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ISOLATION OF PHOSPHODIESTERASE FROM SUGAR BEET LEAVES

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SUMMARY

A phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) from the leaves of sugar beets (*Beta vulgaris*) was purified 680-fold. The separation from the other plant proteins was achieved essentially by preparative electrophoresis in polyacrylamide gel. The enzyme preparation is homogeneous in disc electrophoresis under various conditions. The molecular weight is approximately $1.1 \cdot 10^5$. The isoelectric point is at 3.85. The enzyme is activated by divalent ions and inhibited by EDTA. Reducing agents are also inhibitory, especially cysteine, which gives rise to degradation products that are still enzymatically active after disc electrophoresis. The phosphodiesterase is an exonuclease and produces 5′-nucleotides. The *p*-nitrophenyl esters of the four ribonucleotides are hydrolyzed by the enzyme at not very different rates, whereas the ester of 5′-deoxythymidylic acid is hydrolyzed considerably faster. Pyrophosphates such as ADP, ATP, and NAD are also substrates. Polyribonucleotides, RNA, and denatured DNA are also degraded, whereas native DNA is resistant. The phosphodiesterase is inhibited by its end products, the 5′-nucleotides, most strongly by 5′-adenylic acid and 5′-deoxyadenylic acid.

INTRODUCTION

For the isolation of enzymes animal organs or microorganisms are generally preferred to plants, which frequently contain rather large amounts of phenols and phenol oxidases, the oxidation products of which render the work-up more difficult by tanning the proteins. The occurrence of phenols and phenoloxidases varies between different plants and plant organs. Their most accessible parts, the leaves, are usually

Abbreviations and symbols: nitrophenyl-pU, nitrophenyl-pC, nitrophenyl-pA, nitrophenyl-pG, *p*-nitrophenyl esters of uridine-, cytidine-, adenosine- and guanosine 5′-phosphate, respectively; 2′,3′-*O*-isopropylidenenitrophenyl-pU, 2′,3′-*O*-isopropylideneuridine 5′-*p*-nitrophenyl phosphate; naphthyl-pdT, deoxythymidine 5′-*α*-naphthyl phosphate; nitrophenyl-pdT, deoxythymidine 5′-*p*-nitrophenyl phosphate; 3′-*O*-acetylnitrophenyl-pdT, 3′-*O*-acetyldeoxythymidine 5′-*p*-nitrophenyl phosphate; nitrophenyl-pdTp, deoxythymidine 5′-*p*-nitrophenyl phosphate 3′-phosphate; cyclo-$(pdT)_3$, the cyclic trimer of deoxythymidine 5′-phosphate; A-pU, adenylyl-3′-5′-uridine; U-pA, uridylyl-3′-5′-adenosine.

rich in them and, furthermore, contain large amounts of soluble proteins, which is an additional difficulty for the isolation of a particular enzyme.

However, as we were interested in the enzymes degrading nucleic acids in plant leaves, we attempted to isolate phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) from the leaves of sugar beets (*Beta vulgaris* var. *saccharifera*), which contain the enzyme in a relatively high concentration and are easily available in large quantities, though in working with them one encounters the difficulties mentioned above.

Whereas the phenolic substances could be removed by chromatography on Sephadex, we were unable to separate the phosphodiesterase from the accompanying proteins by the conventional methods of column chromatography with different adsorbents. However, a complete separation could be achieved by preparative gel electrophoresis. This method offers a high degree of resolution and might be suitable for the isolation of other enzymes, particularly those which can be detected after electrophoresis by specific staining.

Since the work was started, the isolation of a phosphodiesterase from carrots[1,2] and a study on a phosphodiesterase in avena leaves[3] have been published.

MATERIALS AND METHODS

Materials

The four 5'-ribonuclotides, 5'-deoxythymidylic acid, ADP, A-pU and U-pA were obtained from Papierwerke Waldhof, Aschaffenburg AG, Mannheim. Uridine 3'-phosphate, NAD, RNA from yeast and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from calf intestine were obtained from Boehringer, Mannheim. Nitrophenyl-pdT, naphthyl-pdT and adenosine 3',5'-monophosphate were products of E. Merck AG, Darmstadt. ATP was purchased from Mann Research Laboratories, New York, highly polymerized yeast RNA from Calbiochem, Luzerne, highly polymerized DNA from calf thymus from Sigma Chemical Co., St. Louis and from Th. Schuchardt, München. The polyribonucleotides were purchased from Miles Research Products, Kankakee, Ill. Sephadex G-10, G-75, G-100 and G-200 was obtained from Deutsche Pharmacia GmbH, Frankfurt. The DEAE-cellulose was Whatman DE 52. Ampholine Carrier Ampholyte was a product of LKB Producter AB, Stockholm. Fast Red RC salt and Visking dialysis tubing were obtained from SERVA, Heidelberg. Acrylamide was purchased from Deutsche Shell Chemie GmbH, Frankfurt. It was recrystallized from chloroform before use. Thin-layer plates were coated with cellulose MN 300 from Macherey and Nagel, Düren. Hydroxylapatite was prepared according to the method of LEVIN[4].

Sugar beet leaves were harvested from the open field late in September. The tissue of the washed leaves was removed from the middle ribs and could be stored in closed polyethylene bags at $-20°$ for several months.

An Ultra-Turrax from Janke and Kunkel, Staufen/Brsg., was used for the homogenization of the leaves and of polyacrylamide gels.

Synthesis of enzyme substrates

Nitrophenyl-pU, nitrophenyl-pC, nitrophenyl-pA and nitrophenyl-pG were synthesized according to published procedures[5], except that no triethylamine was added,

the acetylated nucleotides were esterified with *p*-nitrophenol, the acetyl groups subsequently being removed with ammonia in methanol and chromatography on DEAE-cellulose was performed with a gradient of ammonium bicarbonate (pH 8.2) instead of triethylammonium bicarbonate (pH 8.6). The appropriate peaks were lyophylized and the substances dried over P_4O_{10} in a vacuum desiccator. Nitrophenyl-pdT was synthesized by the same procedure. 3′-*O*-Acetylnitrophenyl-pdT was prepared by acetylation of nitrophenyl-pdT (ref. 6). 2′,3′-*O*-Isopropylidene nitrophenyl-U was prepared according to the procedure published for 2′,3′-*O*-isopropylideneguanosine 5′-*p*-nitrophenyl phosphate[5].

Nitrophenyl-pdTp was synthesized from nitrophenyl-pdT using the phosphorylation method of TENER[7]. To the dry pyridinium salt of cyanoethyl phosphate (2.39 g, 8 mmoles) was added nitrophenyl-pdT (343 mg, 0.75 mmole) and dicyclohexylcarbodiimide (1.65 g, 8 mmoles). The mixture was dissolved in dry pyridine (10 ml). After 4 days in the dark at room temperature water was added (10 ml). After 1 h the precipitate was removed by filtration and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in water (10 ml) and applied to a column of DEAE-cellulose (3 cm × 25 cm) in the bicarbonate form. Elution was performed using a linear gradient system with water (2.3 l) in the mixing flask and 0.16 M ammonium bicarbonate (2.3 l) (pH 8.2) in the reservoir. Fractions (10 ml) were collected at 15-min intervals. Thymidine 5′-*p*-nitrophenyl phosphate 3′-cyanoethyl phosphate was eluted together with cyanoethyl phosphate in Fractions 110–160, which were pooled and lyophylized. A solution of the residue in 12% NH_4OH (50 ml) was heated to 70° for 2 h. The excess ammonia was removed and the solution concentrated to 10 ml by evaporation under reduced pressure. The concentrate was applied to a column of DEAE-cellulose and chromatographed under the same conditions as above.

Nitrophenyl-pdTp was eluted in Fractions 198–278 which were pooled and lyophylized. The residue was dissolved in water (3 ml), passed in three portions through a column (3 cm × 25 cm) of Sephadex G-10 and eluted with water. Fractions (5 ml) were collected at 20-min intervals. Fractions 16–21 were combined and lyophylized after addition of cyclohexylamine (0.1 ml). The cyclohexylammonium salt of nitrophenyl-pdTp (195 mg) was homogeneous by thin-layer chromatography in Solvent (A) (R_F = 0.26), Solvent (B) (R_F=0.18) and thin-layer electrophoresis (mobility = 1.27 relative to dTMP). Anal. calc. for $C_{34}H_{58}N_6O_{13}P_2$: P, 7.55. Found: P, 7.85%. Cyclo-p(dT)$_3$ was synthesized according to the method of KHORANA AND VIZSOLYI[8].

Methods

Chromatography and electrophoresis. The solvents used in thin-layer chromatography were: (A) isopropanol–25% NH_4OH–water (7:1:2, by vol.), (B) 44.2% (w/v) $(NH_4)_2SO_4$–isopropanol (96:4, by vol.) and (C) *n*-butanol–acetone–acetic acid–5% NH_4OH–water (9:3:3:2:4, by vol.). In thin-layer electrophoresis, 0.02 M potassium phosphate (pH 7.1) was used as buffer. A potential of 60 V/cm was applied. Spots were detected by viewing under short-wavelength ultraviolet light or by spraying for phosphate[9].

Determination of phosphodiesterase. The standard assay solution contained in a volume of 3.0 ml; piperazine–HCl buffer (300 μmoles) (pH 9.0), $MgCl_2$ (120 μmoles), nitrophenyl-pU (1.0 μmole) and 0.1 ml of enzyme solution. After incubating at 37° for 30 min the absorbance was read at 400 nm. A molecular extinction coefficient of

18 500 for *p*-nitrophenol was used in the calculations. One unit of enzyme activity is defined as that amount which catalyzes the hydrolysis of 1 μmole substrate per h under the assay conditions. This unit corresponds to approximately 2.6 units as defined by HARVEY *et al.*[2].

Readings were taken against a blank of the same composition, but lacking the enzyme solution. At the early stages of purification, a second blank lacking the substrate only was also run.

Protein was determined according to the method of LOWRY *et al.*[10] after precipitation with trichloroacetic acid. Inorganic phosphate was determined according to the method of BRUCE[11], total phosphorus according to that of MORRISON[12].

The degradation of nucleic acids and polyribonucleotides was assayed by the method of TUVE AND ANFINSEN[13]. DNA was denatured as described by LEHMAN AND NUSSBAUM[14].

Disc electrophoresis was performed in an apparatus as described by DAVIS[15], with glass tubes 5 mm × 80 mm. The buffer systems with Tris–glycine (pH 8.3)[15] and Tris–diethylbarbituric acid (pH 7.0)[16] and gel concentrations of 7% and 7.5%, respectively, were used. 1 mA/tube was applied, until the bromophenol blue had entered the running gel, then 2 mA/tube was applied. The gels were stained for proteins with Coomassie brilliant blue and for phosphodiesterase and nuclease activities as in refs. 17 and 18, respectively.

Electrofocusing was carried out according to the method of WRIGLEY[19] in tubes 5 mm × 100 mm. The gels were polymerized with the addition of Ampholine carrier ampholyte (pH 3–5). The anode vessel contained 0.2% phosphoric acid, the cathode vessel 0.4% ethanolamine. 1 mA/tube was applied for 1 h, and 2 mA/tube for a further 2 h. Two out of twelve gels were stained for phosphodiesterase. The corresponding areas of the other ten were cut out, soaked in water (5 ml) for 4 h and the pH measured in the extract.

Preparative gel electrophoresis was performed in an apparatus as described by KALTSCHMIDT AND WITTMANN[20]. The two rectangular plexiglass chambers were filled with 300 ml of monomer solution for the running gel, which was overlayered with 16 ml of spacer gel solution. The Tris–glycine buffer systems[15] (pH 8.3) and a 5% gel was used in the first electrophoresis. 25 mA were applied until the bromophenol blue had entered the running gel, then 45 mA were applied for about 24 h. The second electrophoresis was performed with the Tris–diethylbarbituric acid buffer system[16] (pH 7.0) and a 7.5% gel. 18 mA were applied for 2 days and 25 mA for another 3 days. Subsequent to electrophoresis, the phosphodiesterase was localized in the gel by specific staining[17] as used after disc electrophoresis. Two preparative gels can be stained with 2 mg naphthyl-pdT and 15 mg Fast Red RC salt in 25 ml of 0.1 M phosphate (pH 7.0).

The molecular weight was determined by gel chromatography as described by ANDREWS[21]. A column (2 cm × 60 cm) of Sephadex G-200 was used.

Enzyme purification

Step I. Frozen tissue of sugar beet leaves (1000 g) was ground at −20° in a cold mortar. The same amount (v/w) of 0.2 M acetate buffer (pH 4.5) was added and the mixture homogenized at room temperature for 15 min. All subsequent operations, except chromatography on hydroxylapatite, were performed in a cold room at 4°.

The homogenate was pressed through two layers of cloth and the resulting green sap adjusted to pH 5.0 with 4 M acetic acid. After centrifugation at 30 000 × *g* for 30 min, 1520 ml of clear brown crude extract were obtained.

Step II. 700 ml of crude extract were applied to each of two columns of Sephadex G-75, equilibrated with 0.05 M acetate (pH 6.0), containing 0.15 M KCl. The columns were eluted with the equilibration buffer, fractions (15 ml) being collected at 8-min intervals. The fractions giving an absorbance of 0.1 or more in the phosphodiesterase assay were pooled (1840 ml). Sugar (200 g) was dissolved in the pooled fractions and the solution concentrated by pressure dialysis according to the method of HOFSTEN AND FALKBRING[22] in two glass tubes (3 cm × 200 cm) against the elution buffer containing 10% (w/v) sugar. The concentrate (97 ml) was dialyzed for 2 h against 2 l of 1:10 diluted electrode buffer (pH 8.3), containing 10% sugar. The precipitated proteins were removed by centrifugation at 20 000 × *g* for 10 min. The supernatant was divided into 10-ml fractions, which were stored frozen until used for electrophoresis.

Step III. For electrophoresis 10 ml of the solution were applied to each of the two electrophoresis chambers[20]. Electrophoresis was stopped when the bromphenol blue had reached the bottom of the gels. They were stained for phosphodiesterase, the coloured bands were cut out with a sharp plexiglass ruler, cut into small pieces and homogenized with 300 ml of 0.05 M piperazine–HCl (pH 5.0). The homogenate was stirred overnight and centrifuged at 20 000 × *g* for 30 min. The residue was stirred with 300 ml of the same buffer for another day. This procedure was repeated 4 times. The combined extracts were concentrated as above, except that glycerol (10%) was added, instead of sugar, to 19 ml. No precipitate occurred at this stage. The concentrated solution was dialyzed against 1:5 diluted electrode buffer (pH 7.0) containing 10% glycerol.

Step IV. The second electrophoresis was performed as above and as described under *Preparative gel electrophoresis*. The extract was concentrated as above, but without addition of sugar or glycerol, to 8.5 ml.

Step V. The concentrate of Step IV was applied to a column (5 cm × 55 cm) of Sephadex G-100, equilibrated with 0.05 M piperazine–HCl (pH 5.0), containing 0.15 M KCl. Fractions (6 ml) were collected at 7-min intervals. The phosphodiesterase activity was eluted in Fractions 20–41, which were pooled and concentrated to 7.8 ml as in Step IV.

Step VI. The concentrate of Step V was applied to a column (1.5 cm × 27 cm) of hydroxylapatite. Elution was performed using a linear gradient system with 0.01 M phosphate (pH 6.8) (250 ml) in the mixing flask and 0.3 M phosphate (pH 6.8) (250 ml) in the reservoir. Fractions (10 ml) were collected at 10-min intervals. The phosphodiesterase activity was found in Fractions 11–20, which were pooled and concentrated, as in Step IV, to 10.5 ml.

RESULTS

Enzyme purification

The steps of a typical purification are summarized in Table I. The total amount of enzyme activity and of protein in the crude extract varied considerably with different extractions, the figures ranging between 17 100 and 44 100 enzyme units, and

800 and 3100 mg of protein per 1400 ml of crude extract. These variations may be partially due to the inhomogeneity of the plant material and also to the different length of storage.

The protein content in the extracts of the preparative electrophoreses, as indicated by the Lowry assay, also varied considerably. These variations are caused by impurities originating from the polyacrylamide gel, which give a positive reaction with the Folin reagent. When a blank electrophoresis in Tris–glycine buffer was run and areas corresponding to a phosphodiesterase zone were cut out, the extracts gave a positive reaction in the Lowry test corresponding to 34 mg of protein.

TABLE I

PURIFICATION OF PHOSPHODIESTERASE

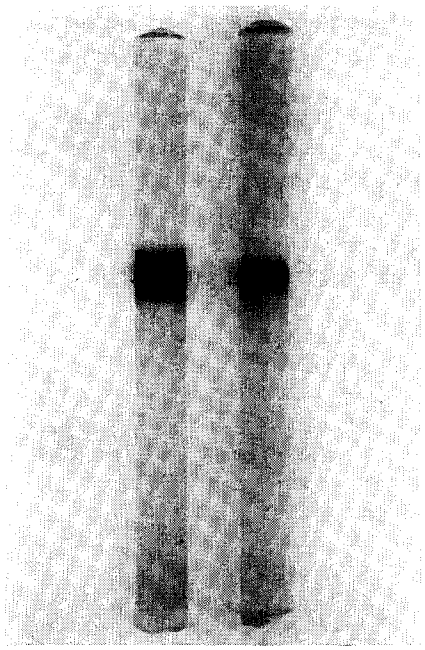
	Step	*Vol. (ml)*	*Total units*	*Protein (mg)*	*Spec. act. (units/mg protein)*	*Yield (%)*	*Purification*
I	Crude extract	1400	27 039	2450	11.1	100	
II	Sephadex G-75, concentration	97	31 623	491	64.5	117	5.8
III	(1) Preparative electrophoresis, concentration	19	20 898	72*	290.4	77.3	26.2
IV	(2) Preparative electrophoresis, concentration	8.5	10 659	16*	666	39.4	60
V	Sephadex G-100, concentration	7.8	8 421	2.9*	2907	31	262
VI	Hydroxylapatite, concentration	10.5	6 348	0.845*	7512	23.8	677

* Interference in the Lowry test by impurities from polyacrylamide. Figures are therefore too high and not representative of true protein content.

The 7.5% gel used in the second preparative electrophoresis apparently has a lower content of these extractable impurities, which are probably due to incomplete polymerization of the acrylamide. All the plant proteins are already removed from the phosphodiesterase after the second electrophoresis. At this stage, there is only one protein band visible after disc electrophoresis, which is stained with Coomassie blue. However, the protein content determined according to the method of Lowry is still too high. The column chromatographic procedures following electrophoresis serve mainly to remove these impurities originating from the gel. The higher protein value obtained after Step VI without precipitation by trichloroacetic acid (0.993 mg instead of 0.845 mg) indicates that this is not completely achieved. Phosphodiesterase (Step VI) stained by different methods after disc electrophoresis is shown in Figs. 1 and 2.

Molecular weight. The determination of the molecular weight of sugar beet phosphodiesterase gave a value of $1.11 \cdot 10^5$. This is in close agreement with the molecular weight of carrot phosphodiesterase[1].

The isoelectric point was found to be at pH 3.85. Enyzme solutions seem to be unstable at or near the isoelectric point. Thus, loss in activity was observed when the crude extract was acidified below pH 5. This may also explain the loss of enzyme



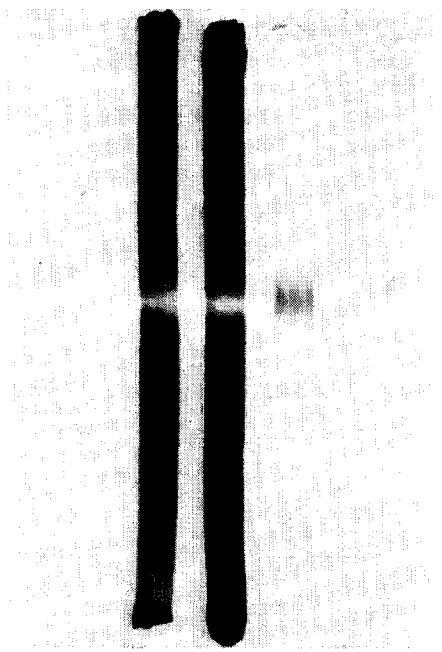


Fig. 1. Disc electrophoresis[15] of phosphodiesterase (Step VI). Cathode at the top. Approx. 4 μg of protein, as determined by the Lowry test, were applied per gel. Gels were stained (from left to right) for phosphodiesterase activity as in ref. 17 and for protein with Coomassie blue.

Fig. 2. Disc electrophoresis[15] of phosphodiesterase. Gels were stained (from left to right) for nuclease activity against DNA[18] (incubation time 2 h), RNA[18] and for phosphodiesterase activity[17].

activity on dialysis of carrot phosphodiesterase at pH 4.5 (ref. 1) and its decreased heat stability at lower pH values.

Stability at high and low temperatures. The enzyme is remarkably stable at higher temperatures at pH 9 (Table II). After heating to 80° for 10 min, about 50% of the activity was lost. The same value was found with the enzyme in carrots[1]. Solutions of purified sugar beet phosphodiesterase can be stored frozen for several months without loss of activity.

The pH-optimum with nitrophenyl-pU as substrate is about pH 9.2 (Fig. 3), in

TABLE II

HEAT STABILITY OF PHOSPHODIESTERASE

The enzyme solution in 0.1 M piperazine–HCl (pH 9.0) was heated to different temperatures for 10 min. Assay with nitrophenyl-pdT under standard conditions.

Temperature (°)	*Remaining activity (%)*
60	94
70	88
80	51
90	—

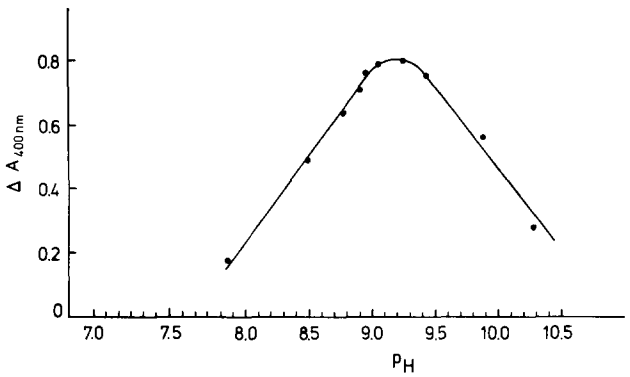


Fig. 3. pH optimum of phosphodiesterase with nitrophenyl-pdT as substrate. 0.1 M piperazine–HCl buffer, containing 0.04 M $MgCl_2$, was used. Assay otherwise under standard conditions.

close agreement with the optima of the two other plant phosphodiesterases[2,3]. Glycine–NaOH buffer proved to be particularly unsuited for the determination, because it depressed the enzyme activity to about 20%, compared to that with piperazine buffer. The optimum is not as pronounced with highly polymerized nucleic acids as substrates.

Effects of ions on phosphodiesterase activity (Table III). The assay was performed at pH 7.0, because at pH 9.0 some of the ions (Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+}) are precipitated as hydroxides. The phosphodiesterase is stimulated by a number of divalent ions, but not generally to a very high degree.

TABLE III

EFFECT OF IONS ON PHOSPHODIESTERASE ACTIVITY

Assay in 0.1 M piperazine–HCl buffer (pH 7.0) with nitrophenyl-dT as substrate. Otherwise standard conditions.

Addition (10^{-2} M)	*Percentage of control*
$MgCl_2$	127
$CaCl_2$	106
$MnSO_4$	150
$CoCl_2$	151
$CuSO_4$	145
$ZnCl_2$	145
$HgCl_2$	40
Iodoacetate	116

In spite of this relatively low stimulation, the phosphodiesterase is strongly inhibited by EDTA at low concentrations (Table IV). This indicates that the enzyme binds the necessary cation and is independent of added cations.

Effect of reducing agents (Table V). The activity of sugar beet phosphodiesterase is depressed by reducing agents. Of the substances tested, cysteine inhibited most strongly. It is interesting that incubation of phosphodiesterase with 10^{-1} M cysteine for 24 h at room temperature caused a degradation of the enzyme to several subunits, which are still enzymatically active after disc electrophoresis, as revealed by staining

TABLE IV

INHIBITION OF PHOSPHODIESTERASE ACTIVITY BY EDTA

Assay with nitrophenyl-pdT under standard conditions, but without Mg^{2+}.

EDTA (M)	*Percentage of control*
10^{-2}	0
10^{-3}	2
10^{-4}	17
10^{-5}	61
10^{-6}	81
10^{-7}	93
—	100

TABLE V

INHIBITION OF PHOSPHODIESTERASE ACTIVITY BY REDUCING AGENTS

Assay with nitrophenyl-pdT under standard conditions.

Addition (10^{-3} M)	*Percentage of control*
Ascorbic acid	92
Glutathione	78
Mercaptoethanol	68
Cysteine	7

TABLE VI

ACTIVITY OF PHOSPHODIESTERASE ON VARIOUS NUCLEOTIDE DERIVATIVES

The substrates (1 μmole) were incubated under standard conditions with 0.146 unit of enzyme.

Substrate	*Rate of hydrolysis (nmoles/h)*
Nitrophenyl-pU	146
2′,3-*O*-Isopropylidenenitrophenyl-pU	155
Nitrophenyl-pA	139
Nitrophenyl-pC	141
Nitrophenyl-pG	160
Nitrophenyl-pdT	272
3′-*O*-Acetylnitrophenyl-pdT	237
Nitrophenyl-pdTp	14
Cyclo-p(dT)$_3$*	—
Adenosine 3′,5′-monophosphate**	—

* 8.4 absorbance units were incubated at 37° for 5 h in a volume of 0.2 ml; containing piperazine–HCl buffer (20 μmoles) (pH 9.0), $MgCl_2$ (2.4 μmoles) and 25 units of enzyme. No degradation could be observed after thin-layer electrophoresis (pH 7.1) and thin-layer chromatography in Solvent A.

** 5 μmoles were incubated at 37° for 2 h in a volume of 0.5 ml; containing piperazine–HCl (50 μmoles) (pH 9.0), $MgCl_2$ (20 μmoles) and 1 unit of enzyme. No AMP could be detected by thin-layer electrophoresis (pH 7.1) and thin-layer chromatography in Solvent B.

for phosphodiesterase activity. The degradation of the enzyme may occur by cleavage of disulfide bridges.

Activity with various nucleotide derivatives (Table VI). The *p*-nitrophenyl esters of the four ribonucleotides are split without marked differences in the rate. From a Lineweaver–Burk plot of the hydrolysis of nitrophenyl-pU (Fig. 4), the $K_m = 1.9 \cdot 10^{-4}$ M and $v_{max} = 8.28$ mmoles/h per mg protein were calculated. The best substrate is nitrophenyl-pdT. Acetylation of its OH group in the 3′ position decreases the rate

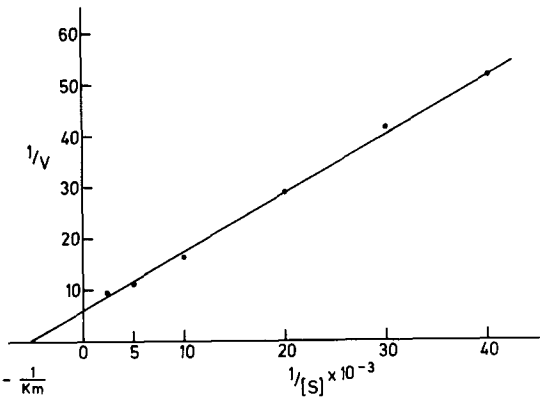


Fig. 4. Lineweaver–Burk plot of the hydrolysis of nitrophenyl-pU by phosphodiesterase. Incubation at 37° in standard assay buffer (2.5 ml), containing 2 ng of protein, as determined by the Lowry test. Substrate concentration in moles/l, velocity in μmoles/h.

somewhat, whereas blocking of the two OH groups of nitrophenyl-pU by the isopropylidene group increases the rate of degradation. Nitrophenyl-pdTp is degraded only very slowly. Cyclo-p(dT)$_3$ and adenosine 3′,5′-monophosphate are not attacked by the phosphodiesterase.

Activity with dinucleoside phosphates and pyrophosphates (Table VII). Dinucleoside phosphates are hydrolyzed at a rate comparable to that of nitrophenyl-pU, whereas ADP is split at a considerably lower rate. AMP was the sole product after incubation of ADP and ATP with phosphodiesterase and NAD was split to AMP and NMN as

TABLE VII

ACTIVITY OF PHOSPHODIESTERASE ON DINUCLEOSIDE PHOSPHATES AND ADP

The substrates (1 μmole) were incubated at 37° in a volume of 0.5 ml; containing piperazine–HCl buffer (50 μmoles), $MgCl_2$ (20 μmoles), alkaline phosphatase (10 μg) and approx. 0.3 unit of phosphodiesterase. Aliquots were withdrawn at different times and analyzed for P_i (ref. 11).

Substrate	*Rate of hydrolysis (nmoles/h)*
A-pU	61.5
U-pA	74.1
ADP*	6.5
Nitrophenyl-pU	111.4

* Without phosphatase.

TABLE VIII

DEGRADATION OF NUCLEIC ACIDS AND POLYRIBONUCLEOTIDES BY PHOSPHODIESTERASE

According to the supplier, the polyribonucleotides had a mean molecular weight of 10^5. Incubation was carried out at 40° for 8 h in a volume of 0.6 ml; containing piperazine–HCl buffer (60 μmoles) (pH 9.0), $MgCl_2$ (24 μmoles), nucleic acid or polyribonucleotide (280 μg) and 15 units of enzyme. The reaction was stopped by the addition of 0.75% uranyl acetate in 25% perchloric acid (0.2 ml) and of water (2.2 ml). After cooling to 0° for 10 min the suspension was centrifuged and the absorbance measured in the supernatant.

Substrate	$A_{260\ nm}$
RNA	0.265
Native DNA	—
Denatured DNA	0.215
Poly (A)	0.190
Poly (C)	0.280
Poly (I)	0.360
Poly (U)	1.180

checked by thin-layer chromatography in Solvent C. Thin-layer chromatography in Solvent B revealed that A-pU and U-pA were split to A and UMP and to U and AMP, respectively.

Degradation of nucleic acids and polyribonucleotides (Table VIII). Highly polymerized RNA and denatured DNA are degraded at nearly the same rate, whereas native DNA is not attacked under the experimental conditions. Of the polyribonucleotides, poly(U) is degraded at the highest rate, followed by poly(I), poly(C) and poly(A). This order is the reverse of the order of the inhibitory action of the corresponding 5′-nucleotides on phosphodiesterase activity. UMP was the sole product of the hydrolysis of poly(U) detectable by thin-layer chromatography with Solvent B.

End product inhibition (Table IX). The enzymatic activity of sugar beet phosphodiesterase on nitrophenyl-pdT is inhibited by 5′-nucleotides. Ribonucleotides and the corresponding deoxyribonucleotides are inhibitory to the same degree. Recently, the inhibition of snake venom phosphodiesterase by 5′-deoxyribonucleotides has also been reported[25].

TABLE IX

END PRODUCT INHIBITION OF PHOSPHODIESTERASE

Nitrophenyl-pdT (1 μmole) and the corresponding 5′-nucleotide (1 μmole) were incubated in a volume of 3.0 ml; containing piperazine–HCl buffer (300 μmoles) (pH 9.0), $MgCl_2$ (120 μmoles) and 0.15 unit of enzyme at 37° for 15 min.

Added inhibitor	*Activity (percentage of control)*
AMP	10
GMP	42
CMP	28
UMP	39
dAMP	10
dGMP	45
dCMP	23
dTMP	45

DISCUSSION

Initial attempts to isolate the phosphodiesterase from sugar beet leaves by conventional column chromatographic techniques were unsuccessful. Column chromatography was performed on DEAE-cellulose, AE-cellulose, CM-cellulose, SE-cellulose, hydroxylapatite and Sephadex G-100. However, no pure enzyme could be obtained by a sequence of these procedures. We then tried preparative electrophoresis with the apparatus described by KALTSCHMIDT AND WITTMANN[20], which they had used for the separation of ribosomal proteins. After only two electrophoretic steps, using different buffer systems and gel concentrations, we obtained a phosphodiesterase preparation which migrated as one band in disc electrophoresis. It should be mentioned here that the resolution by disc electrophoresis is superior to that by preparative electrophoresis and that a different gel concentration was used in disc electrophoresis with Tris–glycine buffer. The impurities present at this stage are not proteins, but substances originating from the polyacrylamide gel, which give a positive reaction in the Lowry test. The specific activity of the enzyme was still increased after subsequent column chromatography on Sephadex G-100 and hydroxylapatite.

The molecular weight of the phosphodiesterase from sugar beet leaves is the same, or not very different, from that of the phosphodiesterase in carrots. The two enzymes, and also the phosphodiesterase from avena leaves, are apparently closely related and are, in all their enzymatic properties, very similar to snake venom phosphodiesterase, whereas a phosphodiesterase purified from malt[23] appears to have somewhat different properties. The experiments on the cleavage of a number of differently substituted nucleotide esters allow some conclusions on the structural features of the substrate required for the binding of the enzyme. The rate of hydrolysis is not markedly influenced by the nature of the base. The slower degradation of nitrophenyl-pU compared with nitrophenyl-pdT is obviously caused by the presence of a 2′-hydroxyl group. In contrast to HARVEY *et al.*[2], we found that acetylation of the 3′-hydroxyl group decreases the rate of hydrolysis somewhat but does not lead to a complete inhibition. The degradation of 3′-*O*-acetyl-nitrophenyl-pdT still proceeds considerably faster than that of nitrophenyl-U. This is in agreement with the findings of RAZZELL AND KHORANA[6] with snake venom phosphodiesterase. 2′,3′-*O*-Isopropylidenenitrophenyl-pU, in which both hydroxyl groups are blocked, is split even faster than nitrophenyl-pU, though not as fast as nitrophenyl-pdT. However, the hydrolysis is severely inhibited by a free phosphate group esterified in the 3′ position, as is also the case with the snake venom[6] and the carrot phosphodiesterase[1].

The inability of the enzyme to split a phosphodiester bond of cyclo-p$(dT)_3$ indicates that it is an exonuclease which needs a free end of an oligonucleotide chain for activity.

Large amounts of phosphodiesterase are necessary to obtain a measurable degradation of highly polymerized nucleic acids. It is therefore unlikely that these are the substrates of the enzyme in the living cell.

The phosphodiesterase of sugar beet leaves is strongly inhibited by its end products, the 5′-nucleotides, especially by 5′-adenylic acid. In contrast to the rate of hydrolysis of the nucleotide *p*-nitrophenyl esters, there is no difference between the ribo- and deoxyribonucleotides in respect to the degree of inhibition. Apparently the sugar moiety does not play the same role in binding the inhibitory end product

to the enzyme as it does in binding the substrate, whereas the nature of the base seems to exert a stronger influence. The phosphodiesterase activity may be regulated under physiological conditions by end product inhibition. Inhibition by ribonucleotides has also been found with a phosphodiesterase from rat liver[24] and with snake venom phosphodiesterase by 5'-deoxyribonucleotides[25], but has not yet been reported from a plant phosphodiesterase.

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