was taken to make $\Sigma_{x=1}{}^{x}F_x$ approach 99.5% of its theoretical limit.

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Properties of 5′-Nucleotidase from *Bacillus cereus* Obtained by Washing Intact Cells with Water†

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ABSTRACT: 5′-Nucleotidase activity can be detected in intact cells of *Bacillus cereus* and is released simply by washing with aqueous media; the release is partly dependent upon storage of cells at −20°, but is scarcely affected by the solute composition of the washing medium. The enzyme preparation obtained by washing cells catalyzes the dephosphorylation of various 5′-mononucleotides: IMP (K_m = 40 μM) is hydrolyzed 5 times faster than AMP (K_m = 4 μM); dAMP (K_m = 7.5 μM) is also dephosphorylated at a higher rate than AMP. The pyrophosphate linkage of ADP-glucose and NAD^+ is also hydrolyzed. ADP and ATP, which are not attacked by *B. cereus* 5′-nucleotidase, exert a strong inhibition on AMP and dAMP hydrolysis (K_i = 2 and 5 μM, respectively); other nucleoside triphosphates are also inhibitory. AMP hydrolysis catalyzed by intact cells of *B. subtilis* is also sensitive to ATP and ADP inhibition. The optimal pH for adenine nucleotide hydrolysis is around 8.4; divalent cations, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}, activate 5′-nucleotidase below pH 8, thus shifting the optimal pH to less alkaline values. Activation by Mg^{2+} and Ca^{2+} at pH 7.2 is abolished in the presence of ATP, whose inhibitory effect is strengthened by the divalent cation; on the other hand, ADP inhibition and divalent cation activation behave as independent effects at pH 7.2. The apparent surface location of the enzyme and its kinetic properties are interpreted as indicating its potential role in the uptake of extracellular nucleic acid material by chemical conversion into compounds which can penetrate the cell membrane, without affecting the cytoplasmic nucleotide pool.

During an investigation on the cell location of 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity in *Bacillus cereus* vegetative cells, we became aware of the fact that when harvested cells were repeatedly washed before use an appreciable loss of enzyme activity in cell homogenates occurred. The presence of 5′-nucleotidase activity in wash fluids was first observed in an attempt to apply the "osmotic shock" technique of Nossal and Heppel (1966); since *B. cereus* vegetative cells are insensitive to lysozyme (McDonald *et al.*, 1963; Felkner and Wyss, 1968), this method was originally thought to be the best to obtain information on the enzyme location.

The present work describes the variety of conditions under which 5′-nucleotidase is released from *B. cereus* cells upon washing in aqueous media, an almost unique property of this species. The kinetic properties of the enzyme preparation thus obtained are also reported. These include strong inhibition by ADP and ATP and activation by divalent cations. The kinetic

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data, in view of the apparent location of the enzyme, suggest, in our opinion, a potential role of 5′-nucleotidase in the utilization of extracellular nucleic acid material.

Experimental Procedure

Materials. Nucleotides were obtained from Sigma Chemical Co. Tris and *p*-nitrophenyl phosphate were purchased from Schuchardt. All other biochemicals were products of Boehringer and Soehne. Highly purified adenosine deaminase (6 mg) from calf intestinal mucosa (300 units/mg) was the kind gift of Dr. A. Lucacchini. All other chemicals were of reagent grade.

Growth of Cultures. The NCIB 8122 strain of *B. cereus* was used. The bacteria were maintained on nutrient agar slants. Cells were grown at 37° with vigorous shaking in a medium of the following composition: Nutrient Broth Difco, 8 g; yeast extract, 3 g; $MnSO_4 \cdot H_2O$, 0.1 g; H_2O, up to 1000 ml. Stationary phase cells (5 ml) were used as inoculum for 600 ml of medium in 2-l. flasks. Growth was followed by reading the optical density (OD) of culture samples at 625 nm. The other species and strains listed in Table III were grown under the same conditions. Cells were harvested by centrifugation in the cold at the beginning of the stationary phase, unless otherwise stated. When harvested cells had to be stored before use, they were kept at −20°.

Cell Washing Procedure. Intact cells were suspended at room temperature in H_2O with a rubber-coated glass rod and immediately centrifuged in the cold at 10,000 rpm for 10 min. The supernatant thus obtained is referred to in the text as "wash fluid" or simply "wash." Any variation in this procedure is described under Results. The wash fluids which showed the highest 5′-nucleotidase activity were pooled; such enzyme preparations were used for most of the kinetic and stability determinations.

Enzyme Assays. All enzyme assays were carried out at 37°. Any variation in the standard experimental conditions is described under Results. One enzyme unit is defined as the amount of enzyme which catalyzes the conversion of 1 μmol of substrate/min under the standard experimental conditions described herein. Specific activity is defined as units/milligram of protein.

Alkaline phosphatase was assayed at pH 8.1 in Tris-Cl buffer (0.05 M) using *p*-nitrophenyl phosphate as substrate (Garen and Levinthal, 1960; Malamy and Horecker, 1961). Aldolase was assayed according to Racker (1947). NADH oxidase was assayed spectrophotometrically following the oxidation of the pyridine coenzyme at 340 nm (Szulmajster and Schaeffer, 1961). 5′-Nucleotidase activity was assayed according to Ipata (1967) when adenine nucleotides were used as substrates; the standard reaction mixture contained, in a 1-cm light-path cuvet, Tris-Cl buffer (0.05 M, pH 8.1), AMP (0.04 mM) (OD at 265 nm, 0.56 ± 0.02), 0.3 unit of adenosine deaminase, and 0.005–0.01 unit of 5′-nucleotidase (contained in a maximal volume of 0.05 ml). The final volume was 2 ml. AMP was omitted in the reference cuvet. Dephosphorylation of ATP, glycerol 2-phosphate, and nonadenine nucleoside monophosphates was measured by the colorimetric determination of inorganic phosphate (Chen *et al.*, 1956). Hydrolysis of the pyrophosphate linkage of ADP, NAD^+, FAD, and ADP-glucose was assayed spectrophotometrically by substituting the opportune substrate for AMP and by adding 0.3 unit of alkaline phosphatase in the reaction mixture described for the 5′-nucleotidase assay. RNase activity was measured both by the spectrophotometric assay of Kunitz (1946) and the acid precipitation method (Klee and Richards, 1957).

Protein was determined according to Warburg and Christian (1941) or by the biuret reaction (Gornall and Hunter, 1943).

Preparation of Crude Extracts. B. cereus cells were suspended in twice their wet weight of Tris-Cl buffer (0.05 M, pH 7.2); glass beads (0.1 mm of average diameter), three times the cells' wet weight, were added to the suspension, which was cooled in an ice bath and eventually homogenized for 1 min in a Braun homogenizer. The homogenate was centrifuged in the cold at 39,000*g* for 15 min; the supernatant thus obtained was used for enzyme assays and is referred to in the text as "crude extract."

Culture Filtrates. For the determination of enzyme activites in the culture medium, samples from growing cultures were passed through a 0.45-μ Millipore filter to remove cells. Enzyme assays were performed using 0.2 ml of culture filtrate.

Disc Gel Electrophoresis. The procedure described by Gabriel (1971) was employed, using a 7.5% acrylamide gel system. Protein bands were revealed with a 1% solution of Coomassie Blue. 5′-Nucleotidase activity was located on the polyacrylamide gels according to Pilcher and Scott (1967).

Results

Enzyme Activities as a Function of Culture Growth. The amount of 5′-nucleotidase and alkaline phosphatase was determined at various times during growth in cell crude extracts and in culture filtrates. The results are summarized in Table I; the increase in 5′-nucleotidase activity, parallel to growth, can be contrasted with the behavior of alkaline phosphatase activity, which reaches a maximum in the late exponential phase and then declines sharply toward zero in the stationary phase. As a consequence, the ratio of the two enzyme activities (last column in Table I) shows nearly a 250-fold increase from expo-

TABLE I: 5′-Nucleotidase and Alkaline Phosphatase Activities of *B. cereus* NCIB 8122 as a Function of Culture Growth.[a]

Time (hr)	OD at 625 nm	A, 5′-Nucleotidase Act. in Crude Extracts	B, Alk. Phosphatase Act. in Crude Extracts	C, Alk. Phosphatase Act. in Culture Filtrates	A/B
0	0.02				
1	0.03				
2	0.16	0.056	0.258	0	0.217
2.3	0.27	0.092	0.300	0	0.306
2.75	0.64	0.120	0.380	0	0.315
3.1	1.40			0.280	
3.25	2.00	0.138	0.190	0.340	0.726
3.5	2.20			0.420	
4	3.20	0.160	0.060	0.405	2.667
4.6	4.50			0.402	
6	6.40	0.196	0.004	0.403	49.000

[a] Assays for 5′-nucleotidase and alkaline phosphatase were carried out, with AMP and *p*-nitrophenyl phosphate as substrates, as described under Experimental Procedure on crude extracts of cells harvested at time intervals during growth, and on culture filtrates. Cells were washed three times immediately after harvesting and homogenized after standing 7 days at −20°. All enzyme activities are expressed as units per gram of cells. 5′-Nucleotidase activity was always found to be absent in culture filtrates.

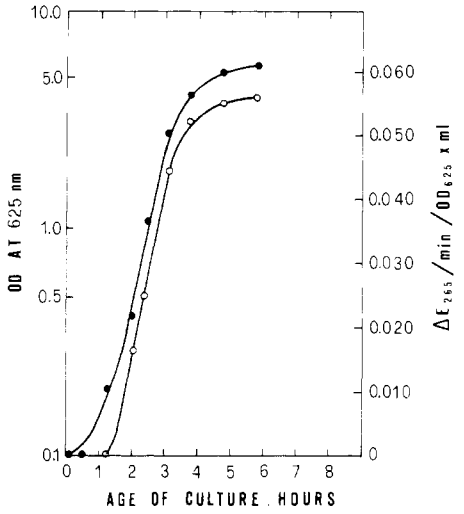

FIGURE 1: 5′-Nucleotidase activity of *B. cereus* NCIB 8122 as a function of culture growth, as assayed on dilute cell suspensions. The spectrophotometric assay was used: the enzyme preparation was substituted by a culture sample (final OD at 625 nm, 0.03–0.04). 5′-Nucleotidase activity was corrected for alkaline phosphatase activity and is expressed as the reaction rate (in arbitrary units) that would occur in the presence of 1 ml of a culture sample absorbing 1 OD unit at 625 nm: (●) OD at 625 nm; (○) 5′-nucleotidase activity.

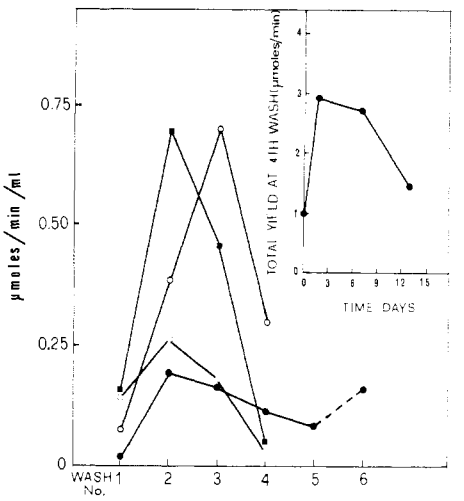

FIGURE 2: The release of 5′-nucleotidase activity from *B. cereus* cells by washing at different times of storage after cell harvesting. Cells were aged as 0.2-g aliquots; each aliquot was washed at least 4 times with 2 ml of H_2O each time. Enzyme activity was assayed by the spectrophotometric method and is expressed as units per milliliter: (●) cell washed immediately after harvesting; (●) the sixth wash, performed after 15 hr standing at 4°; (○) cells aged 2 days; (■) cells aged 7 days; (□) cells aged 13 days. The total units obtained from each cell aliquot by washing 4 times with H_2O are reported in the inset as a function of cell age.

nential to stationary phase cells. Alkaline phosphatase activity appears in the culture medium just when it begins to decline in cell crude extracts, and rapidly reaches a plateau. On the other hand, 5′-nucleotidase activity was never found to be excreted in the culture medium, although it could be assayed on dilute cell suspension. Figure 1 shows 5′-nucleotidase activity assayed on intact cells as a function of culture growth.

Elution of 5′-Nucleotidase from Intact Cells by Washing with H_2O; Effect of Repeated Washing. When *B. cereus* cells from a stationary phase culture are washed several times with constant volumes of distilled water, 5′-nucleotidase activity is found in the washing fluids after removing the cells by centrifugation. Table II shows the results of a typical series of washes; the highest enzyme yield and specific activity are found in the second and third washes. On the other hand, declining values of cell-adsorbed alkaline phosphatase activity are found in the first, second, and third washes. Aldolase and NADH oxidase, taken as controls of the release of intracellular enzymes, were always found to be absent. The crude extracts of 1 g of cells contained 1.2 units of aldolase and 0.826 unit of NADH oxidase.

If cells are ruptured after the fifth wash, a significant amount of 5′-nucleotidase is still found in the crude extract (Table II). However, if an equal amount of cells are ruptured before washing (or after a single wash, which comes almost to the same), they yield fewer enzyme units (nearly 50%) than the sum of five washes plus the crude extract of washed cells.

Aging of Cells. Optimal yields of 5′-nucleotidase were obtained by washing cells stored as a pellet at −20° for 2–7 days (inset in Figure 2). When cells were washed immediately after harvesting, the yield in enzyme units was poor and quite evenly distributed along several washes (Figure 2); indeed, standing at 4° for 15 hr increased the yield of the sixth wash over that of the fifth. If cells are aged over 7 days, enzyme preparations of lower activity are obtained by washing. The same decrease in 5′-nucleotidase activity was observed in crude extracts of unwashed cells.

Cell Dilution. The amount of enzyme released by a given amount of cells is proportional to the volume of H_2O in which they are suspended, up to 10 ml/g of cells; higher dilutions give less active wash fluids.

Duration of Washing Procedure. If cells are stirred in an ice-water slurry immediately after suspension (10%, w/v) and samples are withdrawn and centrifuged at time intervals, the washing fluids show no appreciable increase in 5′-nucleotidase activity.

Osmolarity of Washing Media. Washing cells in Tris-Cl buffer (pH 7.2, 0.01 M) plus NaCl (0.33 M) results in enzyme preparations with specific activities and enzyme units similar to those obtained by washing with distilled water. The effect of increasing concentrations of sucrose (from 5 to 40%, w/v) in the washing media was also investigated; it was found that the

TABLE II: Release of 5′-Nucleotidase and Alkaline Phosphatase from *B. cereus* Cells by Washing with H_2O.[a]

Fraction	Proteins (mg)	5′-Nucleotidase (Units)	Alk. Phosphatase (units)
1st wash fluid	1.00	0.637	0.081
2nd wash fluid	1.75	3.650	0.035
3rd wash fluid	0.95	2.520	0.020
4th wash fluid	1.10	1.190	0.000
5th wash fluid	0.70	0.279	0.000
Crude extract of washed cells	16.00	0.582	0.095

[a] Stationary phase cells (0.5 g, wet wt), stored for 3 days at −20°, were washed at 2° five times, each time with 5 ml of H_2O, as described under Experimental Procedure; washed cells were eventually ruptured and the crude extract was obtained by the described procedure. Assays were carried out as specified in Table I.

TABLE III: Comparison of Some Species and Strains of Spore-Forming Bacilli with Respect to Their Ability to Release 5′-Nucleotidase Activity by Washing with H_2O.[a]

Species	Strain	5′-Nucleotidase Release[b]
Bacillus cereus	NCIB 8122	100
	IP A 30	74
	IP Lille	61
	IP 5127	55
	IP 549	11.6
	IP 5257	10.2
	569 (Pollock)	10
Bacillus megatherium	ATCC 8245	8.5[c]
Bacillus subtilis	ATCC 6633	0
	SB 25 (from 168, Lederberg)	0
	SB 19 (23-X 168, Lederberg)	0
Bacillus stearothermophilus	T_2	0

[a] Cells were grown and harvested according to the described procedure; 0.2 g of cells (wet wt) from each culture, after 1 day of storage at −20°, was washed 3 times with 2 ml of H_2O each time. 5′-Nucleotidase activity was determined on second wash fluids by the spectrophotometric method; corrections were made for alkaline phosphatase activity at 0.04 mM *p*-nitrophenyl phosphate concentration. [b] Relative to that of *B. cereus* NCIB 8122 taken as 100. [c] The third wash gave no 5′-nucleotidase activity.

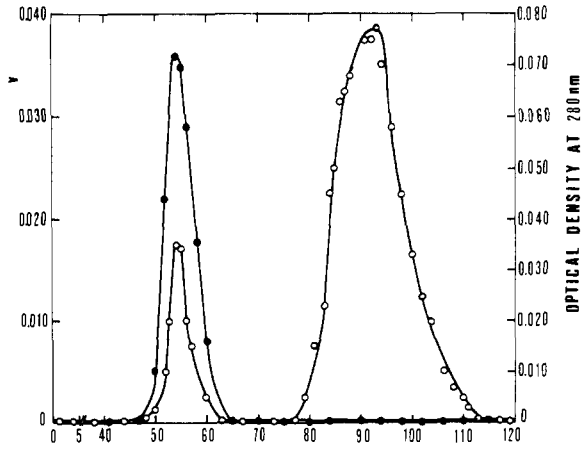

FIGURE 3: Elution pattern of 5′-nucleotidase of the combined second and third wash fluids of *B. cereus* grown for 6 hr. The combined wash fluids (15 ml) were concentrated by lyophilization to contain 0.950 mg of protein/ml. This solution (1.8 ml) was eluted from a Sephadex G-100 column (2.4 × 50 cm) equilibrated with 50 mM Tris-Cl buffer (pH 8.4), at a flow rate of 15 ml/hr. Fractions of 1.5 ml were collected; 0.250 ml of each fraction was used to measure enzyme activity: (●) 5′-nucleotidase activity; (○) optical density at 280 nm. The velocity is expressed at ΔA/minute.

yield in enzyme units is scarcely affected, if at all, by the osmolarity of washing media.

Comparison of Species and Strains. Among some aerobic spore-forming bacilli tested, the ability to release 5′-nucleotidase activity when suspended in aqueous media seemed to be an almost unique property of the *B. cereus* species; this is shown in Table III. Of all other species tested, only *B. megatherium* gave wash fluids endowed with 5′-nucleotidase activity, but the release was over after the second wash. The *B. cereus* strain under investigation (NCIB 8122) appears to be the best source of enzyme by the washing procedure.

Enzymological Properties of 5′-Nucleotidase. Since the enzyme preparation obtained by washing cells had a high specific activity (for instance, the specific activity of the third wash fluid was 331 *vs.* 2.65 for the crude extract) and was devoid of nonspecific phosphomonoesterase activity, kinetic studies were carried out without any further purification. However, a considerable increase of the specific activity of the wash fluids (up to four- to fivefold) could be achieved by fractionation on Sephadex gel filtration as described in Figure 3. In analogy with other 5′-nucleotidases from avian heart (Gibson and Drummond, 1972) and guinea pig skeletal muscle (Magni *et al.*, 1973), chromatography on DEAE Whatman DE 32 and solvent fractionation led to large losses in activity without purification. All efforts to improve the purification have been unsuccessful. Disc gel electrophoresis of the wash fluids revealed a major rapidly moving protein band and at least four minor protein bands. One of the latter did not penetrate the gel, and was associated with the 5′-nucleotidase activity.

Stability. The dilute (0.1 mg of protein/ml) aqueous enzyme preparation, when stored at −20°, showed a 55% loss of activity after 6 days; no further inactivation occurred up to 40 days of storage. A 70% loss of activity was observed over the same period of time when the enzyme was stored in a frozen state in Tris-Cl buffer (0.025 M, pH 8.1) at the same protein concentration. After a week at 4°, the residual activities recovered in dilute enzyme preparations (0.02 mg of protein/ml)kept at pH 6.2, 7.2, and 8.3 were 69, 59, and 56%, respectively; at pH 5, complete inactivation occurred within a few minutes. 5′-Nucleotidase is completely inactivated after 5 min at 37°.

Activation Energy. From the Arrhenius plot of initial reaction rates for AMP hydrolysis at pH 8.1 at various temperatures (ranging from 19 to 45°), an activation energy of 3.36 kcal/mol was evaluated.

Substrate Specificity. Various 5′-mononucleotides are dephosphorylated by the enzyme preparation. The substrate saturation curves follow Michaelis–Menten kinetics. In Table IV, the relative maximal rates of hydrolysis and the K_m values for seven mononucleotides at pH 8.1 are reported. Nucleotide pyrophosphatase activity toward NAD^+ and ADP-glucose is present in the enzyme preparation; FAD is hydrolyzed at a very low rate. The following compounds are not hydrolyzed: 3′-AMP, ADP, ATP, *p*-nitrophenyl phosphate, glycerol 2-phosphate, and RNA. The K_m for AMP at pH 7.2 was 2 μM. As can be noted, 5′-dAMP was hydrolyzed at a higher rate than 5′-AMP; GMP, IMP, CMP, and UMP acted as competitive inhibitors of AMP hydrolysis (Table IV).

Inhibitors. Nucleoside triphosphates were found to exert an inhibitory effect on 5′-nucleotidase activity, the most powerful being ATP; however, ADP was still a stronger inhibitor than ATP itself. In Table IV, K_i values (calculated from Dixon plots) for these two nucleotides are reported. Table V shows the effect of various nucleotides on 5′-nucleotidase activity at pH 8.1; at pH 7.2 the inhibitory effect of ADP and ATP was more pronounced. When ATP and ADP were present together in the reaction mixture, the residual activity measured nearly equalled the product of the residual activities observed in the presence of each adenine nucleotide tested separately. ATP inhibition is of the mixed type when AMP is used as substrate; with dAMP noncompetitive inhibition was observed. ADP, on the other hand, exerted noncompetitive inhibition on AMP hydrolysis (Figure 3).

TABLE IV: Substrate Specificity and Kinetic Constants of *B. cereus* 5′-Nucleotidase.[a]

Compound	V_{max}[b]	K_m (μM)	K_i (μM)[c]
5′-AMP	20.0	4.0	
5′-dAMP	44.0	7.5	
5′-GMP	55.0	16.7	12
5′-IMP	100.0	40.0	34
5′-XMP	44.0	125.0	
5′-CMP	43.0	16.7	9
5′-UMP	55.0	40.0	26
3′-AMP[d]	0.0		
ADP-glucose	4.5	3.3	
NAD^+[d]	5.1		
FAD[d]	0.5		
ADP[d]	0.0		2
ATP[e]	0.0		5
p-Nitrophenyl phosphate	0.0		
Glycerol 2-phosphate[e]	0.0		
Yeast RNA	0.0		

[a] Activity toward the various substrates was determined as described under Experimental Procedure. K_m and K_i values were evaluated from double-reciprocal plots and Dixon's plots, respectively. [b] Relative to that of 5′-IMP, taken as 100. [c] As inhibitors of AMP hydrolysis. [d] Maximal concentration tested, 0.1 mM. [e] Final concentration, 1 mM.

TABLE V: Inhibitory Effect of Various Nucleotides on 5′-Nucleotidase Activity.[a]

Addition	Concn (mM)	Residual Act. (%) Wash Fluids	Intact Cells
None		100	100
GTP	0.025	53	52
ITP	0.025	69	73
XTP	0.025	84	
CTP	0.025	52	57
UTP	0.025	70	
ATP	0.025	35	63
ADP	0.025	23	25
ADP	0.002	60	70
ATP + ADP	0.025 + 0.002	17[b]	45[c]

[a] The standard spectrophotometric assay at pH 8.1 was used; the final AMP concentration was always kept at 0.04 mM. [b] Calculated: 21. [c] Calculated: 44.1.

Dithiobis(nitrobenzoate) inhibited the enzyme activity 50% at 5 μM concentration; with PP_i and P_i the concentrations required to observe 50% inhibition were 3 and 0.1 M, respectively. 3′-AMP was not inhibitory up to 0.1 mM concentration. Inhibition by nucleoside di- and triphosphates could be observed also when the enzyme assay was carried out with intact cells grown for 6 hr, under the experimental conditions described in Figure 1 (Table V).

Effect of pH. Optimal enzyme activity occurs around pH 8.4 in Tris-Cl buffer (0.05 M), both with AMP and dAMP as substrates; 50% of the optimal activity is observed at pH 7.3, while at pH 5.5 (in histidine-Cl buffer, 0.05 M) the relative rate is 10% (histidine had no effect on enzyme activity). A very similar pH profile is found between pH 6.5 and 8.3 in 0.1 M sodium phosphate buffer.

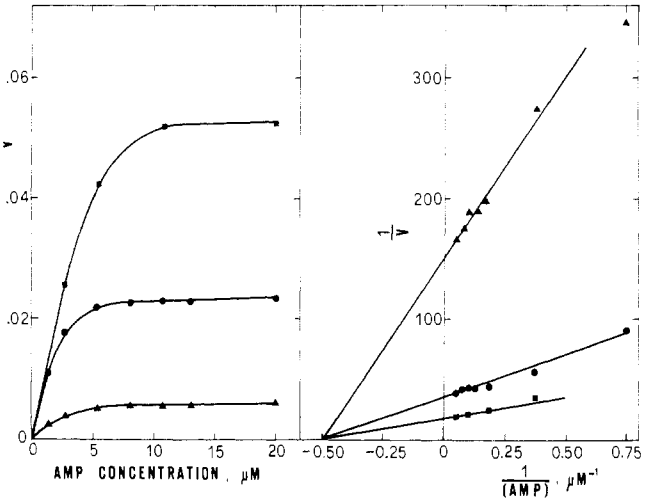

FIGURE 4: The effect of AMP concentration on the initial rate of AMP hydrolysis at pH 7.3 in the absence and in the presence of effectors: (left) direct plot; (right) double-reciprocal plot; (●) no addition; (■) $CaCl_2$ (7.5 mM); (▲) ADP (4 μM). The velocity is expressed as ΔA/minute.

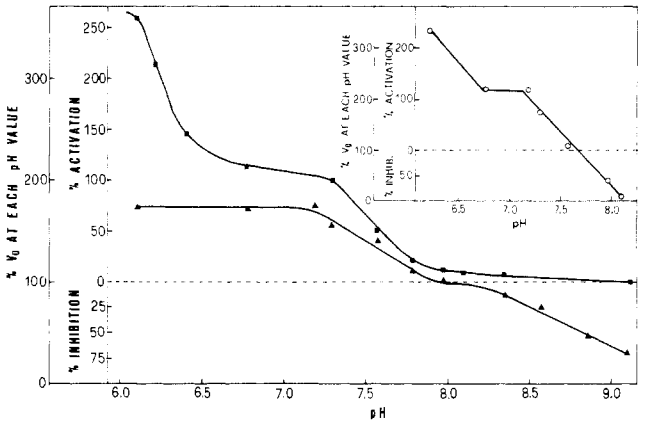

FIGURE 5: The pH dependence of the divalent cation effect on 5′-nucleotidase activity: (■) $CaCl_2$ (1.5 mM); (▲) $MgCl_2$ (1.5 mM); (○) inset, $MnSO_4$ (1.5 mM). For each pH value, the velocity in the presence of the divalent cation is reported as per cent of the velocity in the absence of it. The dotted line, therefore, represents the velocity in the absence of divalent cations at every pH value. The buffers used are: 0.05 M histidine-Cl from pH 6 to 7 and 0.05 M Tris-Cl from pH 7 to 9.1. The spectrophotometric assay was used, at a final AMP concentration of 0.04 mM. The velocity is expressed as ΔA/minute.

Effect of Metal Ions. Although no metal ion requirement was shown by 5′-nucleotidase for full activity at pH 8.1, activation by divalent cations was observed around neutrality; Ca^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}, in order of decreasing effectiveness, were found to accelerate AMP and dAMP hydrolysis between pH 6.0 and 7.5. As a consequence, the optimal pH is shifted from 8.4 to 7.7 in the presence of Ca^{2+} or Mg^{2+} and to pH 7.2 in the presence of Mn^{2+}. In Figure 4 the activating effect of Ca^{2+} at pH 7.2 is reported as a function of AMP concentration; the metal ion has no effect on the K_m. The half-maximal activation was reached at a $CaCl_2$ concentration of 0.25 mM. The pH dependence of the metal ion effect is shown in Figure 5; as can be seen, inhibition is observed in the presence of

TABLE VI: Effect of Magnesium on the Inhibition Exerted by ATP on *B. cereus* 5′-Nucleotidase.[a]

Addition	Rel Rate (%)	
	At pH 7.2	At pH 8.1
None	100	100
$MgCl_2$ (5 mM)	243	105
ATP (0.004 mM)	61	
ATP + $MgCl_2$	1.2	
ATP (0.010 mM)		62
ATP + $MgCl_2$		61

[a] The spectrophotometric assay was used; the final AMP concentration was 0.04 mM. The rate in the absence of effects is taken as 100 at each pH value. ATP hydrolysis in the presence of $MgCl_2$ was always checked and found to be zero.

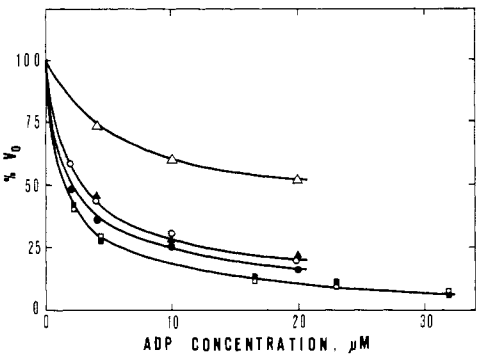

FIGURE 6: The effect of ADP concentration on the initial rate of the AMP hydrolysis catalyzed by *B. cereus* 5′-nucleotidase in the absence (closed symbols) and in the presence (open symbols) of divalent cations: (▲) pH 8.1; (●) pH 7.3; (■) pH 7.18; (△) + $MgCl_2$ 1 mM (pH 8.1); (○) + $MgCl_2$ (1 mM, pH 7.3); (□) + $CaCl_2$ (1 mM, pH 7.18). The final AMP concentration was 0.04 mM. The velocity is expressed for each curve as the per cent of the velocity in the absence of ADP at the pH value indicated and in the presence of the divalent cation used.

Mg^{2+} above pH 8.1 and in the presence of Mn^{2+} above pH 7.7. Ca^{2+} is not inhibitory up to pH 9.1. On the acidic side, activation by Ca^{2+} and Mn^{2+} shows a biphasic relationship, while the Mg^{2+} effect does not increase below pH 6.7. The pH dependence of the cobalt ion effect could not be studied in Tris-Cl and histidine-Cl buffers, since the metal ion forms complexes with the bases of the buffers (Neu, 1968b; Martin, 1964).

5′-Nucleotidase is inhibited by Cu^{2+}; 60% inhibition occurred with AMP as substrate at 0.25 mM metal ion concentration, at pH 7.2. At the same pH value, Zn^{2+} exerted an activating effect (50%) at 0.25 mM, while inhibiting at higher concentration (50% inhibition at 1 mM).

No potentiating effect was seen when two, three, or four activating metal ions were added together in the reaction mixture; thus, in the presence of Ca^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}, each at 1.5 mM concentration, the activation was the same as that observed in the presence of Ca^{2+} at 6 mM concentration, but was very much lower than the sum of the effects observed in the presence of each metal ion tested alone, each at 1.5 mM concentration.

Divalent cations showed a much lower activating effect on the ADP-glucose and NAD^+ pyrophosphatase activity present in the enzyme preparations.

Effect of Metal Ions on ADP and ATP Inhibition. Table VI shows the effect of $MgCl_2$ on ATP inhibition at pH 7.2 and 8.1. At pH 7.2, Mg^{2+} activation is completely abolished in the presence of ATP, even at high relative Mg^{2+} concentration; moreover, ATP inhibition is strengthened in the presence of Mg^{2+}; at pH 8.1, Mg^{2+} neither activates 5′-nucleotidase nor has any effect on ATP inhibition. With ADP as inhibitor, at pH 7.2, Mg^{2+} activates the hydrolysis of AMP and has practically no effect on ADP inhibition (Figure 5); at pH 8.1, a 250-fold excess of Mg^{2+} partly removes ADP inhibition (Figure 5).

Ca^{2+} exerts similar effects at pH 7.2; it enhances ATP inhibition, although to a lesser extent than Mg^{2+}, its activating effect being abolished in the presence of this nucleotide. In the presence of ADP, it still activates AMP hydrolysis, but has no intrinsic effect on ADP inhibition (Figure 6).

Discussion

The data reported in this paper represent a first characterization of 5′-nucleotidase activity obtained from *B. cereus*. The presence of such enzyme activity in this species was first noted by Neu (1968b), while the data of Kohn and Reis (1963) appear to be negative in this respect; this could be due to the peculiar extraction procedure used by these authors or to the fact that 5′-nucleotidase is readily released by *B. cereus* intact cells upon washing (Kohn and Reis washed cells in normal saline). The unusual behavior of this enzyme with respect to cell washing procedure was first observed by us in an attempt to find its cellular location. As the data reported above clearly show, *B. cereus* 5′-nucleotidase activity can be measured in intact cells, and the enzyme protein appears to be loosely bound to cells. As far as we know, the only instance described that could be compared with ours is that of RNA-inhibited DNase from *Escherichia coli* (Neu and Heppel, 1965; Nossal and Heppel, 1966), although the release of this enzyme is far more dependent upon the ionic content of the washing medium (Nossal and Heppel, 1966). The enzyme preparation obtained by washing cells with H_2O is moderately stable and is devoid of 3′-nucleotidase, RNase, and alkaline phosphatase activities. The wash fluid has a protein content similar to that of "shock fluids" obtained from *E. coli* (Neu and Heppel, 1965; Nossal and Heppel, 1966; Neu, 1967a); some material absorbing at 256–260 nm is also present in it.

The release of 5′-nucleotidase from intact *B. cereus* cells by washing in aqueous media can be regarded as a proof of its surface location; whether it can be considered a periplasmic enzyme is still questionable. Surely, it cannot be classified as an exo enzyme, since it is not excreted by the cells in the culture medium during growth. Attempts are currently being made to obtain protoplasts from *B. cereus* by the procedure of Strange and Dark (1957), but preliminary experiments seem to indicate that 5′-nucleotidase is partly inactivated during the incubation of cells in the presence of lytic enzymes. It can be noted here that all microbial 5′-nucleotidases so far investigated have a surface location (Neu and Heppel, 1965; Momose *et al.*, 1964; Mauck and Glaser, 1970; Neu, 1968b; Hayashi *et al.*, 1970; Schwencke *et al.*, 1971); in a few instances, 5′-nucleotidase activities have been described as exo enzymes in the genus *Bacillus* (Demain and Hendlin, 1967; Jacobsen and Rodwell, 1972).

Inhibition by nucleoside triphosphates and by ADP has been reported for 5′-nucleotidases of mammalian origin (Baer *et al.*, 1966; Burger and Lowenstein, 1967, 1970; Ipata, 1968; Murray and Friedrichs, 1969; Bosmann and Pike, 1971). Inhibition by nucleoside triphosphates on *B. subtilis* 5′-nucleotidase has been recently reported (Felicioli *et al.*, 1973). ATP and dATP also inhibit another degradative enzyme (RNase) from the

same microorganism (Yamasaki and Arima, 1967, 1969). These nucleotides are substrates for 5′-nucleotidases from Gram-negative bacteria (Neu, 1967a, 1968b; Hayashi *et al.*, 1970) and yeast (Takei *et al.*, 1969); the enzyme(s) from *B. subtilis*, on the other hand, does (do) not hydrolyze ATP (Felicioli *et al.*, 1973). The data reported herein show that also *B. cereus* 5′-nucleotidase is inactive toward ATP and ADP; besides, these compounds inhibit its activity toward 5′-mononucleotides. It is interesting to note that the 5′-nucleotidase activity of intact cells is also sensitive to nonpermeant nucleoside di- and triphosphates. In Gram-negative bacteria (Enterobacteriaceae), inhibition on 5′-nucleotidase activity was found to be exerted by a cytoplasmic protein (Dvorak *et al.*, 1966; Neu, 1967b, 1968a).

The metabolic role of 5′-nucleotidase in animal tissues and bacteria is far from being clearly established. Current theories on the functional role of 5′-nucleotidase and the periplasmic enzymes in bacteria have been recently discussed by Heppel (1971). For the enzyme from bacilli, the following facts should be considered. (1) It has been shown by Demain and Hendlin (1967) that *B. subtilis* growing cells excrete great amounts of RNA, whose partial degradation also produces extracellular nucleotides. Thus, nucleic acid material appears to be degraded outside the cytoplasm. (2) 5′-Nucleotidase from *B. cereus* and *B. subtilis* (Felicioli *et al.*, 1973) has low K_m values for naturally occurring mononucleotides; therefore, its activity within the cytoplasm would rapidly drain the nucleotide pool of the cell. In fact, this enzyme activity is apparently located at the very surface of the cell, at least outside the cytoplasmic membrane where it could aptly catalyze the dephosphorylation of extracellular mononucleotides (impermeable to the cell membrane) to nucleosides which can penetrate the cell and enter the salvage pathways for nucleotide biosynthesis. (3) The strong inhibition exerted by ADP and ATP on 5′-nucleotidase would hinder the degradation of mononucleotides within the cell, should the enzyme be present in the cytoplasm.

Further studies are required to establish the validity of this hypothesis and the functional role (if any) of the metal ion effect. From the enzymological point of view, it remains to be established whether the nucleotide pyrophosphatase activity found in our enzyme preparations is associated with the same protein which carries the 5′-nucleotidase activity, as was shown to be the case for other microbial enzymes (Neu, 1967a, 1968b; Takei *et al.*, 1969; Mauk and Glaser, 1970).

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