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A 5′-NUCLEOTIDASE FROM *NEUROSPORA CRASSA*

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SUMMARY

The properties of a 5′-nucleotidase (EC 3.1.3.5) which co-purified with the single-strand specific *Neurospora crassa* exonuclease have been studied. The nucleotidase had two pH optima, at pH 5.5 and pH 8.3. The acid and alkaline activities were not separated by polyacrylamide gel electrophoresis at pH 8.9 or at pH 5.5, or by isoelectric focusing, although the nucleotidase activities were clearly separated from the exonuclease in all three procedures. The two nucleotidase activities were equally sensitive to heat inactivation in 0.1 M Tris–HCl buffer at pH 8.6. The enzyme was active on all ribo- and deoxyribonucleoside 5′-monophosphates and on NAD, FAD, and ADP, but not on ATP. Low levels of ATP did not affect the nucleotidase activity with dTMP at pH 8.6. The alkaline activity was stimulated by amino acids, NH_4^+, and phosphate, while the acid activity was unaffected by amino acids and inhibited by phosphate.

INTRODUCTION

A 5′-nucleotidase (EC 3.1.3.5) was shown previously to co-purify with the single-strand specific exonuclease from *Neurospora crassa* conidia up to a final preparative polyacrylamide gel electrophoresis step, in which the two enzymes were separated [1]. We decided to study the properties of the nucleotidase for three reasons: (i) Although many nucleotidases have been characterized from bacteria and vertebrates (see review by Drummond and Yamamoto [2]), the only non-vertebrate eukaryotic nucleotidase that has been studied is that from a yeast, *Saccharomyces oviformis* [2]. (ii) There are a number of observations which suggest that 5′-nucleotidases may not be simple catabolic enzymes, but rather may be under some form of metabolic regulation. For instance, there is a specific protein inhibitor of *Escherichia coli* 5′-nucleotidase [3]; a *Bacillus subtilis* 5′-nucleotidase is inhibited by very low levels of ATP [4] (as are many mammalian nucleotidases [2]) and the activity of this enzyme decreases during sporulation, being completely absent in free spores [4]. We therefore wanted to see if there were any evidence for regulation of the *N. crassa* nucleotidase. (iii) Analysis of digests of denatured DNA with partially purified *N. crassa* exonuclease containing nucleotidase activity revealed that the nucleoside fraction contained

almost entirely deoxythymidine and deoxyguanosine and thus indicated that the nucleotidase might have an unusual base specificity (unpublished observations).

MATERIALS AND METHODS

Materials

Conidia of *N. crassa* (wild type strain ATC 9279) were custom grown by Miles Laboratories, Inc. [5]. Calf thymus DNA was from Worthington Biochemical Corp. [^{3}H]dTMP, [^{3}H]dAMP, and [^{3}H]dCMP were purchased from Schwarz/Mann. [^{14}C]ADP was purified from [^{14}C]ATP (Schwarz/Mann) by chromatography on QAE-Sephadex (Pharmacia). All other nucleotides and nucleosides were from the Sigma Chemical Co. The concentrations of all unlabelled nucleotide and nucleoside solutions were determined by ultraviolet absorbance. β-Glycerol phosphate was a product of Nutritional Biochemicals Corp.

Cyanogum 41 was purchased from Fisher Scientific Co. Thin-layer chromatography was carried out on Eastman Chromagram Sheets, No. 6064 cellulose (Eastman Kodak Co.) and on Polygram Cel 300 PEI (Brinkmann Instruments, Canada).

Preparation of the enzyme

Exonuclease was prepared from *N. crassa* conidia according to the short procedure of Rabin et al. [5]. It was dialyzed into, and stored in, 0.1 M Tris–HCl buffer, pH 7.5. This preparation, used in all of the experiments described in this paper, contained 170 units/ml of exonuclease and 67 units/ml of 5′-nucleotidase. This enzyme preparation did not release P_i from β-glycerol phosphate at pH 5.6 or pH 8.6, indicating that it did not contain any non-specific phosphatases.

Assay of 5′-nucleotidase

Unless otherwise indicated, all assays were performed in 0.1 M Tris–HCl buffer (pH 8.6) or in 0.1 M sodium acetate–acetic acid buffer (pH 5.6) in the presence of 0.01 M $MgCl_2$ at 37 °C. The extent of the reaction was monitored by one of two procedures: (1) P_i was measured colorimetrically as described by Ames and Dubin [6]; (2) Radioactive nucleoside was separated from the substrate by thin-layer chromatography. Nucleotide labelled with tritium in the base was used as substrate. At time points, 5-μl aliquots of the reaction mixture were spotted on cellulose thin-layer plates, on which had been previously spotted the appropriate nucleoside and nucleotide markers. Control experiments showed that no significant further reaction occurred on the plate. The chromatogram was then developed [7] in butanol–methanol–water–NH_4OH (60:20:20:1, by vol.) in which the nucleosides moved with R_F values of 0.4–0.6 while the nucleotides remained close to the origin. Regions containing the markers were scraped, the cellulose collected by suction into elution tubes [8], eluted into scintillation vials with two 400-μl aliquots of water, and counted in toluene–Triton X-100–water (6:3:1, by vol.) containing 4 g of PPO and 50 mg of POPOP per l in a Beckman LS-250 liquid scintillation spectrometer. When [^{14}C]ADP was used as substrate, 5-μl aliquots of the reaction mix were spotted on PEI-cellulose thin-layer plates previously spotted with ADP, AMP, and adenosine as markers. The plates were developed in 0.5 M LiCl, in which ADP, AMP, and adenosine can be resolved [7]. Regions containing the markers were cut out with scissors, placed in scintillation

vials, and counted in toluene–Omnifluor scintillation fluid. Rates were determined from the linear portion of the time-course curves. All straight lines were fitted to the data by the method of least squares. A unit of nucleotidase is defined as that amount which will hydrolyze 1 nmole of dTMP in 1 min in the standard assay.

Assay for pyrophosphatase

$Na_4P_2O_7$, at a concentration of 1 mM, was used as substrate, in a reaction mixture containing 0.1 M Tris–HCl buffer (pH 8.6) and 0.01 M $MgCl_2$. Aliquots of reaction mixture (150 μl) were incubated, with and without enzyme, at 37 °C in Beckman microfuge tubes. After 1 h, 150 μl of 0.05 M $MnCl_2$–0.5 M sodium acetate–acetic acid buffer (pH 5.0) was added to precipitate the unhydrolyzed pyrophosphate [9] and the tubes left on ice for 15 min. They were centrifuged in a Beckman microfuge for 1.5 min. Aliquots of the supernatant (200 μl) were assayed for P_i as previously described.

Disc gel electrophoresis

Nucleotidase preparations were subjected to disc gel electrophoresis according to the method of Davis [10]. For detection of enzyme activity, gels were sliced into 1-mm portions. Each slice was placed either in 200 μl 0.1 M sodium acetate–acetic acid buffer (pH 5.6) or, for alternate slices, in 200 μl 0.1 M Tris–HCl buffer (pH 8.6) and left in the cold overnight. Aliquots (5 μl) of each eluate were assayed in a total volume of 20 μl in the eluting buffer with [^{3}H]dTMP as substrate, for 30 min. Aliquots (30 μl) of each of the pH 8.6 eluates were assayed for exonuclease activity in a total volume of 90 μl as described by Mills and Fraser [11].

Isoelectric focusing

The method of Wrigley [12] was followed, except that the cathode was in the bottom reservoir in 0.2% H_2SO_4, and the anode was in the top reservoir in 0.4% triethanolamine. The gel was pre-run for 30 min, then 100 μl of enzyme solution containing 10 mg sucrose was introduced below the ampholyte layer. The electrophoresis was continued for 3 h more. A blank gel, subjected to the same manipulations as the experimental gel, was sliced and eluted into water for measurement of the pH gradient. The experimental gel was sliced, eluted, and assayed as described in the preceding paragraph.

RESULTS

Variation of 5′-nucleotidase activity with pH

The dependence of 5′-nucleotidase activity on pH, with dTMP as substrate, is shown in Fig. 1. There were two maxima, one at pH 5.5, the other at pH 8.3. When dAMP was the substrate (not shown), a similar profile was obtained, although the acid and alkaline activities were nearly equal. Glycine stimulated the reaction at alkaline pH: this point is discussed below.

The rate of reaction at pH 5.6 was linear for 10–20 min, after which it began to decline. At pH 8.6 there was a lag of 2–5 min, but then the reaction was linear for at least 90 min. At pH 8.6 the rate of reaction was proportional to enzyme concentration in the range 0.13–3.4 units/ml.

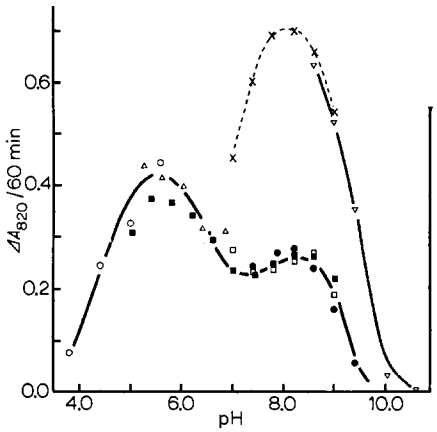

Fig. 1. pH dependence of 5′-nucleotidase activity. Reaction mixtures (1 ml) were incubated at 37 °C and 300-μl aliquots taken for phosphate assay 0 and 60 min after the addition of 5 μl enzyme. All mixtures were 0.01 M in $MgCl_2$. Those denoted by open symbols and crosses were 0.08 M in buffer at the indicated pH and 235 μM in TMP; those denoted by closed symbols were 0.1 M in buffer and 470 μM in TMP. ○, sodium acetate–acetic acid; △, sodium maleate–maleic acid; □, Tris–HCl; ▽, glycine–NaOH; ×, Tris–glycine; ●, glycylglycine–HCl; ■, Tris–maleate.

Attempts to separate the acid and alkaline activities

One explanation for the two pH optima could be the presence of two distinct nucleotidases in the preparation. To test this hypothesis, we attempted to separate the two activities. Fig. 2A shows the profiles of activity with dTMP at pH 5.6 and pH 8.6 and with denatured calf thymus DNA (exonuclease) after electrophoresis in a polyacrylamide gel at pH 8.9. The two 5′-nucleotidase activities with dTMP co-migrated, well separated from the exonuclease. Nucleotidase activities with dAMP and dCMP, assayed at pH 8.6, were optimal in the same fractions as those with dTMP. In a polyacrylamide gel run at pH 5.5 in 0.1 M sodium citrate–citric acid buffer (not shown), the acid and alkaline activities also failed to separate. The ratio of activities at pH 5.6 and pH 8.6 was the same in four different fractions spaced across the peak of nucleotidase activity. In an isoelectric focusing experiment (Fig. 2B), the acid and alkaline activities with dTMP again co-migrated; their pI was 5.0. The exonuclease showed a pI of 5.6.

Heat stability of 5′-nucleotidase activity

Fig. 3 shows the time courses of inactivation of the nucleotidase on heating at 50 °C in 0.1 M Tris–HCl buffer (pH 8.6). Both acid and alkaline activities were inactivated at approximately the same rate. In addition, both rates of inactivation were the same in the presence of 0.01 M Mg^{2+}. Substrate (50 μM dTMP) seemed to protect both activities during short incubations, but was rapidly degraded by the enzyme. Higher concentrations of dTMP were not used for protection because the deoxythymidine produced would have interfered with the subsequent assay (see below).

Substrate specificity

Table I shows the activity of the nucleotidase on various nucleotides and

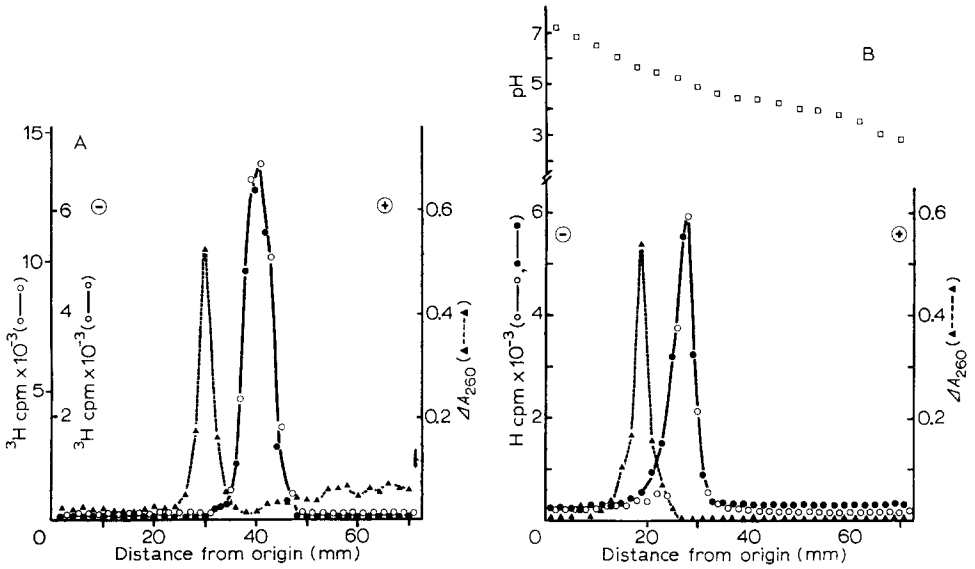

Fig. 2. (A) Disc gel electrophoresis of 5′-nucleotidase. A sample (100 μl) of enzyme solution was electrophoresed on a 7.5-cm polyacrylamide gel as described by Davis [10]. The gel was sliced, eluted, and assayed as described in Materials and Methods. The arrow indicates the position of the tracking dye. (B) Isoelectric focusing of 5′-nucleotidase. A sample (100 μl) of enzyme solution was subjected to isoelectric focusing in a pH 3–10 gradient as described by Wrigley [12]. Gels were sliced, eluted and assayed as described in Materials and Methods except that the pH 5.6 assay was for 15 min and the pH 8.6 assay for 30 min. Adjacent pairs of slices from a parallel, blank gel were eluted in 1 ml water and the pH determined. ○, 5′-nucleotidase assay with dTMP at pH 5.6; ●, 5′-nucleotidase assay with dTMP at pH 8.6; ▲, exonuclease; □, pH.

related compounds. At pH 8.6 the enzyme was active on all deoxyribo- and ribonucleoside 5′-monophosphates, and on ADP, FAD and NAD. There was a singificantly lower K_m for dTMP than for the other nucleotides, while V for dGMP was several-fold higher than for the other nucleotides. At pH 5.6, the nucleotidase was active on all 5′-deoxyribonucleotides and on the one 5′-ribonucleotide tested, 5′-AMP. At pH 5.6 also, the enzyme was active on ADP as well. There was no activity with 2′,3′-AMP, 2′,3′-GMP, or pyrophosphate at pH 8.6, or with ATP or β-glycerol phosphate at either pH.

The activity with ADP at pH 8.6 was investigated in more detail. When [^{14}C]ADP was used as substrate and the products of the nucleotidase reaction analyzed by thin-layer chromatography (see Materials and Methods), radioactivity was transferred quantitatively from ADP to adenosine, with no detectable transient increase in [^{14}C]AMP. When P_i was determined (with or without $MnCl_2$ precipitation in the presence of carrier pyrophosphate as described in Materials and Methods), with ADP as substrate, there was no difference in the amount of P_i released. Taken together with the observation that there was no pyrophosphatase activity in the preparation, this suggests that the phosphates were removed by the nucleotidase from ADP in a stepwise manner.

The failure to observe an increase in [^{14}C]AMP in the earlier experiment could be explained by the AMP not being released from the enzyme before the second

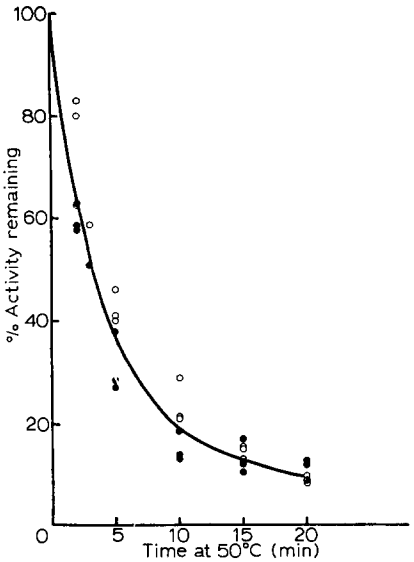

Fig. 3. Heat inactivation of 5′-nucleotidase. Enzyme (20 μl in a final volume of 100 μl) was incubated at 50 °C in 0.1 M Tris–HCl buffer (pH 8.6). At the indicated times, 5-μl aliquots were removed and diluted either with cold 0.1 M sodium acetate–acetic acid buffer (pH 5.6) or into 0.1 M Tris–HCl buffer (pH 8.6) and placed on ice. Each sample was then assayed with dTMP in the diluting buffer as described in Materials and Methods for gel eluates. ○, assayed at pH 5.6 for 10 min; ●, assayed at pH 8.6 for 20 min.

phosphate was removed. This possibility was tested using [^{14}C]ADP and unlabelled AMP as substrates. In this case, the adenosine, AMP, and ADP were separated on a column of QAE-Sephadex (see legend to Table II). The specific activities were determined from absorbance measurements ($A_{260\ nm}$) and scintillation counting after concentration. The results (Table II) indicate that most of the AMP formed by the removal of one phosphate from ADP is released from the enzyme to equilibrate with the pool of free AMP. Again, no buildup of [^{14}C]AMP was seen; it was apparently removed from the AMP pool at a faster rate than it entered, due to much faster dephosphorylation of AMP than of ADP.

Effect of amino acids and other compounds on activity at pH 8.6

Further experiments concentrated on the activity of the nucleotidase at pH 8.6. It was noted in the section on pH dependence that glycine activated the nucleotidase. Table III shows the increase in activity of the nucleotidase at pH 8.6 in the presence of various concentrations of glycine and L-histidine. NH_4Cl also stimulated the reaction, but less effectively than glycine or histidine. The stimulations by 0.05 M glycine and 0.08 M NH_4Cl were additive, but the same concentrations of glycine would not further stimulate the activity in the presence of 0.8 M NH_4Cl. Fig. 4 shows the effect of 0.02 M histidine on the kinetic parameters of the nucleotidase reaction. The K_m for dAMP was unaffected, while V was increased by a factor of 1.8. The pH profile (Fig. 1) shows that the alkaline pH optimum was not shifted in the presence of glycine. The same was true in the presence of histidine. Table IV shows that the

TABLE I

ACTIVITY OF *NEUROSPORA* NUCLEOTIDASE WITH VARIOUS SUBSTRATES

For measurements of relative activities, substrates at final concentrations of 0.5–0.7 mM (except FAD, 0.4 mM, and dCMP, 0.25 mM at pH 5.6), were incubated in 0.1 M glycine–NaOH buffer (pH 8.6) or 0.1 M sodium acetate–acetic acid buffer (pH 5.6) containing 0.01 M $MgCl_2$ and 5 μl of enzyme in a final volume of 1 ml. P_i was assayed colorimetrically as described in Materials and Methods and activities were calculated relative to activity with dTMP. K_m values and V values were determined in 0.1 M Tris–HCl buffer (pH 8.6) or 0.1 M sodium acetate–acetic acid buffer (pH 5.6) containing 0.01 M $MgCl_2$.

Substrate	pH 8.6			pH 5.6		
	Relative activity	K_m (μM)	V (nmoles/30 min for 1 μl enzyme)	Relative activity	K_m (μM)	V (nmoles/30 min for 1 μl enzyme)
5′-dTMP	1.0	15	2.7	1.0	20	3.6
5′-dAMP	0.80	125	2.5	0.92	57	2.0
5′-dCMP	0.89	150	2.5	0.74	95	5.0
5′-dGMP	1.7	83	9.9	1.2	25	7.5
5′-UMP	0.77					
5′-AMP	0.85	130		2.0		
5′-CMP	0.58					
5′-GMP	0.96					
5′-IMP	1.2					
2′,3′-GMP	<0.01*					
2′,3′-AMP	<0.01*					
ADP	0.32			0.25		
ATP	<0.06*			<0.06*		
β-glycerol phosphate	<0.004*			<0.007*		
$Na_4P_2O_7$	<0.015*					
FAD	0.29					
NAD	0.09					

* There was no detectable P_i produced. Numbers indicate the limit of detection.

TABLE II

ACTION OF 5′-NUCLEOTIDASE ON ADP

The reaction mixtures contained 0.1 M Tris–HCl buffer (pH 8.6), 0.01 M $MgCl_2$, 3 mM ADP (24 000 cpm of [^{14}C]ADP), 2.8 mM AMP and 30 μl of enzyme in a total volume of 330 μl. The mixtures were incubated at 37 °C for the indicated time, boiled for 1 min to stop the reaction, and applied to a 0.5 cm × 7 cm column of QAE-Sephadex. The column was washed with 2 ml water and 3 ml 0.001 M HCl; then a linear gradient of 0.001–0.05 M HCl (70 ml each) was applied. $A_{260\ nm}$ was read on each of the 2-ml fractions. Peaks were pooled and the μmoles of each compound determined from the $A_{260\ nm}$. The pooled fractions were evaporated to dryness, taken up in 700 μl water, and counted in toluene–Triton X-100 scintillation fluid.

Compound	Time of incubation (h)								
	0			1			3		
	nmoles	cpm	cpm/nmole	nmoles	cpm	cpm/nmole	nmoles	cpm	cpm/nmole
ADP	954	21 800	23	943	21 500	23	929	21 100	23
AMP	667	2 140	3.2	497	1 840	3.7	250	1 280	5.1
Adenosine	12	—	—	195	690	3.5	488	1 840	3.8

TABLE III

EFFECTS OF VARIOUS COMPOUNDS ON NUCLEOTIDASE ACTIVITY AT pH 8.6
The assays were performed either colorimetrically or by thin-layer chromatography as described in Materials and Methods, in 0.1 M Tris–HCl buffer (pH 8.6)–0.01 M $MgCl_2$.

Compound added to assay mix	Concentration (M)	Relative activity
No addition	—	1.0
Glycine[a]	0.002	1.0
	0.02	1.5
	0.05	2.1
	0.1	2.3
Histidine[a]	0.002	1.3
	0.02	2.4
	0.1	3.0
NH_4Cl[c]	0.08	1.5
	0.80	3.4
0.05 M glycine + NH_4Cl	0.08	2.6
	0.8	3.2
EDTA[b]	0.01	1.1
KCl[c]	0.1	0.90
NaCl[a]	0.1	0.92
K_2HPO_4–KH_2PO_4[c]	$2.5 \cdot 10^{-4}$	1.0
	$2.5 \cdot 10^{-3}$	2.0
	$2.5 \cdot 10^{-2}$	3.2
	$2.5 \cdot 10^{-1}$	2.1
ATP[a]	$6 \cdot 10^{-6}$	1.0
	$6 \cdot 10^{-5}$	0.90

[a] Colorimetric assay, 50 μM dTMP as substrate.
[b] Colorimetric assay, 440 μM dAMP as substrate.
[c] Radioactive assay, 50 μM [^{3}H]dTMP as substrate.

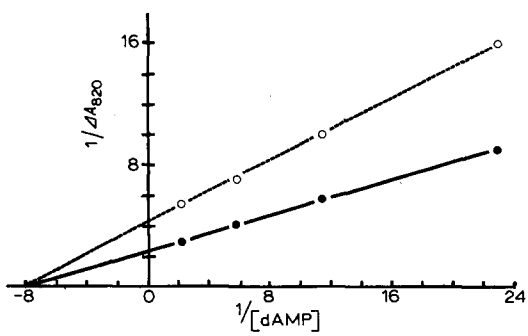

Fig. 4. Lineweaver–Burk plot showing the effect of histidine on the 5′-nucleotidase reaction. The reaction mixtures contained 0.1 M Tris–maleate buffer (pH 8.6), 0.01 M $MgCl_2$, and the indicated concentrations of dAMP, without and with 0.02 M histidine, in a total volume of 1 ml. 5 μl of enzyme solution was added to start the reaction, and at 0 and 30 min 0.3-ml aliquots were withdrawn for P_i assay. ○, no histidine, ●, 0.02 M histidine.

stimulation of activity at pH 8.6 by glycine was dependent on the presence of $MgCl_2$. In the absence of Mg^{2+}, the activities in Tris–HCl and glycine–NaOH buffers were the same.

It can also be seen from Table IV that there was a 35% decrease in activity in Tris–HCl buffer at pH 8.6 when Mg^{2+} was omitted from the reaction mixture, while

TABLE IV

EFFECT OF $MgCl_2$ ON NUCLEOTIDASE ACTIVITY

The amount of P_i produced in 1 h was measured colorimetrically, as described in Materials and Methods. Reaction mixtures contained the indicated buffer (0.1 M), and 50 μM dTMP, $\pm$0.01 M $MgCl_2$.

Buffer	$\Delta A_{820\ nm}$	
	$+Mg^{2+}$	$-Mg^{2+}$
0.1 M sodium acetate–acetic acid (pH 5.6)	0.346	0.348
0.1 M Tris–HCl (pH 8.6)	0.276	0.177
0.1 M glycine–NaOH (pH 8.6)	0.664	0.176

there was no effect on the activity at pH 5.6. When up to 0.01 M EDTA was included with 0.01 M $MgCl_2$ in the reaction mixture (Table III), the activity at pH 8.6 was not inhibited. This was explored further with enzyme that had been dialyzed overnight against two lots of 250 vol. of 1 mM EDTA in 0.1 M Tris–HCl buffer (pH 7.5) and then dialyzed three times against 250 vol. of 0.1 M Tris–HCl buffer (pH 7.5). The enzyme was assayed with dTMP as substrate in 0.1 M Tris–maleate (pH 8.6), with and without various divalent cations. Without divalent cation there was still some residual activity (about 40% of the activity of the undialyzed enzyme). Zn^{2+} (1 mM) completely abolished this activity, while 0.01 M Mg^{2+} restored activity to the level of the undialyzed enzyme. The activity in the presence of 0.01 M Co^{2+} was 125% of that with 0.01 M Mg^{2+}. The optimal concentration of Co^{2+} was about $5 \cdot 10^{-3}$ M. Mn^{2+} (0.01 M) was not as effective as Mg^{2+}, and Ca^{2+} (0.01 M) had a slightly inhibitory effect.

It may also be seen in Table III that 0.1 M KCl or NaCl had little effect on the nucleotidase activity. P_i stimulated the activity at pH 8.6; in contrast, 0.025 M phosphate inhibited the nucleotidase activity at pH 5.6 by 50%. Low concentrations of ATP had no effect on alkaline nucleotidase activity.

Inhibition by nucleosides

Fig. 5 shows the effect of added nucleosides on the dephosphorylation of dTMP and dAMP at pH 8.6. Thymidine and deoxyguanosine inhibited both reactions quite strongly, while deoxyadenosine and deoxycytidine were much less effective. Preliminary experiments at several different concentrations of dTMP (not shown) indicated that thymidine, deoxyguanosine, and deoxyadenosine inhibited the activity with dTMP competitively.

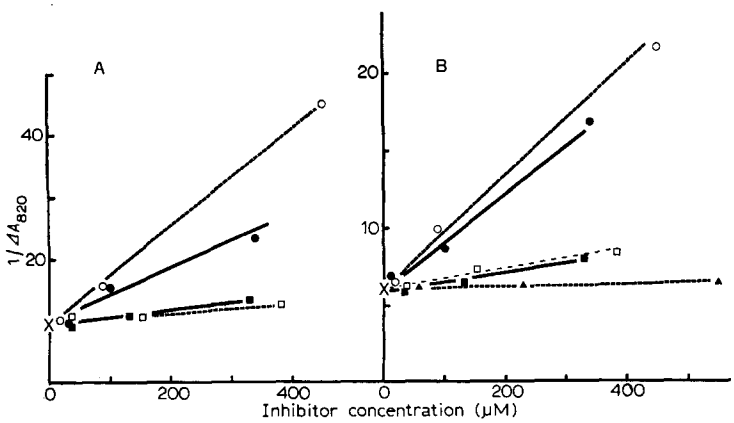

Fig. 5. Inhibition of 5′-nucleotidase activity by nucleosides. (A) Plot of $1/v$ versus [I] for dTMP as substrate. The 1-ml reaction mixture contained 0.1 M glycine–NaOH buffer (pH 8.6), 0.01 M $MgCl_2$, 0.1 mM dTMP, the indicated concentrations of nucleoside, and 5 μl of enzyme. P_i was determined after 15 min incubation. (B) Same as A except the substrate was 0.33 mM dAMP and P_i was determined after 30 min incubation. ○, thymidine; ●, deoxyguanosine; □, deoxyadenosine; ■, deoxycytidine; ▲, deoxyribose.

DISCUSSION

The double pH optimum of this 5′-nucleotidase preparation suggested that there might be two nucleotidases present. All attempts to separate the acid and alkaline activities failed. Electrophoresis at pH 8.9, pH 5.5, and in a pH gradient (isoelectric focusing) resulted in a single band of nucleotidase activity. Both activities were inactivated by heating, in the presence or absence of Mg^{2+}, at the same rate. The results suggest that both activities reside in the same protein, but our failure to separate the activities certainly cannot be taken as proof that there is only one enzyme; further purification and chemical studies are needed to decide this point.

Although no other nucleotidases have been reported to have more than one pH optimum for the same substrate, many show different pH optima with different substrates. Bovine cerebral cortex 5′-nucleotidase [13] and bull seminal plasma nucleotidase [14] both show a shift in the pH optimum in the presence of Mg^{2+}. The *E. coli* 5′-nucleotidase has pH optima of 5.7–6.1 for 5′-AMP, 6.7–7.1 for ATP, and 7–8 for UDPglucose [15]. Since a 5′-nucleotide has one less net negative charge below pH 6 (the pK for dTMP is 6.5), it could be considered as two different substrates at the acid and alkaline pH optima of the *Neurospora* 5′-nucleotidase.

Activation of nucleotidases by amino acids has been observed previously [16, 17]. Bodansky and Schwartz [16] proposed a mechanism for activation involving a complex of amino acid, nucleotide, and divalent cation. It is unlikely that this mechanism is operating here, as the K_m for the nucleotide is unaffected by the addition of amino acid. The activation by amino acids could be an example of general anionic activation, as suggested by Bjork [17]. On the other hand, NH_4^+ also activated, and the activations were additive at a low concentration of NH_4^+ and glycine, but not when the concentration of NH_4^+ was raised. This would suggest that the amino group was specifically involved. It should be noted that the concentration of amino acids

required for stimulation are much higher than those present in vivo: therefore, activation by amino acids is probably not physiologically important.

The K_m values and V values for the 5′-deoxyribonucleotides (Table I) predict that in a mixture of all four nucleotides, such as that produced by digestion of denatured DNA by the *N. crassa* exonuclease, deoxythymidine and deoxyguanosine would be produced preferentially by the *N. crassa* nucleotidase. This, then, explains the apparent base specificity noted earlier.

The analysis of the mode of action of the *N. crassa* 5′-nucleotidase on ADP indicates that most, if not all, of the AMP formed dissociates from the enzyme before the second phosphate is removed. This contrast with the observations of Mauck and Glaser [18] and Glaser et al. [19], who studied the action of *B.subtilis* and *E. coli* 5′-nucleotidases, respectively, on [*uracil*-^{14}C]UDPglucose. The enzyme hydrolyzed UDPglucose to glucose 1-phosphate and UMP, and then dephosphorylated the UMP, yielding uridine and P_i. When the reaction was carried out in the presence of added unlabelled UMP, the uridine formed had a higher specific activity than the UMP, indicating that the UMP formed from UDPglucose did not equilibrate with the unlabelled UMP pool. The reaction can be represented as follows:

$$E + R_1\text{–}P\text{–}P\text{–}R_2 \rightleftharpoons E\text{–}(R_1\text{–}P\text{–}P\text{–}R_2) \rightarrow \begin{matrix} E\text{–}(R_1\text{–}P) + P\text{–}R_2 \\ {}^{1}\swarrow \qquad \searrow^{2} \\ E + R_1\text{–}P \qquad E + R_1 + P_i \end{matrix}$$

where, in the case of UDPglucose, R_1 is uridine and R_2 is glucose; in the case of ADP, R_1 is adenosine and R_2 is –H. The results with the bacterial nucleotidases and UDP-glucose indicate little contribution from Pathway 1, while our results with *N. crassa* nucleotidase and ADP indicate a strong contribution from Pathway 1. It is possible that two different sites on the enzyme could be responsible for the removal of the first and second phosphates from ADP; or, a separate enzyme could remove the first phosphate. Such an enzyme would likely not, however, remove the second phosphate, since only one enzyme appears to be responsible for all observed phosphomono-esterase activities.

Known 5′-nucleotidases fall into two groups with respect to the variety of substrates attacked [2]. Mammalian nucleotidases are active only on ribo- and deoxyribonucleotides, while those from bacteria and yeast may also hydrolyze nucleotide di- and triphosphates, nucleoside diphosphate sugars, and various adenine dinucleotides such as NAD. The *N. crassa* nucleotidase activity at pH 8.6 described here belongs to the latter group, showing activity with ADP, FAD, and NAD, as well as with ribo- and deoxyribonucleotides. Only one other nucleotidase, an enzyme from *B. subtilis*, has been shown to have activity with ADP but not with ATP [20]. It, like the *Neurospora* nucleotidase, co-purified with an exonuclease specific for RNA, but the *B. subtilis* enzyme did not separate from the nuclease by acrylamide gel electrophoresis under conditions which clearly resolved the *N. crassa* exonuclease (a ribonuclease and a deoxyribonuclease) and nucleotidase (Fig. 2A).

Both acid [21] and alkaline [22–24] phosphomonoesterases from *Neurospora* have been well characterized. The acid phosphatase is inactive on dGMP and dTMP [21], while the nucleotidase described in the present work prefers dGMP and dTMP to dAMP and dCMP. One of the constitutive alkaline phosphatases is inactive with ADP [21], which is a substrate for the nucleotidase described here; thus, the

nucleotidase complements activities of the phosphatases. The stimulation of the alkaline nucleotidase activity by P_i facilitates detection of this enzyme in the presence of alkaline phosphatases, since the latter enzymes have K_i values for P_i of the order of 10^{-4} M [22, 24]. For instance, whole cells and crude extracts may be assayed for nucleotidase activity using radioactive substrates in the absence and presence of 0.05 M P_i. Preliminary experiments in our laboratory (Olson and Fraser, unpublished) indicate that one third of the total alkaline nucleotidase activity is released from whole mycelia in 0.1 M Tris–HCl buffer (pH 8.6)–0.01 M $MgCl_2$, while only 10% of the total phosphate-inhibited activity (alkaline phosphatase) is released under the same conditions (total activity was taken as that released by sonication). This indicates that the *Neurospora* nucleotidase may be a periplasmic enzyme, as has been shown for many hydrolytic enzymes in bacteria [25].

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