ENZYMATIC HYDROLYSIS OF CYCLIC NUCLEOSIDE MONOPHOSPHATE IN BARLEY SEEDLINGS[1]

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Abstract: The enzymatic hydrolysis of cyclic nucleoside monophosphate in barley seedlings (*Hordeum vulgare* L., cultivar Himalaya) was investigated. Multiple forms of enzyme activity toward adenosine-2′, 3′-cyclic monophosphate are observed. At least one of these is capable of catalyzing the hydrolysis of adenosine-3′, 5′-cyclic monophosphate. Adenosine is the sole product from the hydrolysis of cyclic AMP because of the presence of nucleotidase activity. Further studies on substrate specificity, sedimentation behavior, pH optimum, metal ion effect, dithiothreitol effect and Km values suggest that the hydrolysis of cyclic nucleoside monophosphate may be due to a nonspecific acid phosphatase.

INTRODUCTION

Adenosine-3′, 5′-cyclic monophosphate (3′, 5′-cyclic AMP) has been implicated as a second messenger in the actions of a variety of a variety hormones (Robison *et al.*, 1968). It is also known to be a mediator of the so-called "glucose effect" in bacteria (Pastan & Perlman, 1970). Although a relationship between this cyclic nucleotide and the action of plant hormones has been postulated (Duffus & Duffus, 1969; Galsky & Lippincott, 1969; Janistyn, 1972; Kamisaka & Masuda, 1970; Lin & Varner, 1972; Salomon & Mascarenhas, 1971), the study of cyclic AMP in higher plants is still at an early stage. Recently, pea cyclic nucleotide phosphodiesterase has been distinguished from nucleotidas, RNase, DNase and p-nitrophenyl phosphate phosphatase, and it has been suggested that the phosphodiesterase is important for sequence degradation of RNA (Lin & Varner, 1972). The fact that pea enzyme is capable of catalyzing the hydrolysis of 2′, 3′-cyclic AMP and 3′, 5′-cyclic AMP has been postulated to be due to a lack of substrate specificity. However, in contrast to pea enzyme, barley cyclic nucleotide phosphodiesteases seem to have nucleontidase activity. Several lines of evidence suggest that the hydrolysis of 2′, 3′-cyclic AMP and 3′, 5′-cyclic AMP is due to a nonspecific acid phosphatase. Some of the studies on the partially purified barley enzyme preparation are presented here.

MATERIALS AND METHODS

Source of Chemicals Nucleotide and nucleoside derivatives, and dithiothreitol were obtained from Sigma Co. Sucrose and ammonium sulfate (special enzyme grade) were purchased from Mann Research Laboratory. Cellulose powder MN300

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Abbreviations: 2′, 3′-cyclic AMP; 2′, 3′-cyclic GMP; 2′, 3′-cyclic UMP and 2′, 3′-cyclic CMP are adenosine, guanosine, uridine and cytidine-2′, 3′-cyclic monophosphate, respectively. 3′, 5′-cyclic AMP; 3′, 5′-cyclic GMP; 3′, 5′-cyclic UMP and 3′, 5′-cyclic CMP are adenosine, guanosine, uridine and cytidine-3′, 5′-cyclic monophosphate, respectively.

(Brinkmann) was obtained from Macherey and Nagel Co.. DEAE-cellulose ion exchanger was purchased from Gallard-Schlesinger Chemical Co. [^{3}H]-3′, 5′-cyclic AMP was obtained from Schwarz BioResearch and purified with a Dowex-50-H$^+$ chromatography (Lin & Varner, 1972). Phytic acid was purchased from Cal Biochem. All standard chemicals were reagent grade.

Growth of Barley Seedlings. Barley seeds (*Hordeum vulgare* L., cultivar Himalaya) were surface-sterilized for 20 minutes in 1% sodium hypochlorite, rinse with sterile distiled water several times, and planted in sterile petri-dishes containing moist sand. After germination at room temperature in the dark for one week, seedlings were removed and rinsed with distilled water.

Enzyme Activity Measurement. The activities of nucleotidase and phytase were assayed by measurement of inorganic phosphate (Pi) released from nucleoside monophosphate and phytic acid, respectively. With a total volume of 0.5 ml, the standard reaction mixture containded 0.1 M buffer (K-actate, pH 5.4 or Tris-acetate, pH 8.0) and 2 mM substrate. The reaction was conducted at 37° for 10 minutes and terminated by adding 0.05 ml of 55% cold TCA (trichloroacetic acid). After standing at 0° for 3 minutes, the precipitate was removed by centrifugation at 2,000 g for 10 minutes. The resulting supernatant was analyzed for P_i by the method of Fiske and Subbarow (1925). One unit of nucleotidase or phytase activity is defined as that amount of enzyme which causes the release of 0.1 μmole of P_i per 10 minutes under the assay conditions described above.

Cyclic nucleotide phosphodiesterase activity was assayed according to the method previously reported (Lin & Varner, 1972). It was not necessary to have additional nucleotidase in the reaction mixture because the present enzyme preparation contained sufficient nucleotidase activity. One unit of enzyme activity is defined as that amount of enzyme which causes an increase of 0.1 μmole of P_i released per hour of incubation at 37°.

RNase and DNase activities were assayed with high molecular weight yeast ribosomal RNA and denatured calf thymus DNA (heated at 100° for 10 minutes and followed by quick cooling) as substrate, respectively (Lin & Varner, 1972). One unit of enzyme activity is defined as that amount of enzyme causing an increase in 0.1 O. D. unit at 260 nm per 30 minutes at 37°. The concentration of protein was determined (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

Sucrose Density Gradient Centrifugation. A 5 to 25% linear sucrose density gradient was prepared (Martin & Ames, 1961). Centrifugation was routinely performed at 4° in a Beckman L-2 65 B ultracentrifuge with a swinging bucket rotor, SW 65 L Ti (Beckman). Upon completion of the run, 10-drop fractions were collected after needle puncture of the bottom of the tube. Assays of enzyme activity were carried out in alternate fractions through the gradient with two different substrates for each set of gradients.

End Product Analysis of the Hydrolysis of [^{3}H]-3′, 5′-Cyclic AMP. The standard reaction mixture containing 1 mM [^{3}H]-3′. 5′-cyclic AMP (4.8×10^6 cpm) and 40 μg of enzyme protein was incubated at 37° for a period of time. At each time, an aliquot of 0.005 ml of the reaction mixture was taken and spotted onto a cellulose-precoated thin-layer plate along with 0.3 μg each of 5′-AMP, 3′-AMP, adenosine, adenine, ADP, ATP and 3′, 5′-cyclic AMP as carriers. The chromatogram was developed with 2-propanol: 0.03 M NH_4HCO_3 pH 8.6 (3:1 v/v) at room temperature for 4 hours. The carriers were located with U. V. light, scraped off and eluted from the cellulose with 0.5 ml of distilled water in 100° for 20 minutes. After cen-

tifugation at 2,000 g for 10 minutes, the supernatnt was poured directly into a scintillation vial to which 15 ml of Bray's solution was added. The radioactivity was determined in a Beckman liquid scintillation spectrometer.

RESULTS

Enzyme Preparation. All procedures were performd at 4° or in ice bath unless otherwise specified. Routinely, 150 g of barley seedlings was homogenized with 200 ml of deionized water for 90 seconds in a Waring blender. The homogenate was squeezed through double-layered cheesecloth, and centrifuged at 4,000 g for 30 minutes. The resulting supernatant was brought to 50% saturation by slowly adding solid ammonium sulfate (29.5 g per 100 ml of filtrate). The solution was stirred for 1 hour, and the precipitate removed by centrifugation at 10,000 g for 20 minutes. The supernatant was then brought to 80% saturation with the addition of 19.7 g of solid ammonium sulfate per 100 ml of solution. After stirring and standing at 0° for at least 2 hours, the precipitate was collected by centrifugation at 10,000 g for 30 minutes and dissolved in 10 mM Tris-acetate, pH 8.0.

The resulting enzyme solution was dialyzed against 20 volumes of 10 mM Tris-acetate, pH 8.0, with constant agitation. After dialysis, the solution containing 140 mg of protein in a volume of 30 ml was applied to a DEAE-cellulose column (2.5×25 cm) pre-equilibrated with 1.0 liter of 10 mM Tris-acetate, pH 8.0. After loading, the column was washed with 200 ml of the buffer previously used for

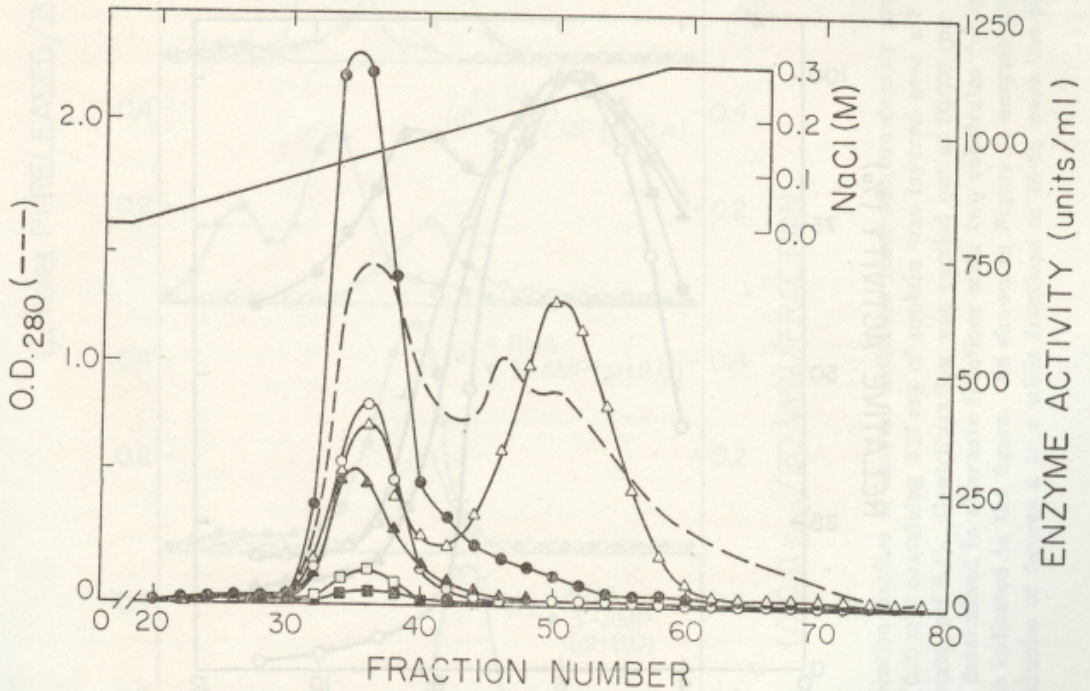

Fig. 1. Elution profiles of protein concentration and enzyme activities from DEAE-cellulose column chromatography. The solid line indicates the salt gradient of NaCl. Dashed line (----) represents the protein concentration as measured by the absorbance at 280 nm. Substrate and pH used for the assay of enzyme activities (units/ml): 3′-AMP, pH 8.0, ●—●; 3′-AMP, pH 5.4, ○—○; RNA, pH 5.4, △—△; DNA, pH 5.4, ▲—▲; 2′, 3′-cyclic AMP, pH 5.4, □—□ and 3′, 5′-cyclic AMP, pH 5.4, ■—■.

equilibration. Proteins were eluted in 6-ml fractions with a linear gradient of NaCl solution, 0.0 to 0.3 M NaCl in 10 mM Tris-acetate, pH 8.0. The elution profiles of protein and enzyme activities are shown in Figure 1. Fractions, from 33 to 39, were combined, dialyzed against 20 volumes of 10 mM Tris-acetate, pH 8.0, and used for the entire experiment. Compared to the activity in the crude homogenate, the enzyme activities (after DEAE-cellulose columm chromatography) toward 3′-AMP, 2′, 3′-cyclic AMP, 3′, 5′-cyclic AMP show 20-fold purification with 65% yield.

Hydrolysis of [^3H]-3′, 5′-Cyclic AMP. During incubation with partially purified enzyme preparation, [^{3}H]-3′, 5′-cyclic AMP is converted to [^{3}H]-adenosine. Although [^{3}H]-3′-AMP is accumulated to some extent during the first 15 minutes of incubation, it is not clear wheter 3′-AMP or 5′-AMP is the actual intermediate product in the hydrolysis of 3′, 5′-cyclic AMP. There is no detectable adenine, ADP, ATP, IMP or 5′-AMP. Heated enzyme preparation (100° for 10 minutes) lacks hydrolytic ability completely.

Multiple Forms of Enzyme. The rate of hydrolysis as a function of pH was studied. As shown in Figure 2, the enzyme has an optimal pH around 5.7 for the hydrolyses of 3′-CMP, 2′, 3′-cyclic AMP and 3′, 5′-cyclic AMP. However, with 3′-AMP as substrate, two pH optima (5.7 and 8.2) are observed. This suggests that at least two nucleotidase activies are involved in the hydrolysis of 3′-AMP. An attempt to separate these activities by gel filtration on Sephadex G-100 is unsuccessful. However, sucrose density gradient centrifugations, as shown in Figure 3, resolve three peaks with nucleotidase activity, also having activity toward 2′, 3′-

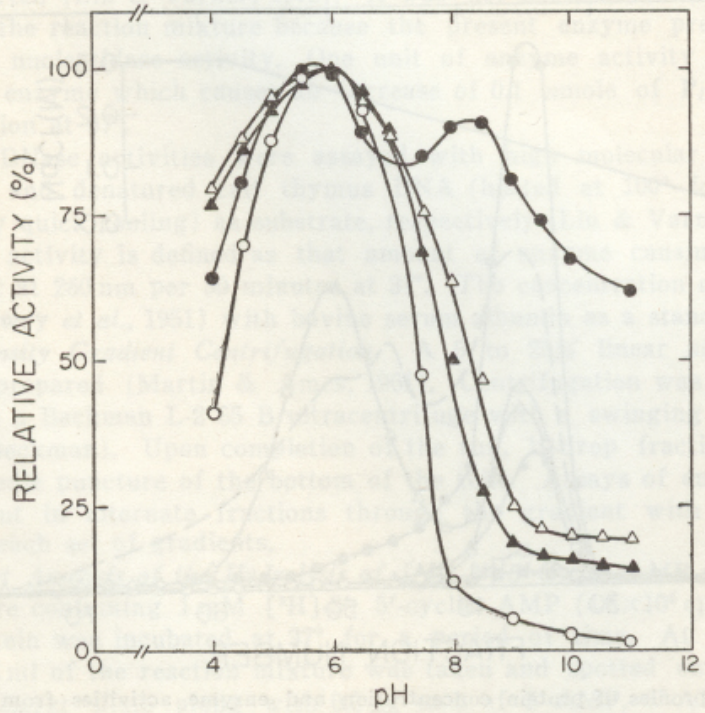

Fig. 2. Effect of pH on the enzyme activity. The reaction mixtur and experimental procedures were as described for the standard assay. 0.1 M K-acetate was used for pH 4–6 and 0.1 M Tris-acetate was used for pH 6–11. Substrate used: 3′AMP, ●—●; 3′-CMP, ○—○; 2′, 3′-cyclic AMP, △—△; 3′.5′-cyclic AMP ▲—▲.

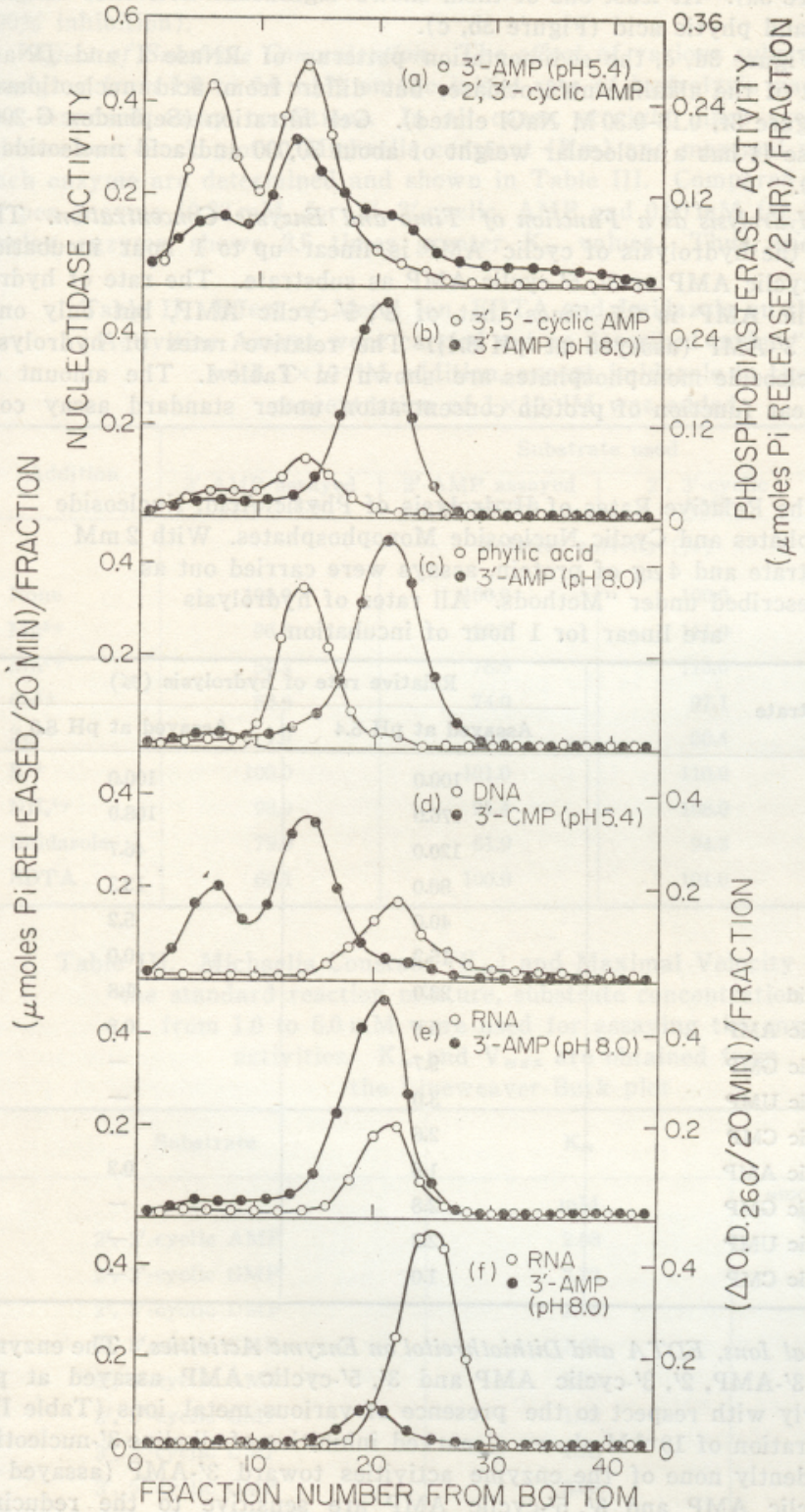

Fig. 3. The elution profiles of enzyme activities from sucrose density gradient centrifugation. A partially purified enzyme preparation (0.25 ml) containing 0.25 mg of protein was layered over a 5 to 25% sucrose density gradient (4.6 ml, prepared in 0.1 M K-acetate, pH 5.4). Centrifugation was carried out at 60,000 rpm for 20 hours as described under "Methods." Enzyme activities were determined in alternate fractions with two substrates for each gradient as shown in the figure. The pH used for assay is also indicated in the figure. As shown in Figure 1, enzyme preparation from fractions of 33-39 was used for the centrifugation studies of figures a to e whils fractions of 46-56 were use for the figure f.

cyclic AMP (Figure 3a). At least one of them shows significant activities toward 3′, 5′-cyclic AMP and phytic acid (Figure 3b, c).

As shown in Figure 3d, e, the sedimentation patterns of RNase I and DNase are similar to that of the alkaline nucleotidase, but differ from acid nucleotidases and RNase II (Figure 3f, 0.15–0.30 M NaCl eluted). Gel filtration (Sephadex G-200) studies show RNase II has a molecular weight of about 30,000 and acid nucleotidase II has about 70,000.

The Rate of Hydrolysis as a Function of Time and Enzyme Concentration. The progress curve of the hydrolysis of cyclic AMP is linear up to 1 hour incubation with either 2′, 3′-cyclic AMP or 3′, 5′-cyclic AMP as substrate. The rate of hydroloysis of 2′, 3′-cyclic AMP is 3.5 times that of 3′, 5′-cyclic AMP, but only one-eighteenth that of 3′-AMP (assayed at pH 5.4). The relative rates of hydrolysis of phytic acid, nucleoside monophosphates are shown in Table I. The amount of hydrolysis is a linear function of protein concentration, under standard assay conditions.

Table I. The Relative Rates of Hydrolysis of Phytic Acid, Nucleoside Monophosphates and Cyclic Nucleoside Monophosphates. With 2 mM substrate and 4 μg of protein, assays were carried out as described under "Methods." All rates of hydrolysis are linear for 1 hour of incubation

Substrate	Relative rate of hydrolysis (%)	
	Assayed at pH 5.4	Assayed at pH 8.0
3′-AMP	100.0	100.0
3′-GMP	176.0	108.0
3′-UMP	120.0	46.7
3′-CMP	80.0	13.3
5′-AMP	40.0	5.2
2′-AMP	15.3	0.0
Phytic acid	23.0	1.8
2′, 3′-cyclic AMP	5.6	0.8
2′, 3′-cyclic GMP	9.7	—
2′, 3′-cyclic UMP	3.0	—
2′, 3′-cyclic CMP	2.6	—
3′, 5′-cyclic AMP	1.6	0.2
3′, 5′-cyclic GMP	2.8	—
3′, 5′-cyclic UMP	2.3	—
3′, 5′-cyclic CMP	1.0	—

Effects of Metal Ions, EDTA and Dithiothreitol on Enzyme Activities. The enzyme activities toward 3′-AMP, 2′, 3′-cyclic AMP and 3′, 5′-cyclic AMP assayed at pH 5.4 behave similarly with respect to the presence of various metal ions (Table II). Zn^{2+} at a concentration of 10^{-2} M shows a marked inhibition of alkaline 3′-nucleotidase activity. Evidently none of the enzyme activities toward 3′-AMP (assayed at pH 5.4), 2′, 3′-cyclic AMP and 3′, 5′-cyclic AMP are sensitive to the reducing

reagent (2×10^{-3} M dithiothreitol) which inhibits the alkaline nucleotidase activity (90% inhibition).

Effect of Substrate Concentration. The effect of various substrate concentrations (ranging from 1.0 to 6.0 mM) on the initial rate of hydrolysis was determined under the standard assay conditions. In all cases, straight lines are obtained for the Linewe-aver-Burk plots. Michaelis constant (Km) and maximal velocity (V_{max}) for each enzyme are determined and shown in Table III. Compared to the K_m values of pea enzyme (0.83 mM for 2′, 3′-cyclic AMP and 0.90 mM for 3′, 5′-cyclic AMP), barley enzyme shows 2.5 times greater K_m values. Thus, the affinity constant

Table II. Effect of Metal Ion, EDTA and Imidazole on the Enzyme Activities Assays were carried out as described under "Methods" with 1×10^{-2}M addition, except imidazole in the concentration of 1×10^{-1}M was added.

Addition	Substrate used			
	3′-AMP assayed at pH 8.0	3′-AMP assayed at pH 5.4	2′, 3′-cyclic AMP	3′, 5′-cyclic AMP
	Relative activity (%)			
None	100.0	100.0	100.0	100.0
Mg^{2+}	96.6	96.7	111.0	101.0
Mn^{2+}	54.4	78.5	113.0	114.0
Co^{2+}	53.8	74.0	97.1	105.0
Zn^{2+}	1.9	50.1	60.4	66.7
K^{1+}	105.0	101.0	110.0	94.1
NH_4^{1+}	98.9	91.4	108.0	90.2
Imidazole	79.9	61.9	94.3	100.0
EDTA	69.1	100.0	101.0	95.1

Table III. Michaelis Constant (K_m) and Maximal Velocity (V_{max}) with the standard reaction mixture, substrate concentration ranged from 1.0 to 6.0 mM were used for assaying the enzyme activities. K_m and V_{max} are obtained from the Lineweaver-Burk plot

Substrate	K_m	V_{max}
	mM	μmole P_i released/hr./ 40 μg of protein
2′, 3′-cyclic AMP	2.08	0.48
2′, 3′-cyclic GMP	2.22	0.83
2′, 3′-cyclic UMP	2.78	0.31
2′, 3′-cyclic CMP	1.43	0.19
3′, 5′-cyclic AMP	2.32	0.13
3′, 5′-cyclic GMP	1.92	0.29
3′, 5′-cyclic UMP	1.78	0.20
3′, 5′-cyclic CMP	2.00	0.08

Substrate	K_m	V_{max}
	mM	μmole P_i released/10 min./6.5 μg of protein
Assayed at pH 5.4:		
3′-AMP	1.25	0.38
3′-GMP	11.10	3.30
3′-UMP	3.70	0.86
3′-CMP	0.83	0.28
Assayed at pH 8.0:		
3′-AMP	0.27	0.33
3′-GMP	6.67	1.47
3′-UMP	7.70	0.71
3′-CMP	5.56	0.11

$(1/K_m)$ for cyclic nucleoside monophosphate to the pea enzyme is 2.5 times greater than that to the barley enzyme.

DISCUSSION

Multiple enzyme activities toward cyclic nucleoside monophosphates and nucleoside monophosphates are found in the partially purified enzyme preparation from barley seedlings. These activities are separable in sucrose density gradient. Three peaks with nucleotidase activity show apparent activity toward 2′, 3′-cyclic AMP while one of them has activity to hydrolyze 3′, 5′-cyclic AMP and phytic acid. Studies on pH optimum and sedimentation benavior indicate at least two acid nucleotidases and one alkaline nucleotidase are present in the enzyme preparation. Further studies on substrate specificity, K_m values and sedimentation pattern in sucrose density gradient suggest acid nucleotidase II with activities toward 2′, 3′-cyclic AMP, 3′, 5′-cyclic AMP and phytic acid is a nonspecific phosphatase. Further purification and characterization is necessary in order to know whether or not these activities are due to a single protein molecule. In the meantime, caution should be taken in interpreting the results from the studies on the effects of plant hormones on the level of the hydrolytic activity toward 3′, 5′-cyclic AMP, especially in the crude extract of plant tissue. The change in the activity of hydrolysis of 3′, 5′-cyclic AMP may be due mainly to a change of a nonspecific phosphatase activity in the barley system, even if there is any specific cyclic nucleotide phosphodiesterase.

Although adenosine is the sole product from the hydrolysis of cyclic AMP under the standard assay conditions, apparently cyclic AMP is first hydrolyzed by a cyclic nucleotide phosphodiesterase, and then further degraded to adenosine by a nucleotidase. However, the present studies suggest that barley 3′-nucleotidase may have cycleotide phosphodisterase activity. Kinetically, barley enzyme is differedt from the corresponding enzymes isolated from *Escherichia coli* B and *Proteus mirabilis* as studied by Anraku (1964) and by Center *et al.* (1968), respectively. It is not clear whether 3′-AMP or 5′-AMP is the actual intermediate product from the hydrolysis of 3′, 5′-cyclic AMP in barley seedling. In the pea system (Lin & Varner, 1972), 3′-AMP is the exclusive product from the hydrolysis of 2′, 3′-cyclic AMP and is the major product from 3′, 5′-cyclc AMP.

Like the enzyme in pea seedlings, the activity toward 2′, 3′-cyclic nucleoside monophosphate of barley enzyme may be important for sequence degradation of RNA (Barnard, 1969; Lin & Varner, 1972). Attempts at separating nucleotidase and cyclic nucleotide phosphodiesterase are presently under study.

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