1 mg of pancreatic DNase and...
FIG. 1. Degradation of RNA synthesized by toluene-treated cells of B. brevis derived from cultures harvested at 150 Klett units was measured as described under "Experimental Procedure" in the absence or presence of cyclic nucleotides (2 mM) or 1,10-phenanthroline (1 mM) as indicated.

FIG. 2. Inhibition of ribonuclease activity in crude extracts by cGMP. Crude extract of B. brevis (10 μg of protein; 0.6 unit) was assayed under standard conditions at various concentrations of cGMP as described under "Experimental Procedure."

The ribonuclease was purified on the basis of its ability to hydrolyze poly(U). However, as will be discussed below, the purified enzyme was also able to hydrolyze other polynucleotides such as poly(A) and denatured DNA, albeit at a considerably lower rate.

TABLE I

| Fraction | Protein concentration (mg/ml) | Total activity (units) | Specific activity (units/mg) | Yield |
|----------|------------------------------|------------------------|-----------------------------|-------|
| I. Crude extract | 20 | 38 | 41,000 | 41 | 100 |
| II. 20,000 × g supernatant | 20 | 34 | 31,700 | 78 |
| III. 150,000 × g supernatant | 16 | 15 | 27,100 | 66 |
| IV. Bio-Gel A-0.5m | 41 | 1.1 | 17,700 | 43 | 18 |
| V. DEAE-cellulose | 30 | 0.71 | 6,250 | 15 | 24 |
| VI. ECTEOLA-cellulose | 30 | 0.085 | 0.200 | 15 | 22 |
| VII. DNA-cellulose | 1 | 0.078 | 900 | 7 | 10 |

*Only one-third of Fraction VI was processed.

The results of the purification procedure are summarized in Table I. Since the DNA-cellulose eluate (Fraction VII) contained some DNA which inhibits the hydrolysis of poly(U) (see below), its activity could not be measured accurately and may be considerably higher than indicated in Table I. The total purification achieved was therefore at least 300-fold with a yield of 7% or higher. Fraction VII, when subjected to electrophoresis on polyacrylamide gels, revealed one major and a minor protein component (Fig. 4A). Upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the major protein component appeared as a sharp doublet band whose molecular weight, estimated by comparison with the electrophoretic mobility of standard proteins, was 60,000 (Fig. 4B). The purified enzyme was relatively unstable. When stored in Buffer A at −10°C, one-half of the enzyme activity was lost in about 2 weeks.

The ribonuclease was purified on the basis of its ability to hydrolyze poly(U). However, as will be discussed below, the purified enzyme was also able to hydrolyze other polynucleotides such as poly(A) and denatured DNA, albeit at a considerably lower rate.
The rate of poly(U) hydrolysis was reduced by only 35%.

of the hydrolysis products of poly(U) and poly(A,C). Even under conditions of incomplete hydrolysis (5 to 10% of the substrate), the sole reaction product was 5'-UMP (Fig. 3), suggesting that a single enzyme acted on all these substrates.

Thus, while 1,10-phenanthroline (0.5 mM) inhibited the hydrolysis of poly(A,C), poly(A), and denatured DNA each by 70%, the hydrolysis of poly(U) was somewhat less sensitive to the chelator than the hydrolysis of other substrates.

lower rate. The ratio of the capacities to hydrolyze poly(U) and poly(A) was relatively constant throughout the purification procedure (Table I). Furthermore, the activities which hydrolyzed polynucleotides required the presence of Mg2+, which could be only partially replaced by Mn2+.

Metal Ion Requirement—As shown in Table II, the hydrolysis of synthetic polynucleotides required the presence of Mn2+, which could be only partially replaced by Mg2+. Nuclease activity was inhibited by 1,10-phenanthroline at concentrations considerably lower than that of Mn2+ present in the incubation mixture, while 1,7-phenanthroline was without effect. It should be noted that the hydrolysis of poly(U) was somewhat less sensitive to the chelator than the hydrolysis of other substrates. Thus, while 1,10-phenanthroline (0.5 mM) inhibited the hydrolysis of poly(A,C), poly(A), and denatured DNA each by 70%, the rate of poly(U) hydrolysis was reduced by only 35%.

The addition of KCl, NaCl, and NH4Cl (0.1 M) had no effect on the activity of the nuclease.

Mode of Action—When polyuridylic acid was hydrolyzed with an excess of enzyme, the sole reaction product was 5'-UMP (Fig. 6). Similarly, the only radioactive product obtained with poly(A,C) labeled with 14C in the adenine moiety was 5'-AMP (not shown). Even under conditions of incomplete hydrolysis (5 to 10%), nucleoside 5'-monophosphates represented more than 90% of the hydrolysis products of poly(U) and poly(A,C).

Table III shows the action of the nuclease on (Ap)70U and (Ap)70Up, labeled with 14C in the adenine moiety and with 3H in uracil. The polymer bearing a 3'-phosphate was a relatively poor substrate and afforded release only of the P4C1 adenylate residues in alcohol-soluble form, while the dephosphorylated polynucleotide was more rapidly hydrolyzed with preferential solubilization of the terminal 3H-ribose residues. The time course of hydrolysis of double labeled (Ap)70U is illustrated in Fig. 7. The hydrolysis of the 3H-labeled 3'-hydroxyl end was considerably more rapid than the over-all hydrolysis of the polynucleotide, measured by the solubilization of the 14C-labeled residues.
Substrate Specificity—Besides poly(U), the purified nuclease preparation could hydrolyze a variety of polynucleotides (Table IV). The observed rates of hydrolysis of poly(A) and denatured DNA were considerably lower than those of poly(U) and poly-(A,C). However, the concentrations of the polynucleotides used were probably not saturating; moreover, the rate of exonucleolytic degradation will be a function of the concentration of 3'-termini rather than of nucleotide residues. Consequently, since the average chain lengths of the various polynucleotides had not been determined, it is not possible to evaluate their relative effectiveness as substrates. Nevertheless, the rates of hydrolysis of denatured and native DNA can be directly compared since they come from the same DNA preparation, and it is clear that denatured DNA is hydrolyzed much more rapidly.

Table V shows the effect of unlabeled polynucleotides on the hydrolysis of labeled substrates. An equivalent amount of poly(A) completely inhibited the hydrolysis of [3H]poly(U), suggesting that the poly(U)-poly(A) complex is resistant to hydrolysis. Denatured, but not native DNA inhibited the hydrolysis of polyribonucleotides while poly(U) inhibited the hydrolysis of denatured DNA, supporting the idea that ribonuclease and deoxyribonuclease activities are attributes of a single enzyme. It should be noted that ribonuclease activity was inhibited at a very low DNA:RNA ratio whereas a considerable excess of RNA was required to inhibit DNA hydrolysis, suggesting that the enzyme has a higher affinity for denatured DNA than for polyribonucleotides.

In order to test whether cGMP is hydrolyzed by the nuclease, [3H]cGMP (20 nmoi; 0.5 μCi) was incubated for 15 min with 1 unit of nuclease under standard assay conditions in a volume of 10 μl. When the reaction mixture was subjected to chromatography...

**Table IV**

Hydrolysis of various polynucleotides

The hydrolysis of polynucleotides, at the concentration indicated, by the nuclease of B. brevis (Fraction VI) was assayed as described under “Experimental Procedure,” except with DNA as substrate, where precipitation with 0.3 N trichloroacetic acid was used.

| Substrate          | Concentration | Relative rate of hydrolysis |
|--------------------|---------------|-----------------------------|
| Poly(U)            | 800           | 100                         |
| Poly(A,C)          | 350           | 19                          |
| Poly(A)            | 430           | 2                           |
| Denatured T7 DNA  | 11            | 0.3                         |
| Native T7 DNA      | 11            | <0.001                      |

**Table V**

Effect of unlabeled polynucleotides on hydrolysis of labeled substrates

The hydrolysis by nuclease (Fraction VI) of the substrates indicated was assayed in the presence of unlabeled polynucleotides as described under “Experimental Procedure,” except that 0.3 N trichloroacetic acid was used as the precipitant when DNA was the substrate. Unlabeled DNA was derived from salmon sperm.

| Labeled substrate | Unlabeled polynucleotide | Inhibition % |
|-------------------|---------------------------|--------------|
| 0.89 mM poly(U)   | 1.0 mM poly(U)            | 55           |
| 1.0 mM poly(A)    | 0.015 mM denatured DNA    | 51           |
| 0.43 mM poly(A)   | 0.015 mM native DNA       | 5            |
| 0.43 mM poly(A)   | 0.015 mM denatured DNA    | 35           |
| 0.011 mM denatured DNA | 1.0 mM poly(U)             | 52           |

Fig. 7 (left). Time course of hydrolysis of (Ap)₃U. Nuclease was incubated with (Ap)₃U, labeled with [¹⁴C]adenine and [³H]uracil, as described in Table IV. A, percentage of [¹⁴C] and [³H] units rendered alcohol-soluble as a function of time; B, solubilization of [³H] as a function of [¹⁴C] release.

Fig. 8 (right). Effect of cGMP on the hydrolysis of polynucleotides. Assays were carried out as described under “Experimental Procedure” at various concentrations of cGMP with substrates and amounts of enzyme as follows: 0.89 mM [¹⁴C]poly(U) with 1.4 units of Fraction VI; 0.35 mM [¹⁴C]poly(A,C) with 2.2 units of Fraction VI; 0.43 mM [¹⁴C]poly(A) with 5 units of Fraction VII; and 0.011 mM [³H]labeled denatured phage T7 DNA with 5 units of Fraction VII.
Inhibition of poly(U) hydrolysis by nucleotides

Table VI

| Nucleotide                        | Inhibition by 0.1 mM | Inhibition by 1.0 mM |
|-----------------------------------|----------------------|----------------------|
| cGMP                              | 69                   | 98                   |
| cAMP                              | 26                   | 38                   |
| cIMP                              | 7                    | 74                   |
| 2':3'-cGMP                        | 11                   | n.t.                 |
| 3':GMP                            | 24                   | n.t.                 |
| 5'-GMP                            | 28                   | 44                   |
| 5'-GDP                            | 3                    | 24                   |
| 5'-GTP                            | 9                    | 22                   |
| Guanosine 3',5'-bisphosphate      | 16                   | 56                   |
| Guanosine 3'-diphosphate 5'       | 0                    | n.t.                 |

n.t., not tested.

Effect of pH—The hydrolysis of poly(U) by the nuclease of B. brevis occurred over a broad range of pH, with more than maximal activity between pH 7.1 and 10.6 (Fig. 10). However, the sensitivity to inhibition by cGMP declined in the higher pH range, so that the pH optimum was shifted upward in the presence of the cyclic nucleotide. The nuclease activity observed at high pH did not seem to be due to a different enzyme, since it showed the same substrate specificity as that observed at pH 7.1 and produced 5'-mononucleotides as the only product (not shown). Nevertheless, in order to assure maximum sensitivity to inhibition by cGMP, most of our studies were done at pH 7.1, where the enzyme exhibited about half-maximal activity.

Levels of Cyclic Nucleotides in B. brevis—We made attempts to measure the intracellular levels of cGMP and cAMP in samples of early and late exponential as well as early and late sporulating cultures of B. brevis. Although cyclic nucleotides added as internal standards were recovered in good yield, no endogenous cGMP and cAMP was found. The sensitivity of the radioimmunoassay used was such that intracellular concentrations of 5 × 10^{-8} M cGMP and 5 × 10^{-7} M cAMP would have been detected.

**DISCUSSION**

Although the turnover of mRNA in bacteria has been widely studied, little is known about the factors that regulate this process. It was therefore of considerable interest that the degradation of newly synthesized RNA in toluene-treated cells of B. brevis was markedly inhibited by the addition of cGMP, suggesting the presence of a nuclease whose activity is regulated by the cyclic nucleotide. This was confirmed by the observation that most of the ribonuclease activity in cell-free extracts of B. brevis could be inhibited by cGMP (Fig. 2). Moreover, RNA degradation in toluene-treated cells and the ribonuclease in cell-free extracts were similarly inhibited by 1,10-phenanthroline.

After partial purification, the cGMP-sensitive enzyme could be characterized as an exonuclease because it produced ribonucleoside 5'-monophosphates as the sole products even during the very early stages of hydrolysis of synthetic polynucleotides (Fig. 4). Moreover, polynucleotides bearing a 3'-phosphate were relatively resistant to the action of the enzyme (Table III). Their slow hydrolysis did not lead to the release of the 3'-terminal nucleotide and may have been due to a small amount of endo-
nuclease contaminating our partially purified enzyme preparation. Studies on the time course of hydrolysis of the double labeled polynucleotide (Ap)35U showed a much more rapid solubilization of the 3′ terminal uridylate than of the adenylate residues (Fig. 7), a result consistent with random exonuclease degradation from the 3′ end.

The purified exonuclease preparation from _B. brevis_ could hydrolyze a variety of single-stranded polynucleotides. Several lines of evidence suggest that the action on the various substrates is the manifestation of a single enzyme. (a) The ratio of the capacities to hydrolyze poly(U) and poly(A) was relatively constant during the purification procedure (Table I); (b) the activities that hydrolyzed poly(U) and denatured DNA eluted in a common peak from ECTEOLA-cellulose (Fig. 3); (c) the ribonuclease activity was adsorbed to denatured DNA bound to cellulose; (d) the hydrolysis of poly(U) and poly(A) was inhibited by denatured DNA, while the hydrolysis of DNA was inhibited by polynucleotides (Table V); and (e) all activities were inhibited to some extent by cGMP and 1,10-phenanthroline, even though the relative sensitivities to these agents did depend on the nature of the substrate.

A striking property of the nuclease from _B. brevis_ is its sensitivity to inhibition by cGMP. Two possible mechanisms could account for this phenomenon. First of these is the direct combination of the cyclic nucleotide with the active site of the enzyme, either as a substrate or as a substrate analog. This possibility is unlikely, since no hydrolysis of cGMP by the enzyme could be detected and since the inhibition was strictly noncompetitive (Fig. 9). Moreover, specificity studies showed that nucleotides not containing a phosphodiester linkage, such as 5′-GMP and guanosine 3′,5′-bisphosphate, were also somewhat inhibitory, and that cUMP and cGMP were weaker inhibitors than cGMP, in spite of the fact that the enzyme preferred to hydrolyze polynucleotides containing pyrimidine residues. We must therefore consider a second possibility, namely that the inhibitors do not bind to the active site of the enzyme but to an allosteric site. Strictly speaking, the occurrence of noncompetitive inhibition is sufficient evidence for the existence of an allosteric site, but, ordinarily, the dissociation of catalytic activity from inhibition is regarded as a stronger criterion. Such a phenomenon was indeed observed at high pH, where the hydrolysis of poly(U) still proceeded at a relatively rapid rate but was insensitive to inhibition by cGMP (Fig. 10). Since the ribonuclease activities assayed at pH 7.1 and 10.6 eluted in a single peak from ECTEOLA-cellulose (Fig. 3) and the catalytic properties at pH 7.1 and 10.6 were very similar, it is unlikely that these activities are due to two different enzymes. Rather, it appears that at high pH the enzyme has selectively lost the ability to respond to allosteric inhibition. Moreover, the differences in sensitivity to inhibition by cGMP of the hydrolysis of different substrates (Fig. 8) can be more readily understood in terms of an allosteric mechanism than in terms of binding of the cyclic nucleotide to the active site. Finally, it should be noted that the molecular weight of the nuclease from _B. brevis_, 360,000, is very much higher than that of other ribonucleases known and suggests that the enzyme is composed of subunits, a property characteristic also of allosteric enzymes.

The physiological function of the exonuclease of _B. brevis_ is still uncertain, but the experiments with toluene-treated cells suggest that the enzyme may be involved in the degradation of mRNA. An important question concerns therefore the nature of the substance that inhibits the ribonuclease under physiological conditions. Our analyses revealed that the intracellular levels of cGMP1' and cAMP in _B. brevis_ are at least several orders of magnitude lower than the concentrations that produce 50% inhibition of the ribonuclease. Other workers have also been unable to detect cAMP in either _Bacillus licheniformis_ (17) or _Bacillus megaterium_ (18), while the level of cGMP in _B. licheniformis_ has been found to be less than 8 × 10−9 M (17). Therefore, it is unlikely that cGMP is the agent that modulates the activity of the ribonuclease in vivo, in spite of the fact that it was the most inhibitory of the substances that we have examined with the purified enzyme. A naturally occurring inhibitor of the nuclease of _B. brevis_, perhaps structurally related to cGMP, thus remains to be identified.

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