Higher-Plant Cyclic Nucleotide Phosphodiesterases

RESOLUTION, PARTIAL PURIFICATION AND PROPERTIES OF THREE PHOSPHODIESTERASES FROM POTATO TUBER

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1. Three phosphodiesterases that are capable of hydrolysing 3′:5′-cyclic nucleotides were purified from potato tubers. 2. The phosphodiesterases were fractionated by $(NH_4)_2SO_4$ precipitation and CM-cellulose chromatography. The phosphodiesterases were resolved from each other and further purified by gel filtration in high- and low-ionic-strength conditions. 3. All three enzymes lacked significant nucleotidase activity. 4. Enzymes I and II had mol.wts. 240000 and 80000 respectively, determined by gel filtration, whereas enzyme III showed anomalous behaviour on gel filtration, behaving as a high- or low-molecular-weight protein in high- or low-ionic-strength buffers respectively. 5. All enzymes hydrolysed 2′:3′-cyclic nucleotides as well as 3′:5′-cyclic nucleotides. The enzymes also had nucleotide pyrophosphatase activity, hydrolysing NAD^+ and UDP-glucose to various extents. Enzymes I and II hydrolyse cyclic nucleotides at a greater rate than NAD^+, whereas enzyme III hydrolyses NAD^+ at a much greater rate than cyclic nucleotides. All three enzymes hydrolysed the artificial substrate bis-(*p*-nitrophenyl) phosphate. 6. The enzymes do not require the addition of bivalent cations for activity. 7. Both enzymes I and II have optimum activity at pH6 with 3′:5′-cyclic AMP and bis-(*p*-nitrophenyl) phosphate as substrates. The products of 3′:5′-cyclic AMP hydrolysis were 3′-AMP and 5′-AMP, the ratio of the two products being different for each enzyme and varying with pH. 8. Theophylline inhibits enzymes I and II slightly, but other methyl xanthines have little effect. Enzymes I and II were competitively inhibited by many nucleotides containing phosphomonoester and phosphodiester bonds, as well as by P_i. 9. The possible significance of these phosphodiesterases in cyclic nucleotide metabolism in higher plants is discussed.

Although cyclic nucleotides are of major importance in the regulation of growth and metabolism in micro-organisms and animal cells, considerable doubt exists as to their role in higher plants. 3′:5′-Cyclic AMP has been demonstrated in higher plants by a variety of analytical procedures [for discussion see Lin (1974) and Amrhein (1974*b*)], but adenylate cyclase has yet to be demonstrated convincingly in cell-free higher-plant extracts (Lin, 1974). Nevertheless, several proteins that have a high affinity for cyclic nucleotides have been isolated from higher plants.

3′:5′-Cyclic GMP at micromolar concentrations stimulates polypeptide synthesis in the cell-free translation system derived from wheat embryo. This effect is due to promotion of GTP binding to elongation factor 1 (Lanzani *et al.*, 1974). A highly specific 5′-nucleotidase that is competitively inhibited by 3′:5′-cyclic AMP and other cyclic nucleotides has been purified from wheat seedlings (Polya & Ashton, 1973; Polya, 1974*a*) and from potato (Polya, 1974*b*, 1975). These higher-plant 5′-nucleotidases have

micromolar values for K_i constants for 3′:5′-cyclic AMP and 3′:5′-cyclic GMP. The potato 5′-nucleotidase is similar in molecular weight, subunit composition, K_D values for 3′:5′-cyclic AMP and 3′:5′-cyclic GMP and in purification behaviour to the *Escherichia coli* 3′:5′-cyclic AMP receptor protein (Polya, 1975). 3′:5′-Cyclic AMP-binding activity has been demonstrated in crude extracts of oats (Anderson & Pastan, 1973), but the nature of the protein(s) involved is unknown. Protein kinases have been detected in higher plants (Elliott, 1973; Keates, 1973), but these enzymes are not activated by cyclic nucleotides.

If cyclic nucleotides are to act as intracellular 'messengers' in higher plants as in other eukaryotic cells (Bitensky & Gorman, 1973), there must be phosphodiesterases present capable of rapidly diminishing intracellular cyclic nucleotide concentrations. Phosphodiesterases capable of hydrolysing 3′:5′-cyclic AMP have been detected in crude extracts of various higher-plant tissues (for references see Lin, 1974).

Phosphodiesterases have been partially purified from several plant sources (Lin & Varner, 1972; Brewin & Northcote, 1973). These enzymes have acidic pH optima, do not require bivalent cations for activity and are insensitive to methyl xanthines. These properties are in contrast with those of phosphodiesterases from other eukaryotic organisms (Appleman *et al.*, 1973).

During large-scale purification of a cyclic nucleotide-regulated 5'-nucleotidase from potato (Polya, 1974*b*, 1975) multiple forms of phosphodiesterases that can hydrolyse 3':5'-cyclic nucleotides were detected. Two phosphodiesterases of differing molecular size have been reported to be present in potato tubers (Shimoyama *et al.*, 1972). The present paper reports a procedure for extensive purification of three potato phosphodiesterases capable of hydrolysing cyclic nucleotides, and the kinetic properties of these enzymes.

Experimental

Enzyme assays

All enzyme assays were conducted as a routine at 30°C and were corrected by the use of appropriate zero-time controls. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

3':5'-Cyclic AMP or 3':5'-cyclic GMP phosphodiesterase assays were conducted in a final volume of $30\,\mu l$ containing: 50mM-2-(N-morpholino)ethanesulphonate (Na$^+$ salt, pH6.0); 1mM-3':5'-cyclic nucleotide; 3':5'-cyclic [8-^{3}H]nucleotide (final specific radioactivity 8.3Ci/mol). After incubation in the presence of enzyme the reaction was stopped by spotting a $20\,\mu l$ portion of the reaction mixture on PEI (polyethyleneimine)-cellulose thin-layer sheets. The thin-layer sheets were first developed by ascending chromatography with distilled water in which nucleosides move (e.g., for adenosine, $R_F = 0.7$), but nucleotides remain at the origin. After drying, the thin layers were developed by ascending chromatography in either solvent A (5%, w/v, boric acid–0.5M-ammonium acetate, pH7.5) or solvent B (0.5M-ammonium acetate, pH7.5). Solvent A resolves 5'-nucleotides from 3'-nucleotides as well as from 3':5'-cyclic nucleotides. Thus final R_F values in the water–solvent A system were 0.11 (5'-AMP), 0.29 (3'-AMP), 0.62 (3':5'-cyclic AMP) and 0.90 (adenosine). Solvent B resolves cyclic nucleotides from 5'-nucleotides and 3'-nucleotides. Final R_F values in the water–solvent B system were 0.51 (3':5'-cyclic AMP), 0.07 (3'-AMP and 5'-AMP) and 0.93 (adenosine). Marker nucleotides on the thin-layer chromatograms were detected under u.v. light and the appropriate spots cut out and added directly to scintillation vials containing 6ml of a scintillation fluid {0.5% PPO (2,5-diphenyloxazole)

and 0.03% POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] in toluene}. Samples were counted at 6% efficiency in a Packard model 3003 scintillation spectrometer. The data were corrected by means of appropriate zero-time controls.

Non-radiochemical assays of rates of hydrolysis of 2':3'-cyclic AMP, 8-bromo-3':5'-cyclic AMP and 6-N,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate were determined by carrying out reactions in the same assay medium described above but with the inclusion of 4–6mM substrate. After resolution of the products by PEI-cellulose chromatography as described above (with appropriate markers included in adjoining slots), the products were eluted from the thin layers with 1ml of 0.7M-MgCl$_2$ in 2mM-Tris–HCl buffer, pH7.5. Nucleotide concentrations were determined spectrophotometrically. The data were corrected by the use of appropriate zero-time controls. UDP-glucose pyrophosphatase activity was determined non-radiochemically by the procedure described above, but UMP ($R_F = 0.42$) was separated from UDP-glucose ($R_F = 0.82$) by a single development of the thin layers with 0.05M-LiCl as solvent.

Phosphodiesterase with bis-(p-nitrophenyl) phosphate as substrate was assayed in a 1ml reaction mixture containing 0.1M-maleate (Na$^+$ salt), pH6.0, and 1mM-bis-(p-nitrophenyl) phosphate. The reaction was initiated by the addition of enzyme and was terminated by the addition of 2ml of 0.1M-NaOH. The release of p-nitrophenol was measured by absorbance at 400nm (Andersch & Szcypinski, 1947). Phosphatase was assayed similarly by using p-nitrophenyl phosphate as substrate. Nucleotidase was assayed in a 1ml reaction mixture containing 0.1M-maleate (Na$^+$), pH6.0, and 1mM nucleotide. The reaction was terminated by the addition of 2ml of 9% (w/v) HClO$_4$. The phosphate released was measured by the method of Allen (1940).

NAD$^+$ pyrophosphatase activity was measured in a reaction mixture of 0.5ml containing 0.1M-maleate (Na$^+$), pH6.0, and 1mM-NAD$^+$. The incubation was terminated by placing the reaction tubes in a boiling-water bath for 3min. The amount of NAD$^+$ remaining was measured essentially by the method of Ciotti & Kaplan (1957). Sodium pyrophosphate (2.5ml of 0.1M) in 0.5M-ethanol was added to the killed reaction mixture, followed by $50\,\mu g$ of yeast alcohol dehydrogenase. The absorbance at 340nm was measured after incubation at room temperature (22°C) for 30min. Ribonuclease (EC 3.1.4.22) was assayed in a medium containing 0.1M-succinate (Na$^+$), pH6.0, and 5mM-MgCl$_2$ in a final volume of 0.5ml by measuring the release of acid-soluble nucleotides as described by Shortman (1961).

Molecular-weight determinations

Molecular weights were estimated by gel filtration

on a column (5 cm^2 × 58 cm) of Sephadex G-150 equilibrated with 50 mM-KCl, 1 mM-EDTA (Na$^+$ salt), 10 mM-Tris–HCl, pH 7.5, at 4°C. The column was calibrated with cytochrome c (12400) ovalbumin (45000), hexokinase (102000), lactate dehydrogenase (140000), catalase (250000), γ-globulin (160000) and horse serum cholinesterase (336000). Thyroglobulin (670000) was used as a void-volume marker. Calibration curves of relative elution volume versus log(molecular weight) were obtained, following the procedure of Andrews (1965).

Materials

Potato tubers (*Solanum tuberosum*), grown in the Kinglake district of Victoria, were purchased locally and stored at 4°C before processing. All nucleotides, nucleosides, phosphate esters, bovine serum albumin and yeast alcohol dehydrogenase (twice recrystallized) were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. 3′:5′-Cyclic [8-^{3}H]AMP (27.5 Ci/mmol) and 3′:5′-cyclic [8-^{3}H]-GMP (13.0 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. CM-cellulose (Cellex-CM, 0.695 mequiv/g) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Sephadex G-150 was obtained from Pharmacia, Uppsala, Sweden. All other reagents were of analytical-reagent grade. Miracloth was obtained from Calbiochem, San Diego, Calif., U.S.A. PEI-cellulose thin layers were prepared on plastic sheets by the method of Randerath & Randerath (1966). A 50% solution of polyethyleneimine (Polymin P) was supplied by B.A.S.F., North Melbourne, Australia, and Avicel PH-101 cellulose was obtained from J. Beith and Co., Melbourne, Australia. The thin layers (0.5 mm-thick) were washed by procedure 3 of Randerath & Randerath (1966). The layers were then scored into 1 cm-wide × 9 cm-long slots, each slot being used for a separate sample.

Results

Purification of phosphodiesterases

It was found that the potato phosphodiesterases were all capable of hydrolysing bis-(p-nitrophenyl) phosphate as well as cyclic nucleotides. Hydrolysis of this substrate was accordingly used as a convenient means of following the purifications of the phosphodiesterases. Pre-chilled potatoes were peeled, washed in tap water and distilled water and weighed. All subsequent procedures were carried out at 4°C. The potatoes (approx. 5 kg) were homogenized in a National fruit-juice extractor and the juice was immediately adjusted to 10 mM-Tris–HCl, 1 mM-EDTA and 4 mM-2-mercaptoethanol, pH 8.0. The juice was left for 5 min to permit starch to settle and then filtered through Miracloth. The filtered homogenate was taken as the starting point for the

purification. The homogenate (approx. 2000 ml) was adjusted to 40% $(NH_4)_2SO_4$ saturation. The precipitate was collected by centrifugation at 4800g for 30 min in the 981 rotor of the International PR 6000 centrifuge. The supernatant was then adjusted to 70% $(NH_4)_2SO_4$ saturation and the precipitate collected by centrifugation as before. The pellet was resuspended as a slurry and dialysed against 3 × 10 litres of buffer containing 10 mM-Tris–HCl, 1 mM-EDTA, 4 mM-2-mercaptoethanol, pH 8.0 (buffer A). After dialysis the non-diffusible solution had a conductivity equivalent to that of 3 mM-$(NH_4)_2SO_4$ in buffer A. The enzyme preparation was made 10 mM with respect to succinate and adjusted to pH 6.0 with 0.1 M-HCl. The solution was clarified by centrifugation at 12000g for 15 min and then applied to a column (16 cm^2 × 10 cm) of CM-cellulose (25 g dry wt. of Cellex CM, 0.695 mequiv./g) equilibrated with buffer containing 10 mM-succinate (Na$^+$ salt), 1 mM-EDTA (Na$^+$ salt) and 4 mM-2-mercaptoethanol, pH 6.0. The column was then washed with 300 ml of this buffer and a linear gradient (0–100 mM-$(NH_4)_2SO_4$ in the succinate buffer) was applied to the column at a flow rate of 120 ml/h. Fractions (20 ml) were collected. The elution profile (Fig. 1) shows that a broad peak of phosphodiesterase was eluted with the gradient. The trailing edge of this phosphodiesterase peak was enriched with NAD$^+$ pyrophosphatase activity. Fractions containing this enriched NAD$^+$ pyrophosphatase (fractions 46–62 in Fig. 1) were pooled and designated fraction CM2. Fractions containing most of the bis-(p-nitrophenyl) phosphate phosphodiesterase activity (fractions 28–45 in Fig. 1) were also pooled and designated fraction CM1. Both fractions were concentrated by $(NH_4)_2SO_4$ precipitation.

These fractions were then further purified and three phosphodiesterases resolved completely from one another by gel filtration through Sephadex G-150. It was found that the elution volume of the NAD$^+$
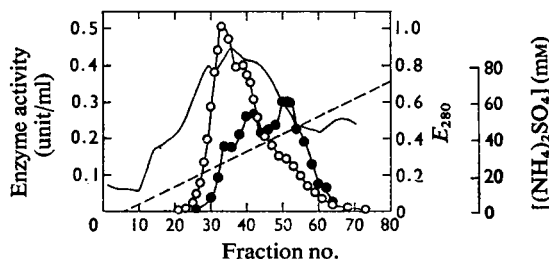

Fig. 1. *Elution of potato phosphodiesterases from a CM-cellulose column*

Details of column and buffers are given in the Experimental section. ——, E_{280}; – – – –, $(NH_4)_2SO_4$ concentration; ○, bis-(p-nitrophenyl) phosphatase; ●, NAD$^+$ pyrophosphatase.
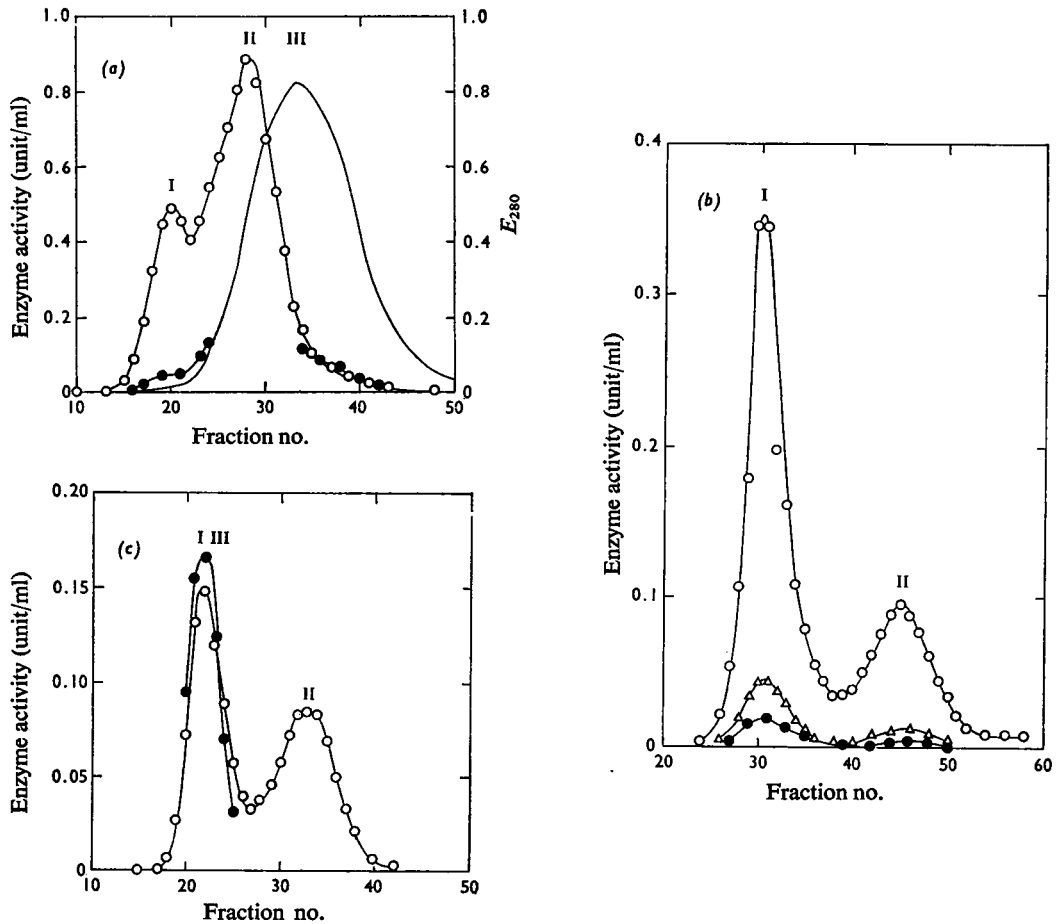
Fig. 2. *Elution profiles of phosphodiesterases (derived from fraction CM1) on a column of Sephadex G*-150

(*a*) Elution of fraction CM1 from a column of Sephadex G-150 equilibrated with buffer A. (*b*) Elution of enzymes I and II [obtained from first peak of (*a*)], from a column of Sephadex G-150 equilibrated with buffer A. (*c*) Elution of second peak of (*a*) from a column of Sephadex G-150 equilibrated with 100mM-KCl in buffer A. ——, E_{280}; ○, bis-(*p*-nitrophenyl) phosphatase; ●, NAD$^+$ pyrophosphatase; △, *p*-nitrophenyl phosphatase.

pyrophosphatase decreased when the ionic strength of the column buffer was increased by the inclusion of 0.1 M-KCl in the buffer. This behaviour was utilized in the subsequent resolution of the NAD$^+$ pyrophosphatase (phosphodiesterase III), from two other phosphodiesterases, one of high molecular weight (phosphodiesterase I) and the other of low molecular weight (phosphodiesterase II).

The concentrated fraction CM1 was applied to a column (5 cm$^2 \times$ 58 cm) of Sephadex G-150 equilibrated and eluted with buffer A. Two peaks of phosphodiesterase activity were observed, with NAD$^+$ pyrophosphatase being associated with the lower-molecular-weight-phosphodiesterase fraction (Fig. 2*a*). The fractions containing the high-mole-

cular-weight phosphodiesterase and very little NAD$^+$ pyrophosphatase were pooled, concentrated by ultrafiltration through a UM-10 membrane and rechromatographed on the Sephadex G-150 column with buffer A as eluent. This rechromatography substantially resolved a high-molecular-weight enzyme (phosphodiesterase I) and a low-molecular-weight enzyme (phosphodiesterase II) (Fig. 2*b*). Peaks of phosphodiesterase, determined with bis-(*p*-nitrophenyl) phosphate as substrate, coincide with peaks of acid phosphatase, measured with *p*-nitrophenyl phosphate as substrate (Fig. 2*b*). Peaks of phosphodiesterase measured with 2':3'-cyclic AMP, 3':5'-cyclic AMP and NAD$^+$ as substrates also correspond exactly to the phosphodiesterase
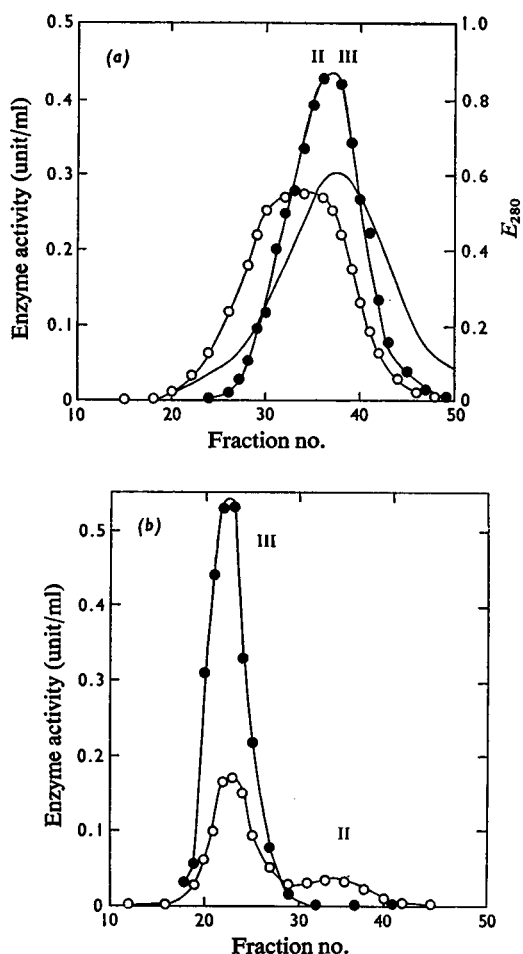
Fig. 3. *Elution profiles of phosphodiesterases (derived from fraction CM-2), on a column of Sephadex G-150*

(a) Elution of fraction CM-2 from a column of Sephadex G-150 equilibrated with buffer A. (b) Elution of main peak of (a) from a column of Sephadex G-150 equilibrated with 100mM-KCl in buffer A. ——, E_{280}; ○, bis-(p-nitrophenyl) phosphatase; ●, NAD$^+$ pyrophosphatase.

peaks determined with bis-(p-nitrophenyl) phosphate as substrate. The peak fractions were pooled, concentrated by ultrafiltration and used for enzymic studies. More of phosphodiesterase II can be recovered from fractions from the first Sephadex G-150 elution containing phosphodiesterases II and III (fraction 25–35, Fig. 2a). These fractions were pooled, concentrated and eluted from Sephadex G-150 with buffer A containing 0.1M-KCl. In these conditions of higher ionic strength the NAD$^+$ pyrophosphatase (phosphodiesterase III) is eluted in a high-molecular-weight form together with residual phosphodiesterase I. Phosphodiesterase II is satisfactorily resolved from enzymes I and III by this procedure (Fig. 2c).

Phosphodiesterase III can be resolved from the other phosphodiesterases by gel filtration of the CM-cellulose fraction CM2 (fractions 45–62, Fig. 1). Passage of fraction CM2 through Sephadex G-150 at low ionic strength in buffer A resolves low-molecular-weight phosphodiesterases II and III from residual high-molecular-weight phosphodiesterase I (Fig. 3a). The low-molecular-weight phosphodiesterase fractions (fractions 29–44, Fig. 3a) were pooled, concentrated and eluted from Sephadex G-150 at high ionic strength (buffer A containing 0.1M-KCl) to resolve the low-molecular-weight phosphodiesterase II from the NAD$^+$ pyrophosphatase (phosphodiesterase III) which, as described above, is eluted in a high molecular-weight form in these conditions (Fig. 3b). A further rechromatography of the peak of enzyme III under the same conditions yields uncontaminated enzyme III.

A summary of the purifications of phosphodiesterases I and II is shown in Table 1. The details of the purification of phosphodiesterase III are presented in Table 2. The amounts of protein in the final phosphodiesterase preparations are extremely small. Since there was insufficient protein for protein determinations by the method of Lowry *et al.* (1951), estimates of protein concentration in the final preparations were derived from absorbance measure-

Table 1. *Purification of enzymes I and II from potato with 3′:5′-cyclic AMP as substrate*

Values are corrected for sampling losses. One unit of enzyme activity is defined as 1 μmol of substrate hydrolysed/min at 30°C.

Fraction	Total activity (munits)	Total protein (mg)	Specific activity (munits/mg)	Recovery (%)	Purification (fold)	Activity with cyclic AMP Activity with bis-(p-nitrophenyl) phosphate
Homogenate	5441	24182	0.225	100	—	0.034
(NH₄)₂SO₄ ppt.	11801	14462	0.816	217	3.6	0.064
Cellulose CM-1	6255	1188	5.265	115	23.4	0.143
Sephadex G-150 (1)	2228	3.3	675	40.9	3000	0.332
Sephadex G-150 (2) EI	497	0.05	9940	9.1	44178	0.311
Sephadex G-150 (2) EII	93	0.08	1162	1.7	5164	0.282

Table 2. *Purification of enzyme III* (*NAD$^+$ pyrophosphatase*) *with NAD$^+$ as substrate*

Values are corrected for sampling losses. One unit is defined as 1 μmol of substrate hydrolysed/min at 30°C.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)	Activity with NAD$^+$ Activity with bis-(p-nitrophenyl) phosphate
Homogenate	300	24182	0.0124	100	—	1.88
(NH$_4$)$_2$SO$_4$ ppt.	132	14462	0.0091	44	0.7	0.71
Cellulose CM-2	58.3	490	0.119	19.4	9.6	4.33
Sephadex G-150 (low I)	272	277	0.098	9.1	7.9	5.13
Sephadex G-150 (high I)	12.4	2.3	5.39	4.1	435	3.39
Sephadex G-150 (high I)	11.0	0.05	220	3.7	17742	11.22

ments at 205 nm with a Zeiss PMQ III spectrophotometer and assuming an $E^{1\%}_{1cm} = 300$ (Scopes, 1974). It is readily seen from Tables 1 and 2 that large losses of all activities, especially of phosphodiesterases I and II, occurred during the final gel-filtration stages of the purifications.

The overall recovery of phosphodiesterase I is approx. 9% on the basis of the total initial homogenate 3′:5′-cyclic AMP phosphodiesterase, or 1% on the basis of homogenate phosphodiesterase assayed with bis-(p-nitrophenyl) phosphate. Since phosphodiesterase I is derived only from the CM-cellulose fraction CM1 in this procedure, one can correct these estimates of the recovery upwards by a factor of approx. 2. The specific activities of the final pooled phosphodiesterase I fractions from the purification are 9.9 μmol hydrolysed/min per mg of protein or 32 μmol hydrolysed/min per mg of protein with 3′:5′-cyclic AMP or bis-(p-nitrophenyl) phosphate respectively as substrates. The maximum specific activity of peak fractions from the final gel filtration of phosphodiesterase I is 40 μmol of 3′:5′-cyclic AMP hydrolysed/min per mg of protein with 1 mM-3′:5′-cyclic AMP as substrate, assayed in the conditions described in the Experimental section.

The recovery of phosphodiesterase II is very low [1.7 and 0.2% respectively on the basis of 3′:5′-cyclic AMP or bis-(p-nitrophenyl) phosphate as substrates], but these estimates are based on the yield of very high specific activity phosphodiesterase II from the processing of the CM-cellulose fraction CM1 (Fig. 2) and should be revised upwards by a factor of approx. 6 to account for removal of other phosphodiesterases. The specific activities of phosphodiesterase II from this procedure were 1.2 and 4.1 μmol hydrolysed/min per mg of protein with 3′:5′-cyclic AMP or bis-(p-nitrophenyl) phosphate as substrates respectively. The maximum specific activity of the peak fractions from the final gel filtration of phosphodiesterase II is 24 μmol of 3′:5′-cyclic AMP hydrolysed/min per mg of protein. The phosphodiesterase II recovered from processing of the CM-cellulose fraction CM-2 was of low specific activity and was not purified further. The large losses

of both phosphodiesterases I and II in the final stages of the purifications may well derive from the extremely small amounts of protein being processed. The overall purifications of phosphodiesterases I and II are approx. 44000- and 5000-fold respectively with respect to 3′:5′-cyclic AMP phosphodiesterase activity (Table 1).

The NAD$^+$ pyrophosphatase (phosphodiesterase III)-purification schedule is shown in Table 2. The overall purification of phosphodiesterase III (estimated from NAD$^+$ pyrophosphatase activity) is about 4% and the final preparations have extremely high specific activities: 220 and 7.2 μmol hydrolysed/min per mg of protein with NAD$^+$ and bis-(p-nitrophenyl) phosphate respectively. The overall purification of NAD$^+$ pyrophosphatase was approx. 18000-fold (Table 2).

Removal of enzymic contaminants in the purifications

The homogenate and (NH$_4$)$_2$SO$_4$-precipitated fractions both contained high amounts of acid phosphatase [orthophosphoric monoester phosphohydrolase (acid optimum) EC 3.1.3.2] and 5′- and 3′-nucleotidases (5′- and 3′-ribonucleotide phosphohydrolases, EC 3.1.3.5 and EC 3.1.3.6 respectively). Because of the presence of these hydrolases the major product of 3′:5′-cyclic AMP hydrolysis catalysed by these fractions was adenosine. After the CM-cellulose step the nucleotidase activity is negligible. The potato nucleotidase, previously described by Kornberg & Pricer (1950) and Heppel & Hilmoe (1951), is very similar to the wheat seedling 5′(3′)-ribonucleotide phosphohydrolase (Polya & Ashton, 1973; Polya, 1974a). The potato nucleotidase has micromolar K_m values for 3′- and 5′-AMP, hydrolyses both nucleotides at comparable rates at pH 5.0 (Polya, 1974b, 1975) and is tightly bound to CM-cellulose at pH 8.0.

CM-cellulose chromatography also removes a large amount of acid phosphatase. Acid phosphatase in the phosphodiesterase fractions is decreased to approx. 25% of the activity with bis-(p-nitrophenyl) phosphate as substrate. This residual acid phosphatase was not decreased during further purification. The acid phosphatases co-purifying with all three

phosphodiesterases have similar pH optima to the phosphodiesterases and are inhibited by 3':5'-cyclic AMP. This suggests that the residual acid phosphatase activities detected derive from hydrolysis of p-nitrophenyl phosphate as well as bis-(p-nitrophenyl) phosphate by the phosphodiesterases.

The purification procedures described above resolve the three phosphodiesterases from each other. The apparent change in molecular weight of phosphodiesterase III depended on the ionic strength of the gel-filtration eluting buffer and ensured resolution of phosphodiesterases I and II free from NAD$^+$ pyrophosphatase.

Molecular weights of the phosphodiesterases

Molecular weights of the phosphodiesterases were estimated by gel filtration on a Sephadex G-150 column calibrated with proteins of known molecular weight as described in the Experimental section. In the eluting conditions used [eluting buffer 50 mM-KCl, 1 mM-EDTA (Na$^+$ salt) and 10 mM-Tris–HCl, pH 7.5, at 4°C], the elution volumes of phosphodiesterases I and II corresponded to mol.wts. of 240 000 and 82 000 respectively. The elution volume of phosphodiesterase III, eluted in the same conditions but with 100 mM-KCl in the eluting buffer, corresponded to a mol.wt. of approx. 300 000. The molecular weight of phosphodiesterase III in conditions of low ionic strength is much lower than at high ionic strength (e.g., see Fig. 3a). The elution volume of phosphodiesterase III, eluted at 4°C from the calibrated Sephadex G-150 column in buffer A, corresponded to a mol.wt. of 60 000.

pH optima of the phosphodiesterases

Phosphodiesterases I and II hydrolyse bis-(p-nitrophenyl) phosphate at comparable rates over a wide range of pH values between pH 4 and 7. The pH optima for phosphodiesterases I and II with this substrate are approx. 6.2 and 6.1 respectively. Phosphodiesterase III has a pH optimum of approx. 3.3 but in addition a second peak of activity occurs at approx. pH 6.1 with bis-(p-nitrophenyl)

phosphate as substrate. Conversely phosphodiesterase III hydrolyses NAD$^+$ most effectively at high rather than low pH values.

With 3':5'-cyclic AMP as substrate phosphodiesterases I and II both have relatively sharp pH optima at approx. pH 5.5.

Products of cyclic nucleotide hydrolysis by the phosphodiesterases

The products of the hydrolysis of 3':5'-cyclic nucleotides by the phosphodiesterases (5'- and 3'-nucleoside monophosphates) were resolved by chromatography on PEI-cellulose thin layers developed with water and then with solvent A as described in the Experimental section. Hydrolysis of 3':5'-cyclic AMP or 3':5'-cyclic GMP by all three phosphodiesterases yields both 5'-AMP and 3'-AMP or both 5'-GMP and 3'-GMP respectively. However, the ratio of nucleoside 5'-monophosphate to nucleoside 3'-monophosphate resulting from hydrolysis of cyclic nucleotides by the phosphodiesterases at pH 6 varies for each enzyme and also depends on the base moiety of the cyclic nucleotide substrate (Table 3). With phosphodiesterase I the ratio of 3'-nucleoside monophosphate/5'-monophosphate product is approx. 2.5 after hydrolysis of 3':5'-cyclic AMP, 3':5'-cyclic GMP and 8-bromo-3':5'-cyclic AMP.

This 3'-NMP/5'-NMP ratio of the nucleoside monophosphate product is higher with phosphodiesterase II with 3':5'-cyclic AMP as substrate and the product of hydrolysis of 3':5'-cyclic GMP by phosphodiesterase II is predominantly 3'-GMP at pH 6. With phosphodiesterase III, 3'-AMP and 5'-AMP are produced in equal amounts from hydrolysis of 3':5'-cyclic AMP, but with 3':5'-cyclic GMP as substrate over three times more 5'-GMP than 3'-GMP is produced (Table 3).

The pH of the assay medium has a marked effect on the ratio of the products of hydrolysis of 3':5'-cyclic AMP by phosphodiesterases I and II. Above the pH optimum for hydrolysis of 3':5'-cyclic AMP for both enzymes (pH 5.5), the ratio 3'-AMP/5'-AMP increases markedly. At pH values below the pH optimum the ratio of 3'-AMP/5'-AMP is relatively constant for the reactions catalysed by both phosphodiesterases I and II (Fig. 4). We do not at present know the chemical basis for this marked change of product ratio at pH values close to the pH optimum.

The formation of both 3'- and 5'-AMP from 3':5'-cyclic AMP in reactions catalysed by higher-plant phosphodiesterases have been reported (Lin & Varner, 1972; Vandepeute *et al.*, 1973; Amrhein, 1974*a*). Lin & Varner (1972) suggested that the two products are formed by different enzymes, or that a phosphotransferase may be present that is capable of catalysing the interconversion of 3'- and 5'-AMP. However, no such phosphotransferase could be

Table 3. *Ratio of 3'-nucleoside monophosphate to 5'-nucleoside monophosphate in products of hydrolysis of several 3':5'-cyclic nucleotide substrates by phosphodiesterases*

For details see the text.

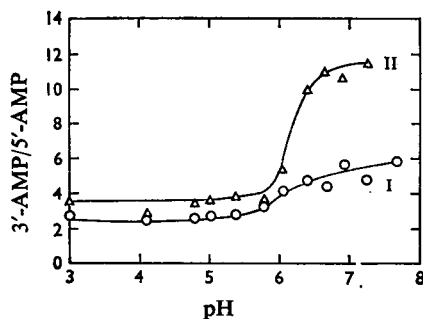| Enzyme | Ratio 3'-NMP/5'-NMP | | |
	3':5'-Cyclic AMP	3':5'-Cyclic GMP	8-Bromo-3':5'-cyclic AMP
I	2.7	1.4	2.4
II	3.8	15	—
III	1	0.3	—

Fig. 4. *Effect of pH on the ratio of the products, 3'-AMP and 5'-AMP, of 3':5'-cyclic AMP hydrolysis*

The assays were conducted as described in the Experimental section but with 100 mM-acetate (Na^+) buffer (pH 3–5) and 100 mM-Tris–maleate buffer (pH 5.3–8.0). $\bigcirc$, Enzyme I; $\triangle$, enzyme II.

Table 4. *Substrate specificities of the potato phosphodiesterases I, II and III*

Rates of hydrolysis were determined as described in the Experimental section, with 1 mM final concentration for all substrates except for 2':3'-cyclic AMP (4 mM) and RNA (5.8 mg/ml). Rates of hydrolysis are given relative to the rates of hydrolysis of 3':5'-cyclic AMP (=100).

Substrate	Enzyme I	Enzyme II	Enzyme III
3':5'-Cyclic AMP	100	100	100
3':5'-Cyclic GMP	68	77	28
2':3'-Cyclic AMP	126	58	1770
Bis-(p-nitrophenyl) phosphate	859	404	9000
p-Nitrophenyl phosphate	107	55	2430
NAD^+	44	55	451000
5'-AMP	<2	<2	—
3'-AMP	<2	<2	—
2'-AMP	<2	<2	—
RNA	<1	<1	

detected by these workers (Lin & Varner, 1972; Vandepeute *et al.*, 1973). Addition of either 5'- or 3'-AMP to the phosphodiesterase reactions does not alter the product ratio obtained with potato phosphodiesterases I and II, further suggesting that no phosphotransferase is involved. If separate phosphodiesterases are responsible for the mixture of products then they must have identical properties since the 3'-AMP/5'-AMP product ratio remains constant in the presence of various inhibitors and is not affected by changes in substrate concentration.

Substrate specificities of the phosphodiesterases

The relative rates of hydrolysis of a variety of nucleotides by the three phosphodiesterases are shown in Table 4. Phosphodiesterase III differs radically from phosphodiesterases I and II in that it hydro-

lyses NAD^+ at a much greater rate than any other substrate tested. In addition the rate of hydrolysis of 3':5'-cyclic nucleotides by phosphodiesterase III is much lower than for other hydrolysable substrates such as 2':3'-cyclic AMP or bis-(p-nitrophenyl) phosphate; the rates of hydrolysis of these latter substrates by phosphodiesterases I and II are of the same order of magnitude as the rates of hydrolysis of 3':5'-cyclic AMP catalysed by these enzymes. Phosphodiesterase III hydrolyses both 3':5'-cyclic AMP and 3':5'-cyclic GMP at very low rates compared with hydrolysis of 2':3'-cyclic AMP. Of the most effective substrates for phosphodiesterase III, NAD^+ > bis-(p-nitrophenyl) phosphate > p-nitrophenyl phosphate and 2':3'-cyclic AMP with respect to relative rates of hydrolysis at pH 6 (Table 4).

The pattern of substrate hydrolysis by phosphodiesterase I is very similar to that for phosphodiesterase II. For both enzymes rates of hydrolysis of bis-(p-nitrophenyl) phosphate are greater than for other hydrolysable substrates. As an approximation, 3':5'-cyclic AMP, 3':5'-cyclic GMP, 2':3'-cyclic AMP, NAD^+ and p-nitrophenyl phosphate are hydrolysed at comparable rates by both phosphodiesterases I and II. Hydrolysis of 2'-AMP, 3'-AMP, 5'-AMP and RNA by phosphodiesterases I and II was not detectable at the levels of detectability indicated in Table 4.

In the standard assay system at pH 6 containing 4.1 mM-6-N-2'-O-dibutyryl 3':5'-cyclic AMP, 5.5 mM-8-bromo-3':5'-cyclic AMP or 1.7 mM-UDP-glucose, the rates of hydrolysis catalysed by phosphodiesterase I were 41, 27 and 14.5% respectively of the rates of hydrolysis of equivalent concentrations of 3':5'-cyclic AMP in the same conditions.

Effects of cations on the phosphodiesterases

All three phosphodiesterases are active in the absence of added bivalent cations. The addition of 10 mM-EDTA (Na^+ salt) did not cause inhibition. With bis-(p-nitrophenyl) phosphate as substrate, 10 mM-Mn^{2+} activated phosphodiesterases I and II slightly (14–21%), but the addition of 10 mM-Na^+, -K^+, -Li^+, -NH_4^+, -Mg^{2+}, -Ca^{2+}, -Ba^{2+} or -Pb^{2+} failed to activate or inhibit either enzyme, but 10 mM-Zn^{2+}, -Cu^{2+} and -Fe^{2+} inhibited phosphodiesterases I and II. With 3':5'-cyclic AMP as substrate, 10 mM-Mn^{2+} activates phosphodiesterases I and II by 50 and 140% respectively; 10 mM-Fe^{3+} activates phosphodiesterase I by 80%. However, other bivalent cations have little effect on hydrolysis of 3':5'-cyclic AMP by phosphodiesterases I and II. The activation of phosphodiesterases I and II by Mn^{2+} involves an increase in $V_{max.}$ with no alteration in the K_m value for 3':5'-cyclic AMP (results not shown).

Inhibitors of phosphodiesterases I and II

Theophylline (10 mM) inhibits phosphodiesterases I

Table 5. *Effects of cations and various compounds on the activity of phosphodiesterases I and II with bis-(p-nitrophenyl) phosphate and 3':5'-cyclic AMP as substrates*

All assays were conducted as described in the Experimental section. The compounds added were all 10mM final concentration.

Compound added	Activity with bis-(p-nitrophenyl) phosphate (% of control)		Activity with 3':5'-cyclic AMP (% of control)	
	Enzyme I	Enzyme II	Enzyme I	Enzyme II
EDTA (Na+ salt; pH6.0)	101	100	91	121
MgCl₂	98	100	97	123
MnCl₂	114	121	150	238
ZnSO₄	53	55	106	43
CuSO₄	41	0	63	74
FeSO₄	74	66	76	47
FeCl₃	—	—	180	81
Theophylline	89	89	82	70
Theobromine	90	89	93	84
Caffeine	89	89	98	96
Sodium dodecyl sulphate	73	16	94	65
Sodium phosphate (pH6.0)	24	14	6	11
Sodium pyrophosphate (pH6.0)	59	36	18	20
Imidazole	93	96	95	116
NaF	90	82	89	45

and II by 20–30%, but other methyl xanthines show little inhibition of 3':5'-cyclic AMP or bis-(p-nitrophenyl) phosphate hydrolysis (Table 5). Phosphodiesterase II is inhibited more severely by sodium dodecyl sulphate and Cu²⁺ than is phosphodiesterase I. Both enzymes are inhibited to a comparable extent by Zn²⁺, Fe²⁺, P_i and PP_i. Imidazole, which activates various mammalian phosphodiesterases, and F⁻, which inhibits phosphodiesterases from several sources, failed to inhibit phosphodiesterases I and II (Table 5).

Phosphate and a variety of nucleotides can act as competitive inhibitors of phosphodiesterases I and II with either 3':5'-cyclic AMP or bis-(p-nitrophenyl) phosphate as substrates (Table 6). Yeast RNA and calf thymus DNA competitively inhibit 3':5'-cyclic AMP hydrolysis by enzyme I. The K_t values were 0.3mg/ml for RNA and 75mM (nucleotide equivalent) for DNA.

K_m values

The K_m value for 3':5'-cyclic AMP determined at 30°C in the standard assay medium was 0.6mM for both phosphodiesterases I and II. The K_m values for

bis-(p-nitrophenyl) phosphate in the same conditions were in the range 0.2–0.4mM for phosphodiesterases I and II. The K_m for NAD⁺ of the NAD⁺ pyrophosphatase (phosphodiesterase III) is 0.2mM.

Discussion

A major problem encountered in the purification of higher-plant cyclic nucleotide phosphodiesterases has been the resolution of these enzymes from phosphatases that can further hydrolyse the products of cyclic nucleotide hydrolysis. Lin & Varner (1972) separated the pea-seedling cyclic nucleotide phosphodiesterase from nucleotidases by sucrose-density-gradient centrifugation. However, Brewin & Northcote (1973) encountered co-purification of phosphodiesterases and phosphatases and suggested the existence of a multienzyme complex containing both types of activities. Gel filtration alone or ion-exchange chromatography on DEAE-cellulose or phospho-

Table 6. *K_t values of phosphodiesterases I and II for competitive inhibitors with the substrates shown*

For details see the text.

Inhibitor	Inhibitor concn. (mM)	Enzyme	K_t (mM)
Bis-(p-nitrophenyl) phosphate			
Sodium phosphate (pH6.0)	1.0	I	0.9
Sodium phosphate (pH6.0)	4.0	I	1.3
3':5'-Cyclic AMP	1.0	I	1.4
3':5'-Cyclic AMP	4.0	I	3.5
3':5'-Cyclic AMP	1.0	II	2.1
3':5'-Cyclic AMP	4.0	II	2.2
3':5'-Cyclic AMP			
3':5'-Cyclic GMP	4.44	I	0.3
2':3'-Cyclic AMP	0.94	I	0.03
8-Bromo-3':5'-cyclic AMP	5.49	I	0.9
6-N,2'-O-Dibutyryl 3':5'-cyclic AMP	4.14	I	0.5
Sodium phosphate (pH6.0)	2.0	I	0.3
NAD⁺	3.22	I	1.6
ATP	1.22	I	0.1
5'-AMP	0.98	I	0.1
3'-AMP	0.1	I	0.06
2'-AMP	0.66	I	0.09
UDP-glucose	1.73	I	4.9
3':5'-Cyclic GMP	4.44	II	1.5
2':3'-Cyclic AMP	0.94	II	
8-Bromo-3':5'-cyclic AMP	5.49	II	0.5
6-N,2'-O-Dibutyryl 3':5'-cyclic AMP	4.14	II	1.5
Sodium phosphate (pH6.0)	2.0	II	0.1
NAD⁺	3.22	II	6.9
ATP	1.22	II	0.2
5'-AMP	0.98	II	0.08
3'-AMP	1.0	II	0.04
2'-AMP	0.66	II	0.04

cellulose did not adequately resolve the potato phosphodiesterases from contaminating phosphatases. However, CM-cellulose chromatography does resolve the potato phosphodiesterases from the bulk of the phosphatase as well as from apyrase and 5′-nucleotidase. All three phosphodiesterases co-purify with a low acid phosphatase activity that appears to be a catalytic capability of these enzymes. A phosphodiesterase–phosphomonoesterase from the fungus *Fusarium* hydrolyses *p*-nitrophenyl phosphate as well as bis-(*p*-nitrophenyl) phosphate and cyclic nucleotides (Yoshida, 1973; Hamagishi & Yoshida, 1974). However, although the *Fusarium* enzyme catalyses the hydrolysis of a variety of nucleoside monophosphates, *p*-nitrophenyl phosphate is the only phosphomonoester that we have been able to show to be hydrolysed by the potato phosphodiesterases.

Phosphodiesterase III catalyses the hydrolysis of cyclic nucleotides but differs from phosphodiesterases I and II in its specificity for NAD^+ and corresponds to the potato NAD^+ pyrophosphatase of Kornberg & Pricer (1950). The specific activities of the potato phosphodiesterases I and II for 3′:5′-cyclic AMP are two orders of magnitude greater than that of phosphodiesterase III and cyclic nucleotides are among the best physiological substrates for phosphodiesterases I and II that we have found.

The potato phosphodiesterases I and II are similar to cyclic nucleotide phosphodiesterases from pea seedlings (Lin & Varner, 1972), soya bean (Brewin & Northcote, 1973) and barley (Vandepeute *et al.*, 1973) in having acid pH optima, lack of a requirement for bivalent cations and in the lack of substantial inhibition by methyl xanthines. The products of hydrolysis of 3′:5′-cyclic AMP catalysed by these higher-plant phosphodiesterases are 3′-AMP and 5′-AMP, whereas 5′-AMP is the product with animal 3′:5′-cyclic AMP phosphodiesterases (Appleman *et al.*, 1973). The potato phosphodiesterases I and II and other higher-plant phosphodiesterases catalyse the hydrolysis of 2′:3′-cyclic nucleotides as well as 3′:5′-cyclic nucleotides, as does a phosphodiesterase isolated from rat liver (Campbell & Pearce, 1973) and from *Fusarium* (Hamagishi & Yoshida, 1974).

The K_m values for 3′:5′-cyclic AMP of phosphodiesterases I and II are 0.6mM, three orders of magnitude greater than the reported concentrations of 3′:5′-cyclic AMP in higher plants (Raymond *et al.*, 1973; Lundeen *et al.*, 1973; Giannattasio *et al.*, 1974). Cyclic nucleotide phosphodiesterases from *E. coli* and *Saccharomyces cerevisiae* have K_m values of 0.5mM and 0.25mM respectively (Nielson *et al.*, 1973; Fujimoto *et al.*, 1974). Phosphodiesterases from pea seedlings, soya bean and barley have K_m values for 3′:5′-cyclic AMP of the same order of magnitude (Lin & Varner, 1972; Brewin & Northcote, 1973; Vandepeute *et al.*, 1973). Although similar high K_m values have been reported for phosphodiesterases from animal sources, some animal phosphodiesterases have micromolar K_m values for 3′:5′-cyclic AMP (Appleman *et al.*, 1973).

The specific activities of the highly purified potato phosphodiesterases I and II with 3′:5′-cyclic AMP as substrate are 10-fold greater than for purified animal cyclic nucleotide phosphodiesterase (Goren & Rosen, 1972). This suggests the possibility that, despite the relatively high K_m values for 3′:5′-cyclic AMP, these plant phosphodiesterases may have a physiological role in maintaining steady-state low amounts of 3′:5′-cyclic AMP *in vivo*. Highly active cyclic nucleotide phosphodiesterases such as enzymes I and II are required if transient increases in cyclic nucleotide concentrations are able to serve as intracellular signals in higher plants.

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