Nucleoside Phosphomonoesterases during Growth Cycle of *Bacillus subtilis*†

Romano Angelo Felicioli, Sonia Senesi, Franco Marmocchi, Giuseppe Falcone, and Pier Luigi Ipata*

ABSTRACT: The levels of 5′-nucleotidase and 3′-nucleotidase activities have been determined in spore-forming *Bacillus subtilis*. Both 3′- and 5′-nucleotidases were detectable in vegetative forms and in forespores. In free spores, however, 3′-nucleotidase, but not 5′-nucleotidase, was detectable. Sephadex G-100 gel filtration of cell-free extracts obtained from cultures grown for 6, 14, and 17 hr led to the elution of two peaks with 3′-nucleotidase activity and one peak with 5′-nucleotidase activity. One of the two peaks with 3′-nucleotidase activity and the peak with 5′-nucleotidase activity were absent in the eluates of free-spore extracts. The 5′-nucleotidase was competitively inhibited by nucleoside triphosphates, the concentration required for 50% inhibition being lower than 0.5 μM. The two 3′-nucleotidases were uncompetitively inhibited by nucleoside triphosphates, and were by far less sensitive to nucleoside and triphosphates inhibition than 5′-nucleotidase. Furthermore, the inhibition curves for 3′-nucleotidases showed sigmoidal shapes. It is postulated that the levels of nucleoside triphosphates during the growth cycle of spore-forming *B. subtilis* might regulate the dephosphorylation of 3′- and 5′-mononucleotides.

In recent years considerable interest has been focused on the regulatory properties of nucleoside phosphomonoesterases in view of the potential role of these enzymes as modulators of the levels of 5′-mononucleotide pools (Fritzson, 1967, 1971) or in nucleotide interconversion (Ipata, 1968).

The mammalian and avian 5′-nucleotidases (EC 3.1.3.5) are strongly inhibited by ATP and other nucleoside triphosphates, as shown for 5′-nucleotidase from sheep brain (Ipata, 1968), rat heart (Baer *et al.*, 1966; Edwards and Maguire, 1970; Sullivan and Alpers, 1971), avian heart (Gibson and Drummond, 1972), and rat cerebellum (Bosmann and Pike, 1971). The bacterial 5′-nucleotidases appear to be a somewhat heterogeneous group of enzymes differing in substrate specificity and metal requirements (see Drummond and Yamamoto, 1971, for review). On the other hand, 3′-nucleotidases (EC 3.1.3.6) have attracted comparatively little attention. In bacteria 3′-nucleotidases have first been described by Kohn and Reis in 1963. However, no data are available on their regulatory properties.

In this paper we describe the inhibition of *Bacillus subtilis* 5′-nucleotidase by ATP and other nucleoside triphosphates, and two distinct 3′-mononucleotidases, much less sensitive to ATP inhibition.

These and other properties of *B. subtilis* nucleoside phosphomonoesterases, together with the change of their relative levels during the sporulating cycle, suggest a major role of these enzymes both in the control of 5′-mononucleotide pools and in the production of free nucleosides for the "salvage" synthesis of nucleoside triphosphates occurring during germination of spore forming bacteria (Setlow and Kornberg, 1970b).

Experimental Section

Materials. Nucleosides and nucleotides were obtained either from Sigma Chemical Co. or from Boehringer und Soehne. Adenosine deaminase was obtained from Boehringer und Soehne. 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 nucleotidase reaction was carried out as previously described by Ipata (1967, 1968) with 3′-AMP or 5′-AMP as substrate in the presence of excess adenosine deaminase. The adenosine formed was determined spectrophotometrically at 265 mμ in a recording Beckman DB spectrophotometer at 36°. The molar spectral change for AMP disappearance was taken as 8.1×10^3 ODU, the difference between adenosine and inosine absorption (see Møllering and Bergmeyer, 1962).

The reaction was carried out in a final volume of 2.0 ml, containing 75 mM Tris-Cl (pH 7.1), 0.1 unit of adenosine deaminase, and various concentrations of substrate, inhibitors, and metal ions. The molarities of all substrate and inhibitor solutions were measured spectrophotometrically from the extinction coefficients at 260 mμ at pH 7 (Cohn, 1955).

Preparation of Crude Extracts and Sephadex G-100 Fractionation. B. subtilis, strain ATCC 6633, was grown on a solid medium of the following composition: 5 g of peptone, 3 g of beef extract, 3 g of yeast extract, 0.1 g of $MnSO_4$, 20 g of agar, and H_2O to 1000 ml. Cultures incubated at 37° for 6, 10, 14, 17, and 24 hr were used in our experiments. Sporulation was followed by phase-contrast microscopy; only vegetative forms were present at the 6th hr, sporulating forms first appeared at the 7th hr, and only free spores were present at the 24th hr. The cells were harvested, washed three times with water by centrifugation at 2°, weighed, suspended in two volumes of 50 mM Tris buffer (pH 7.0), and disintegrated with glass beads in a Brawn disintegrator flushed continuously with CO_2. The extracts were centrifuged, and a 20% solution of streptomycin sulfate (0.07 ml/10 mg of protein) was added

† From the Institute of Biological Chemistry, University of Camerino (F. M., P. L. I.), 62032 Camerino, Italy, from the Laboratory for the Study of Physical Properties of Biomolecules and Cells of C. N. R. (R. A. F.), 56100 Pisa, Italy, and from the Institute of Microbiology, University of Pisa (S. S., G. F.), 56100 Pisa, Italy. *Received July 3, 1972.* This work was supported by a grant from the Italian C. N. R.

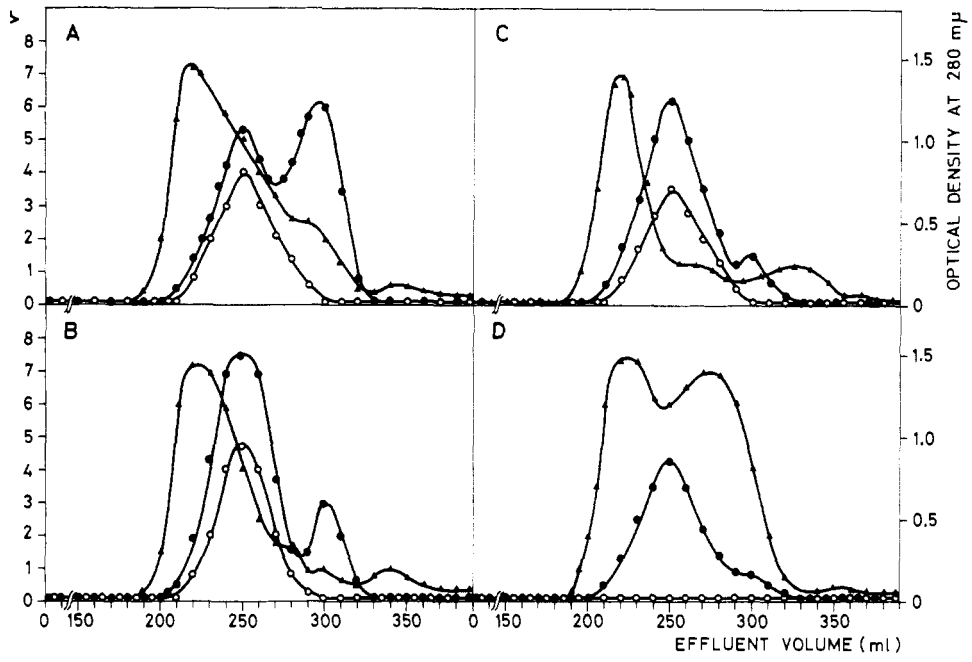

FIGURE 1: Elution pattern of nucleoside phosphomonoesterases of *B. subtilis* grown for different time intervals: 8 ml of the 80% ammonium sulfate fractions dissolved in 50 mM Tris-Cl buffer (pH 7.4) containing *ca.* 15 mg of protein/ml was eluted from Sephadex G-100 columns (3 × 120 cm) with the same buffer, at a flow rate of 15 ml/hr. Fractions of 5 ml were collected; 0.2 ml of each fraction was used to measure enzyme activities. (○) 5′-Nucleotidase activity. (●) 3′-Nucleotidase activity. (▲) Optical density at 280 mμ. Cultures were grown for 6 hr (A), 14 hr (B), 17 hr (C), and 24 hr (D). The velocity is expressed as nmol of adenosine formed per min.

slowly to the clear supernatant. After standing at 0° for 120 min, the suspension was centrifuged at 25,000*g* for 15 min. The supernatant fluid was brought to 80% saturation with ammonium sulfate at pH 7.4, left overnight at 4°, and centrifuged. No nucleoside phosphomonoesterase activity could be detected in the supernatant fluid. The precipitate was dissolved in 50 mM Tris-Cl buffer (pH 7.4), applied to a Sephadex G-25 column (3 × 14 cm), and eluted with the same buffer; the bulk of proteins is referred to as crude extract.

The kinetic properties of 3′- and 5′-nucleoside phosphomonoesterases were studied in partly purified preparations derived from cultures grown for 14 hr. Partial purification was achieved by Sephadex G-100 gel filtration under the experimental conditions described in the legend to Figure 1.

Proteins were determined spectrophotometrically according to Warburg and Christian (1942).

TABLE I: Specific Activities of Nucleoside Phosphomonoesterases of *B. subtilis* during Sporulation.[a]

	Time of Incubation				
	6 hr	10 hr	14 hr	17 hr	24 hr
3′-Nucleotidase	18.4	23.6	17.5	15.9	15
5′-Nucleotidase	7.4	10.6	9.4	7.2	0

[a] 3′- and 5′-mononucleotidase activities were measured spectrophotometrically in crude extracts as described under Experimental Section. The final 3′- or 5′-AMP concentration was 50 μM. The data are given as units of nucleoside phosphomonoesterase per mg of protein. One enzyme unit equals the amount of enzyme catalyzing the liberation of 1 nmol of adenosine/min.

Results

Table I shows the specific activity of 3′- and 5′-nucleotidases in crude extracts of *B. subtilis*. It can be seen that 3′-nucleotidase activity is detectable throughout the spore forming cycle, while 5′-nucleotidase activity could not be detected in extracts obtained from free spores. The absence of 5′-nucleotidase activity in spores was confirmed by incubating crude extracts of disrupted spores in the presence of 1 mM 5′-AMP, in the same experimental conditions described for the spectrophotometric assay. The reaction was stopped with 8% perchloric acid, the precipitate was discarded by centrifugation, and inorganic phosphate was measured on the supernatant fluid according to Fiske and Subbarow (1925). When assayed with 3′-AMP, the same crude spore extract catalyzed the liberation of 6 nmol of P_i/mg wet wt per hr.

The absence of 5′-nucleotidase in spore extracts of *B.* subtilis was also demonstrated by fractionation of crude spore extracts by G-100 Sephadex gel filtration as reported in Figure 1D. Figure 1A–C shows the elution pattern of 5′- and 3′-nucleotidase activities of cell-free extracts of *B. subtilis* cultures grown for 6, 14, and 17 hr, respectively. Sephadex gel filtration led to the appearance of two peaks with 3′-nucleotidase activity, the faster moving one (3′-nucleotidase A) almost superimposable with the 5′-mononucleotidase, the slower one (3′-nucleotidase B) at about 300 ml of elution volume. ATP at 15 μM almost completely abolished the 5′-mononucleotidase activity and had no effect on the two 3′-mononucleotidases. It must be emphasized that under our experimental conditions no dephosphorylation of *p*-nitrophenyl phosphate was observed in fractions with 3′- or 5′-mononucleotidase activity.

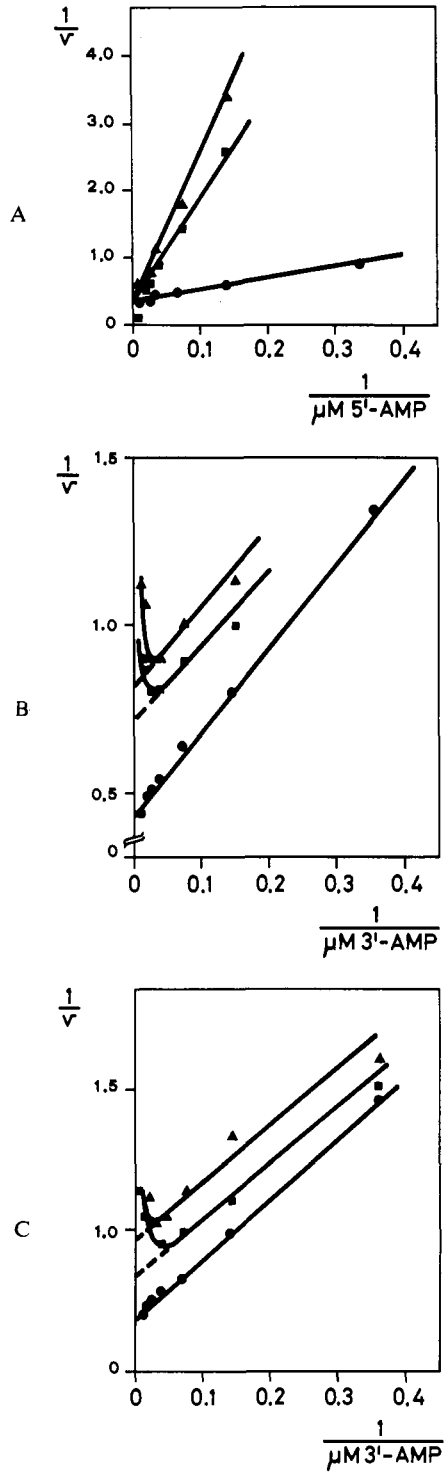

FIGURE 2: Lineweaver–Burk plots (1934) $1/v$ *vs.* 1/[S] of nucleoside phosphomonoesterases assayed in the absence and presence of nucleoside triphosphates. (A) 5′-Nucleotidase assayed in the absence of nucleoside triphosphates (●), and in the presence of 0.376 μM CTP (■), or 0.263 μM ATP (▲). (B) 3′-Nucleotidase A measured in the absence of nucleoside triphosphates (●) and in the presence of 152 μM CTP (■), or 106 μM ATP (▲). (C) 3′-Nucleotidase B measured in the absence of nucleoside triphosphates (●), and in the presence of 106 μM ATP (▲), or 152 μM CTP (■).

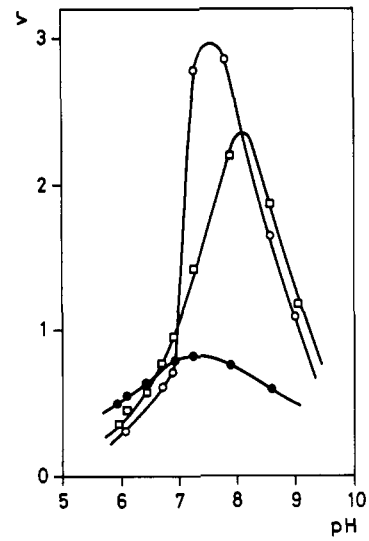

FIGURE 3: Optimal pH for catalytic activity of 5′-nucleotidase (●) and of 3′-nucleotidase A (□) and B (○). The assays were carried out in 75 mM Tris–acetate buffer. v is expressed as in Figure 1.

Reaction Kinetics. The B. subtilis nucleoside phosphomonoesterases display normal Michaelian kinetics. With 3′- and 5′-adenosine monophosphates as substrates, apparent K_m values of 4.5×10^{-6} M for the 5′-mononucleotidase (Figure 2A) and of 4.78×10^{-6} and 6×10^{-6} M for 3′-mononucleotidases A and B, respectively (Figure 2B,C), were calculated. These K_m values are significantly lower than those found for the other bacterial 5′-nucleotidases and 3′-nucleotidases so far studied (see Drummond and Yamamoto, 1971).

pH Optimum. In Tris–acetate buffer optimal activity occurred at about pH 7.1 for 5′-nucleotidase, and at about pH 8.1 and 7.6 for 3′-mononucleotidases A and B, respectively (Figure 3).

Inhibitors. The nucleoside phosphomonoesterases are inhibited to different extent by nucleoside triphosphates. For instance 54% inhibition is observed for 5′-mononucleotidase at 0.2 μM ATP, while 87 μM ATP resulted in 41 and 36% inhibition, respectively, for 3′-mononucleotidases A and B. Furthermore, as shown in Figure 2, 5′-nucleotidase is competitively inhibited by nucleoside triphosphates, while the inhibition of the 3′-mononucleotidases apparently is of the "uncompetitive type"; moreover, when assayed in the presence of nucleoside triphosphates, the two 3′-nucleotidases show appreciable substrate inhibition (Figure 2).

In the experimental condition of the spectrophotometric assays described under "Enzyme Assay Procedure" no dephosphorylation of nucleoside tri- and diphosphates could be observed.

Effects of Divalent Cations and of EDTA (Table II). The inclusion of Co^{2+}, Ca^{2+}, and Mg^{2+} in the concentrations shown in Table II had no significant effect on *B. subtilis* nucleoside phosphomonoesterases, while 2 mM Zn^{2+} completely abolished the three activities. On the contrary inclusion of EDTA resulted in significant loss of the three activities.

Effects of Divalent Cations on ATP Inhibition (Table III). The inhibition of phosphomonoesterases by ATP was differently affected by inclusion of divalent cations. The addition of Mg^{2+}, Ca^{2+}, and Co^{2+} in the concentration shown resulted in reversal of inhibition exerted by 0.263 μM ATP on 5′-nucleotidase, while the inhibition exerted on the 3′-nucleotidases by 106 μM ATP was slightly affected.

5′-Mononucleotidase. The inhibition curves by nucleoside triphosphates (Figure 4) are asymptotic to finite values and apparently do not suggest interaction between inhibitor molecules; when the data were plotted according to Hill n'

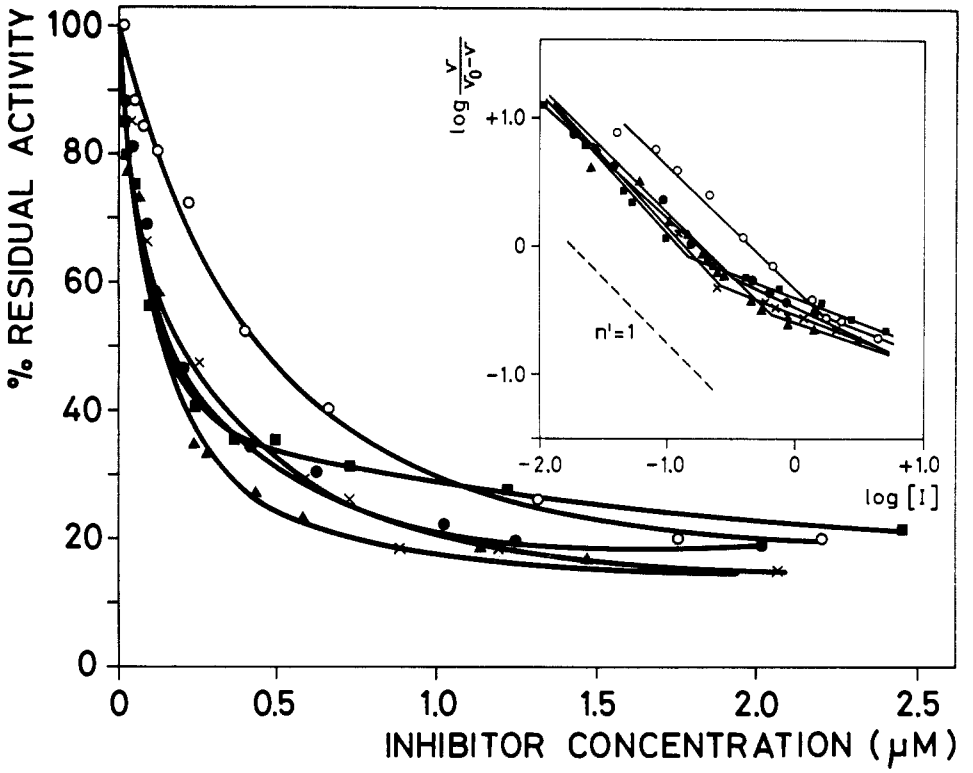

FIGURE 4: Per cent residual activity of 5′-nucleotidase of *B. subtilis* as a function of increasing ATP (●), CTP (▲), UTP (○), GTP (×), and ITP (■) concentrations. The final 5′-AMP concentration was 54 μM.

values of 1 were observed up to concentrations ranging between 0.1 and 0.45 μM for CTP, GTP, ATP, and ITP, and up to 1 μM for UTP; at higher inhibitor concentrations n' values considerably lower than 1 were observed suggesting a negative cooperative effect between inhibitor molecules, as first described by Conway and Koshland (1968) for the binding of NAD to 3′-phosphoglyceraldehyde dehydrogenase from rabbit muscle.

3′-Mononucleotidases. The inhibition curves by ATP, CTP, UTP, and GTP on 3′-nucleotidase A show sigmoidal shapes with n' values considerably higher than 1 (Figure 5), indicating a positive cooperativity between inhibitor molecules. Similar results were obtained with the 3′-nucleotidase B. Cooperativity between different inhibitor molecules is also

TABLE II: Effect of Divalent Cations and EDTA on Nucleoside Phosphomonoesterases of *B. subtilis*.[a]

Addition (mM)	% Residual Activity		
	5′-Nucleotidase	3′-Nucleotidase A	3′-Nucleotidase B
None	100	100	100
$MgSO_4$ (2)	108.5	114	9.87
$MgSO_4$ (6)	112.9	119.5	113.0
$MgCl_2$ (11)		108	
$MgCl_2$ (16)	114	108	98.0
$ZnCl_2$ (0.08)	61.7	78.2	69.5
$ZnCl_2$ (0.2)		32.6	
$ZnCl_2$ (2)	0	0	0
$CaCl_2$ (4.5)	93.6	110.8	87.8
$CaCl_2$ (13.5)	117	114.1	97.8
$CoCl_2$ (4)	106.4	89.1	86.9
$CoCl_2$ (12)	106.4	70.6	73.9
EDTA (1.5)	85.0	59.7	58.6
EDTA (4)	55.3	10.8	0

[a] Cations were added simultaneously with substrates at initiations of reactions. EDTA was included in the assay mixture, which was incubated for 5 min before AMP was added to 50 μM.

TABLE III: Effect of Mg^{2+}, Ca^{2+}, and Co^{2+} on ATP Inhibition of *B. subtilis* Nucleoside Phosphomonoesterases.[a]

Addition	% Residual Act. of 5′-Nucleotidase	% Residual Act. of 3′-Nucleotidase A	% Residual Act. of 3′-Nucleotidase B
None	100	100	100
ATP (0.263 μM)	35.8		
ATP (106 μM)		35.5	37
ATP + $MgSO_4$ (6 mM)	88.8	45.2	51
ATP + $CaCl_2$ (12 mM)	100	48.4	51
ATP + $CoCl_2$ (10 mM)	94.4	35.5	37

[a] 5′- and 3′-mononucleotidase activities were measured spectrophotometrically as described under Experimental Section. The final 5′- or 3′-AMP concentration was 50 μM.

TABLE IV: Cooperative Inhibition of 3′-Nucleotidases by ATP and CTP.[a]

	Concn (μM)			Predicted Inhibn
	ATP	CTP	Inhibn (%)	(%)
3′-Nucleotidase A	53	0	5.5	
	0	114	41	
	0	152	45	
	53	114	71	45
	53	152	100	48
3′-Nucleotidase B	53	0	16	
	106	0	61	
	0	76	35.5	
	0	114	47	
	53	114	74	56
	106	76	100	75

[a] The predicted inhibition is obtained from the product of the residual activities in the presence of each inhibitor alone, as described by Ipata (1968). The final 3′-AMP concentration was 50 μM.

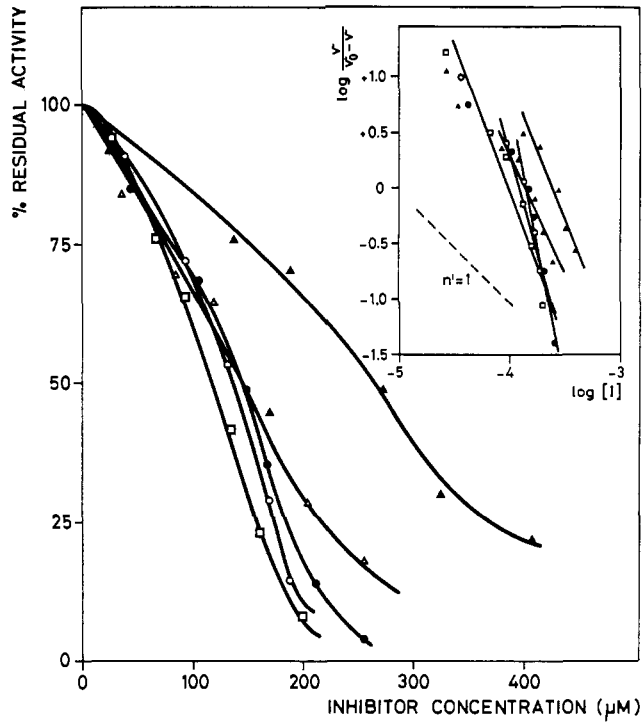

FIGURE 5: Effect of varying concentrations of ATP (□), CTP (●), UTP (▲), GTP (Δ) and ITP (○) on the initial velocity of the 3′-mononucleotidase A. The final 3′-AMP concentration was 56 μM. The velocity is expressed as per cent of the velocity in the absence of inhibitors.

shown by the results obtained with pairs of nucleoside triphosphates. When ATP and CTP were used together, the observed inhibition was higher than the sum of the individual inhibitions (Table IV).

Discussion

Bacterial enzyme activities hydrolyzing 3′- or 5′-mononucleotides have been first described in a large number of microorganisms including *Bacillus* and *Clostridium* by Kohn and Reis in 1963. Since then, 5′-mononucleotidases from *E. coli* and *Proteus* have been thoroughly studied (Neu and Heppel, 1964; Neu, 1967), while 3′-mononucleotidases have received scant attention.

The present study discloses for the first time that ATP and other nucleoside triphosphates are inhibitors of at least some bacterial 5′- and 3′-nucleotidases. However, the 3′-nucleotidases of *B. subtilis* are less sensitive to nucleoside triphosphates inhibition than 5′-nucleotidase of this organism, the concentration required for 50% inhibition being about 700-fold higher.

Extreme sensitivity to nucleoside triphosphates has been described for 5′-nucleotidases of mammalian sources. The nature of the observed inhibitions is different for the 5′-nucleotidases so far studied. Inhibition of 5′-nucleotidase from nerve tissue is formally of the mixed competitive and noncompetitive type and shows a strong positive cooperativity effect between inhibitor molecules (Ipata, 1967, 1968). 5′-Nucleotidases from heart muscle and tumor ascites on the other hand are competitively inhibited by nucleoside triphosphates and do not show cooperative effects (Murray and Friedrichs, 1969; Sullivan and Alpers, 1971).

Our results suggest that inhibition by nucleoside triphosphates may be a general property of 5′-nucleotidases from different sources. The extreme sensitivity of *B. subtilis* 5′-nucleotidase to nucleoside triphosphates resembles that of the nerve tissue enzyme, but no positive cooperativity is observed. As shown in Figure 4, n' values approaching 1 were obtained up to 0.1, 0.25, 0.3, 0.45, and 1 μM concentration for CTP, GTP, ATP, ITP, and UTP, respectively.

Our experimental results show that the 3′-nucleotidase activities found in *B. subtilis* differ for molecular weight and for pH optimum. However, they have similar inhibitory properties: (a) the inhibition by nucleoside triphosphates is formally of the uncompetitive type; (b) the inhibition curves show sigmoidal shapes, with n' values higher than 1 indicating a positive cooperative effect. The allosteric nature of the inhibition of the 3′-mononucleotidases is also supported by the cooperative inhibition by pairs of nucleoside triphosphates. This cooperative inhibition cannot be explained by interaction of nucleoside triphosphate molecules at a single binding site and indicates that two ribonucleotides can be bound simultaneously by the enzyme and that the binding of one inhibitor molecule increases the likelihood of binding of the second.

Similarly to 5′-nucleotidase from sheep brain (Ipata, 1968) and rat heart (Sullivan and Alpers, 1971), no effect of divalent cations on *B. subtilis* nucleoside phosphomonoesterase activities could be observed. However, divalent cations were effective in removing the inhibition exerted by nucleoside triphosphates on 5′-nucleotidase, but not that exerted on 3′-nucleotidases.

The different mode and sensitivity of control of nucleoside monophosphoesterases by nucleoside triphosphates in *B. subtilis* may have a potential role in the germination process.

It has been shown by Setlow and Kornberg (1970a,b) that in the dormant spore purine and pyrimidine bases, nucleosides and 5′-mononucleotides must be readily available for the "salvage" biosynthesis of nucleoside triphosphates for the RNA synthesis in the early stages of germination, since the synthetic *de novo* pathways are not complete in the dormant spore. The problem therefore arises about the possible sources

of these RNA precursors. We have recently studied the development of 5′- and 3′-nucleoside monophosphate producing phosphodiesterases and of nucleotidases during the sporulation period of *B. subtilis* and have found that in free spores the 3′-monophosphate producing phosphodiesterases and 5′-nucleotidase are absent (Felicioli *et al.*, 1972a,b). During bacterial growth the extreme sensitivity of 5′-nucleotidase to nucleoside triphosphates renders the 5′-mononucleotides directly available for nucleoside diphosphates and nucleoside triphosphates synthesis, avoiding their breakdown to degradation products. It must be emphasized in this regard that even the low concentrations of nucleoside triphosphates found in sporulating forms (Nelson and Kornberg, 1970) are still quite sufficient to inhibit 5′-nucleotidase, but not the 3′-nucleotidases. From a speculative point of view it can therefore be postulated that at nucleoside triphosphate concentrations present in the log growth phase both 3′- and 5′-nucleotidases might be inhibited; concomitantly with the dramatic fall of the nucleoside triphosphate concentrations occurring during the sporulating period the inhibition of 3′-nucleotidases but not that of 5′-nucleotidase could be removed, thus rendering all purine and pyrimidine rings available for the "salvage" synthesis of RNA precursors in the early germination periods. The differences between 3′- and 5′-nucleotidases in sensitivity to control by nucleoside triphosphates would therefore play a major role in allowing the efficient recycling of nucleoside monophosphates.

References

Baer, H. P., Drummond, G. I., and Duncan, L. (1966), *Mol. Pharmacol. 2*, 67.

Bosmann, H. B., and Pike, G. Z. (1971), *Biochim. Biophys. Acta 227*, 402.

Cohn, W. E. (1955), *Methods Enzymol. 3*, 740.

Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.

Drummond, G. I., and Yamamoto, M. (1971), *Enzymes 4*, 337.

Edwards, M. J., and Maguire, H. M. (1970), *Mol. Pharmacol. 6*, 641.

Felicioli, R. A., Ipata, P. L., Senesi, S., and Falcone, G. (1972b), *Biochim. Biophys. Acta 276*, 584.

Felicioli, R. A., Senesi, S., Ipata, P. L., Falcone, G. (1972a), *Ital. J. Biochem. 21*, 93.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Fritzson, P. (1967), *Eur. J. Biochem. 1*, 12.

Fritzson, P. (1971), *Biochim. Biophys. Acta 235*, 128.

Gibson, W. B., and Drummond, G. I. (1972), *Biochemistry 11*, 2.

Ipata, P. L. (1967), *Anal. Biochem. 20*, 30.

Ipata, P. L. (1968), *Biochemistry 7*, 507.

Kohn, J., and Reis, J. L. (1963), *J. Bacteriol. 86*, 713.

Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.

Møllering, H., and Bergmeyer, H. U., Ed. (1962), Methoden der Enzymatischen Analyse, Weinheim Verlag Chemie, p 491.

Murray, A. W., and Friedrichs, B. (1969), *Biochem. J. 111*, 83.

Nelson, D. L., and Kornberg, A. (1970), *J. Biol. Chem. 245*, 1137.

Neu, H. C. (1967), *J. Biol. Chem. 242*, 3905.

Neu, H. C., and Heppel, L. A. (1964), *Biochem. Biophys. Res. Commun. 17*, 215.

Setlow, P., and Kornberg, A. (1970a), *J. Biol. Chem. 245*, 3637.

Setlow, P., and Kornberg, A. (1970b), *J. Biol. Chem. 245*, 3645.

Sullivan, J. M., and Alpers, J. B. (1971), *J. Biol. Chem. 246*, 3057.

Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.