Inhibition of Phosphodiesterases from *Bacillus subtilis* by Nucleoside Triphosphates†

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ABSTRACT: Both phosphodiesterase I and phosphodiesterase II (PDase I and PDase II, respectively, according to W. E. Razzell's terminology, (1961), *J. Biol. Chem. 236*, 3028), are detectable in spore-forming *Bacillus subtilis*. Sephadex G-100 gel filtration of cell-free extracts obtained from cultures grown for 14 hr led to the elution of two peaks with PDase II and PDase I activities, respectively. PDase II is competitively inhibited by nucleoside triphosphates, the concentration required for 50% inhibition ranging between about 0.07 μM for CTP and 0.5 μM for UTP. PDase I is uncompetitively inhibited by nucleoside triphosphates and is by far less sensitive to nucleoside triphosphate inhibition than PDase II. Taken together with the previously reported regulatory properties of *Bacillus subtilis* nucleoside phosphomonoesterases (R. A. Felicioli *et al.* (1973), *Biochemistry 12*, 547), these observations suggest that in *Bacillus subtilis* the enzymatic breakdown of polynucleotides to nucleoside monophosphates and free nucleosides might be modulated by the levels of nucleoside triphosphates.

In the early stages of germination of spore-forming bacteria the nucleotide "de novo" synthesis is not operative (Nelson and Kornberg, 1970), and the rapid increase of nucleoside triphosphate levels (Setlow and Kornberg, 1970) apparently occurs by utilization of the preformed nucleosides and 5′-mononucleotides of the dormant spore.

Our previous results (Felicioli *et al.*, 1972a, 1973) indicate that in *Bacillus subtilis* the difference between 3′- and 5′-mononucleotidases in sensitivity to control by nucleoside triphosphates plays a major role in the modulation of the relative levels of free nucleosides, 3′- and 5′-mononucleotides. This conclusion is mainly based on the observation that the 3′-mononucleotidase is about 1000-fold less sensitive to nucleoside triphosphate inhibition than the 5′-mononucleotidase.

To our knowledge, the only mechanism for the formation of 3′-mononucleotides is the action of phosphodiesterase I (PDase I)[1] on polynucleotides. In this paper we show that this enzyme activity in *B. subtilis* is subjected to a powerful inhibition by nucleoside triphosphates, while PDase I is much less sensitive to nucleoside triphosphate inhibition.

These and other properties of *B. subtilis* PDase I and PDase II, together with the change in their relative levels during the growth cycle (Felicioli *et al.*, 1972b), and with the regulatory properties of 3′- and 5′-mononucleotidases reported previously (Felicioli *et al.*, 1973), provide a clear picture of the regulation of nucleosides and mononucleotides production from RNA for the *salvage* synthesis of nucleoside triphosphates occurring during germination.

Experimental Section

Materials. Dinucleoside monophosphates and nucleotides were obtained either from Sigma Chemical Co. or from Boehringer und Soehne. Adenosine deaminase from intestinal mucosa (10 mg/ml) was obtained from Boehringer und Soehne and was diluted 500-fold with water before use. Nucleoside phosphorylase (1 mg/ml), guanase (2 mg/ml), and xanthine oxydase (10 mg/ml) were obtained from Boehringer GmbH (Mannheim, Germany). Tris (Sigma) was used as a buffer in most experiments. Other chemicals were of reagent grade or of the highest quality available.

Enzyme Assay Procedure. The phosphodiesterase reaction was carried out by previously described spectrophotometric methods (Ipata and Felicioli, 1969), using the following dinucleoside monophosphates as substrates: adenylyl-(3′-5′)-adenosine (ApA); adenylyl-(3′-5′)-guanosine (ApG); cytidylyl-(3′-5′)-adenosine (CpA); guanylyl-(3′-5′)-adenosine (GpA); adenylyl-(3′-5′)-cytidine (ApC).

The phosphodiesterase assay procedure is based on the enzymatic measure of the adenosine or guanosine specifically released from either end of the dinucleoside monophosphates used as substrates. The adenosine formed is detected spectrophotometrically at 265 nm by addition of commercial adenosine deaminase, in a recording Hitachi-Perkin-Elmer double beam spectrophotometer at 37°; the molar spectral change for adenosine disappearance was taken as 8.1×10^3 ODU, the difference between adenosine and inosine absorption (Møllering and Bergmeyer, 1962). The guanosine released was detected at 290 nm, following its conversion to uric acid by the combined action of a "guanosine revealing system" (Felicioli *et al.*, 1970), composed of nucleoside phosphorylase, guanase, and xanthine oxidase. The molar spectral change for guanosine disappearance was taken as 12.0×10^3 ODU, the difference between guanosine and uric acid absorption (Felicioli *et al.*, 1970).

The reaction was carried out in a final volume of 2 ml, containing Tris-Cl buffer (pH 7.38), 50 mM, different amounts of ApA, GpA, CpA, or ApC, and adenosine deaminase (0.13 unit) to reveal adenosine as released nucleoside. To reveal guanosine as released nucleoside, ApG and GpA were used as substrates in the presence of guanase, nucleoside phosphorylase, and xanthine oxidase (0.1 unit each).

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[1] The designations "PDase I" and "PDase II" are for convenience only, and do not imply analogous properties with respect to the enzymes from snake venom or spleen.

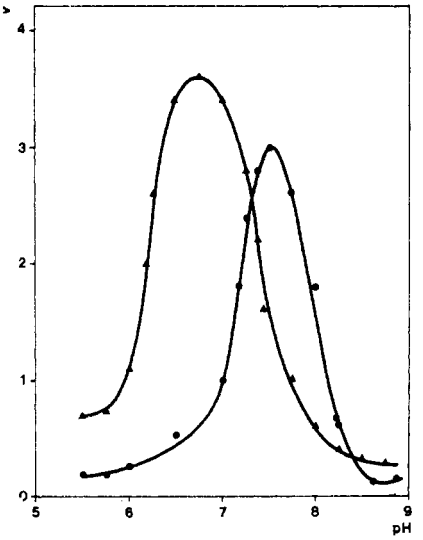


FIGURE 1: pH–activity curves for PDase II (●) and PDase I (▲). Substrates used were CpA (final concentration 47 μM) and ApC (final concentration 50 μM) for PDase II and PDase I, respectively. The assays were carried out in Tris-acetate buffer, using 300 μg of protein preparation and adenosine deaminase as ancillary enzyme. v is expressed as nanomoles of adenosine formed per minute.

At the concentrations used, none of the compounds exerting inhibition on the phosphodiesterase activities had any inhibitory or activatory effect on adenosine deaminase or on the "guanosine revealing system."

The molarities of all substrate and inhibitor solutions were measured spectrophotometrically from the extinction coefficients at 260 nm at pH 7.0 (Cohn, 1955).

Preparation of Crude Extracts and Sephadex G-100 Fractionation. Bacillus subtilis, strain ATCC 6633, was grown on a solid medium as previously described (Falcone, 1961; Felicioli *et al.*, 1973). Sporulation was followed by phase contrast microscopy. The crude extracts were prepared as previously described (Felicioli *et al.*, 1973) and their protein content, measured with the biuret method of Gornall *et al.* (1949), ranged between 15 and 20 mg/ml.

PDase II and PDase I were separated and partially purified from crude extracts of cultures grown for 14 hr by Sephadex G-100 gel filtration following the procedure described elsewhere (Felicioli *et al.*, 1972b).

The protein content of the active eluates was determined according to Warburg and Christian (1942).

Results

Optimum pH. The two phosphodiesterases show different optimum pH as shown in Figure 1. In Tris-acetate buffer the optimum pHs are 7.5 and 6.7 for PDase I and PDase II, respectively. It must be pointed out that adenosine deaminase, used as ancillary enzyme, is active over a wide range of pH (Brady and O'Connell, 1962), and that at each pH value tested it was present in excess. Therefore, the velocities of the reaction are determined by the rate at which adenosine is released from dinucleoside monophosphates (see also Ipata, 1967).

Reaction Kinetics. The substrates saturation curves for both PDase I and PDase II of *B. subtilis* show hyperbolic shapes. The apparent K_m values of the two enzymes for the various dinucleoside monophosphates used as substrates were calculated from double reciprocal plots, and are reported in Table I. These values are close to those previously reported for snake venom and spleen phosphodiesterases (Ipata and Felicioli, 1969). Both PDase I and PDase II of *B. subtilis* are inhibited by nucleoside triphosphates. However, they markedly differ in sensitivity and mode of inhibition.

TABLE I: K_m Values of *Bacillus subtilis* PDase I and PDase II Using Various Dinucleoside Monophosphates as Substrates.

	Substrate	K_m (10^{-6} M)[a]
PDase I	ApA[b]	7.1
	CpA[b]	10.0
	GpA[b]	12.5
	ApG[c]	8.2
PDase II	ApA[b]	5.5
	ApC[b]	4.0
	ApG[b]	4.4
	GpA[c]	8.6

[a] The K_m values were calculated from double reciprocal plots $1/v$ *vs.* 1/[S]. [b] Adenosine deaminase was used as ancillary enzyme (Ipata and Felicioli, 1969) to measure the enzyme activity. [c] "Guanosine revealing ancillary system" (Felicioli *et al.*, 1970) was used to measure the enzyme activity.

Phosphodiesterase II. PDase II is competitively inhibited by ATP, CTP, UTP, and GTP (Figure 2A). The inhibition curves by nucleosides triphosphates are reported in Figure 3. It can be seen that CTP is the most powerful inhibitor, the concentration required for 50% inhibition being 0.07 μM. The same extent of inhibition is observed with GTP, ATP, and UTP at 0.125, 0.300, and 0.525 μM, respectively.

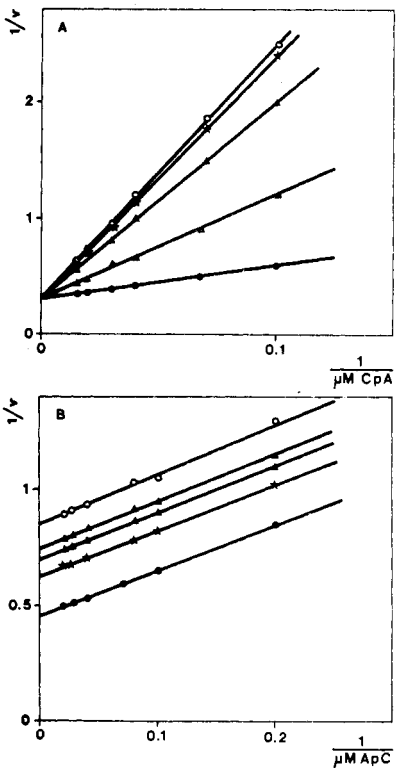


FIGURE 2: Lineweaver–Burk plots (1934), $1/v$ *vs.* 1/[S], of phosphodiesterases assayed in the absence and presence of nucleoside triphosphates. (A) PDase II assayed in the absence of nucleoside triphosphates (●), and in the presence of 0.3 μM ATP (▲), 0.3 μM UTP (△), 0.2 μM GTP (○), or 0.1 μM CTP (★). (B) PDase I in the absence of nucleoside triphosphates (●) and with 420 μM ATP (○), 250 μM UTP (△), 260 μM CTP (▲), or 175 μM GTP (★).

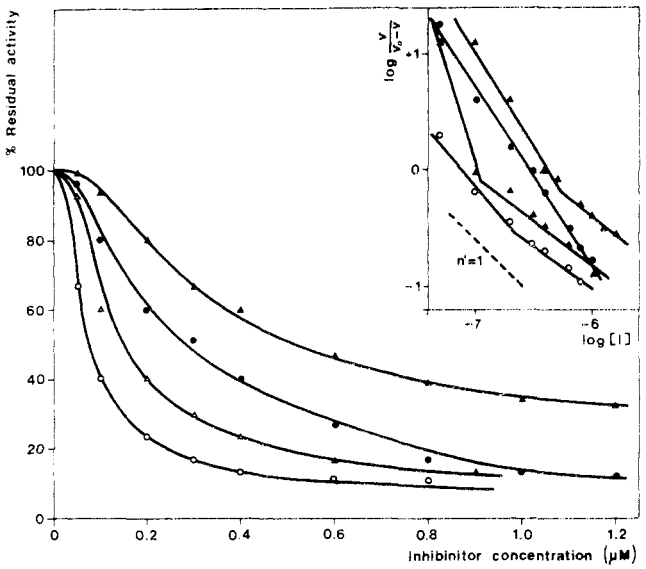


FIGURE 3: Per cent residual activity of PDase II as a function of increasing concentrations of UTP (▲), ATP (●), GTP (△), and CTP (○). The final CpA concentration was 47 μM. The velocity is expressed as per cent of the velocity in the absence of inhibitor. The inset shows the Hill plots.

Phosphodiesterase II. PDase II is competitively inhibited by ATP, CTP, UTP, and GTP (Figure 2A). The inhibition curves by nucleosides triphosphates are reported in Figure 3. It can be seen that CTP is the most powerful inhibitor, the concentration required for 50% inhibition being 0.07 μM. The same extent of inhibition is observed with GTP, ATP, and UTP at 0.125, 0.300, and 0.525 μM, respectively.

When the inhibition data for UTP, GTP, and CTP from Figure 4 were plotted according to Hill (1910), n' values higher than 1 at low inhibitor concentrations and n' values lower than 1 at high inhibitor concentrations were observed. For ATP an n' value of 1.8 was constantly found throughout the entire range of inhibitor concentrations tested.

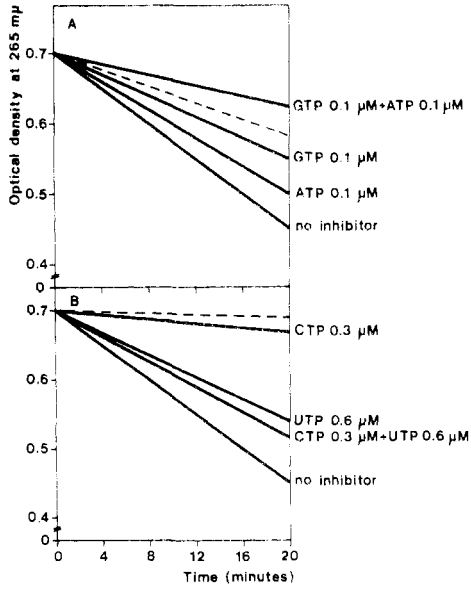


FIGURE 4: Time course of the change in absorbance at 265 nm during optical test for PDase II of *Bacillus subtilis*. (A) Inhibitor concentrations in the range of positive cooperativity as shown in Figure 4. (B) Inhibitor concentrations in the range of negative cooperativity as shown in Figure 4. (- - - -) The predicted inhibition in experiments with pairs.

TABLE II: Cooperative Inhibition of *Bacillus subtilis* PDase II by Nucleoside Triphosphates.[a]

Concn (μM) Nucleoside Triphosphates				Inhibn.	Predicted Inhibn.
ATP	CTP	GTP	UTP	(%)	(%)
0.1	0	0	0	20	
0	0.05	0	0	34	
0	0	0.1	0	40	
0	0	0	0.3	36	
0.1	0.05	0	0	60	47
0.1	0	0.1	0	71	52
0.1	0	0	0.3	73	49
0	0.05	0.1	0	69	60
0	0.05	0	0.3	71	58
0	0	0.1	0.3	77	62
0.2	0	0	0	44	
0	0.3	0	0	78	
0	0	0.2	0	56	
0	0	0	0.6	56	
0.2	0.3	0	0	81	88
0.2	0	0.2	0	71	75
0.2	0	0	0.6	67	75
0	0.3	0.2	0	58	90
0	0.3	0	0.6	51	90
0	0	0.2	0.6	62	81

[a] PDase II was measured spectrophotometrically as described in Figure 4 in the presence of either each nucleoside triphosphate alone or a mixture of two nucleoside triphosphates at the reported concentrations. The predicted residual activity is obtained from the product of the residual activities obtained in the presence of each inhibitor alone. For example, there was 80% residual activity in the presence of 0.1 μM ATP and 64% residual activity in the presence of 0.3 μM UTP. The predicted activity in the presence of 0.1 μM ATP and 0.3 μM UTP is (0.80 × 0.64) × 100, or 51%. CpA (final concentration 46 μM) was used as substrate.

The biphasic Hill plots apparently suggest that, at least with CTP, UTP, and GTP, either positive or negative cooperativity between inhibitor molecules can occur, depending on the inhibitor concentration. This point has been further investigated by using pairs of inhibitors. Table II summarizes the results of experiments in which pairs of inhibitors at concentrations in the range of either negative or positive cooperativity are used. It can be seen that when pairs of nucleoside triphosphates were made by using each member at concentrations in the negative cooperativity range, the observed inhibition is lower than that expected. *Vice versa*, when concentrations of the nucleoside triphosphates used in pairs are in the range of positive cooperativity, the observed inhibition is higher. Figure 4 shows the results of the time course of some typical reactions in which the effect of pairs of nucleotides was studied.

Phosphodiesterase I. The inhibition exerted by nucleoside triphosphates on PDase I of *B. subtilis* is of the uncompetitive type (Figure 2B).

The PDase I is by far less sensitive to nucleoside triphosphate inhibition. 50% inhibition being observed at concentrations ranging from about 300 μM for GTP, UTP, and

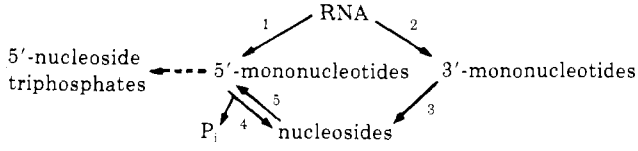
ATP to about 450 μM for CTP. The inhibition curves are reported in Figure 5.

It can be seen that the four curves show sigmoidal shape and that the n' values are constantly higher than 1. These data suggest positive cooperativity between inhibitor molecules.

Discussion

The results presented in this paper show that the production of 3′- and 5′-mononucleotides by the action of *B. subtilis* phosphodiesterases is subjected to a complex regulation by nucleoside triphosphates.

Even though the phosphodiester bonds of RNA can be hydrolyzed in the 3′ and 5′ positions, our data suggest that, in vegetative forms of *B. subtilis*, where relatively high nucleoside triphosphate concentrations are present (Nelson and Kornberg, 1970), the production of 3′-mononucleotides, which cannot be *directly* utilized for nucleoside triphosphates synthesis, might be prevented by the strong inhibition exerted by nucleoside triphosphates on the PDase II; 50% inhibition has been observed at about 0.5 μM nucleoside triphosphates, a concentration by far lower than that generally found in aerobic spore formers (Setlow and Kornberg, 1970b). PDase I is also inhibited by ATP; 50% inhibition, however, is observed at about 0.5 mM nucleoside triphosphates, a "physiological" concentration. Only in the final stages of sporulation, when a dramatic fall in ATP concentration occurs (Nelson and Kornberg, 1970), the inhibition exerted on the "spleen type" phosphodiesterase should be relieved, to yield 3′-mononucleotides. The different sensitivity to the control by nucleoside triphosphates of the two phosphodiesterase activities in *B. subtilis* should be considered in conjunction with the different sensitivity to the control exerted by the same compounds on 3′- and 5′-mononucleotidases of the same organism (Felicioli *et al.*, 1972b). The following scheme summarizes our results on the regulation of RNA breakdown and on nucleoside monophosphates recycling in *B. subtilis*. Enzyme 2 (PDase II)



and enzyme 4 (5′-mononucleotidase) are inhibited by micromolar nucleoside triphosphate concentration. Enzyme 1 (PDase I) and enzyme 3 (3′-mononucleotidase) are inhibited by millimolar nucleoside triphosphate concentration. As a consequence in vegetative forms of *B. subtilis*, RNA breakdown should mainly produce 5′-mononucleotides, whereas in sporulating forms both 3′- and 5′-mononucleotides should be produced.

It must be emphasized that the free nucleosides are phosphorylated at the 5′ position by the action of nucleoside kinase (enzyme 5).

From a speculative point of view it can be postulated that at nucleoside triphosphate concentrations present in the log growth phase (Nelson and Kornberg, 1970), the PDase II and the 5′-mononucleotidase might be inhibited: as a consequence, only production of 5′-mononucleotides should be allowed, its rate depending on the fluctuations of the nucleoside triphosphate concentrations.

The intracellular divalent cation levels might also contribute to the regulation of phosphodiesterase activities, by

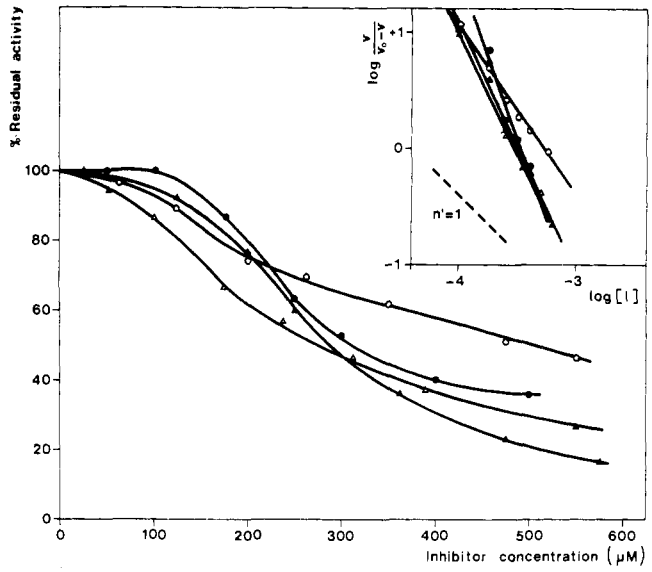


FIGURE 5: Per cent residual activity of PDase I as a function of increasing ATP (●), CTP (○), GTP (△), and UTP (▲) concentrations. The final ApC concentration was 50 μM.

forming complexes with the inhibitory nucleotides: preliminary results not reported here have indicated that these complexes are also inhibitory, even if to a minor extent.

The differences between PDase I and PDase II and between 3′- and 5′-mononucleotidases to control by nucleoside triphosphates would therefore play a major role in allowing the efficient recycling of nucleoside monophosphates and in the regulation of the rapid RNA turnover is sporulating *B. subtilis*.

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