NUCLEOLYTIC ENZYMES OF RAPE SEEDLINGS
II. UNSPECIFIC ACID PHOSPHOMONOESTER HYDROLASE

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Aqueous extracts of rape seedlings (*Brassica napus* L.) display phosphomonoesterase activity. The enzyme was purified gradually by chromatography on tert-butyl ester of Cellex P, affinity chromatography on Sepharose 4 B with linked *p*-aminophenyl(phenyl)thiophosphate or N^6-(6-aminohexyl)adenosine 2′(3′)-thiophosphate (final degree of purification 269×). The enzyme displayed activity toward various ribo- and 2′-deoxyribonucleotides, with preference for 3′-ribonucleotides, and to aryl or alkyl esters of phosphoric acid, ADP, ATP, inorganic pyrophosphate, as well as to nucleotide analogues containing acids of phosphorus with a small substituent on the phosphorus atom. The internucleotidic linkages of RNA and DNA are resistent. The enzyme is classified as an orthophosphate monoester hydrolase (EC 3.1.3.2).

In the preceding paper[1] the isolation and the characterization of two main nucleolytic activities of neutral aqueous extract of rape seedling homogenate (*Brassica napus* L.) has been described: uridine-(cytidine-)specific decyclizing 2′-ribonucleotidyl transferase (EC 2.7.7) and the unspecific decyclizing 2′,3′-phosphodiesterase. The third main nucleolytic activity of this material is a phosphomonoesterase which was identified on the basis of the cleavage of *p*-nitrophenyl phosphate and adenosine 3′-phosphate. In this paper the purification and the characterization of this as yet undescribed enzyme is described.

EXPERIMENTAL

Material

Unless stated otherwise, 2′(3′)- and 5′-ribonucleotides and 2′-deoxy-5′ribonucleotides were purchased from Calbiochem (U.S.A.). Ribonucleoside 3′-phosphates were prepared on cleavage of corresponding 2′,3′-cyclophosphates with ribonuclease A or T2 and isolated in the form of lithium salts. Cytidine 2′-phosphate was isolated from a mixture of isomers by chromatography on Dowex 1X2 in 0·02M formic acid (see[2]). *p*-Nitrophenyl phosphate, phenyl phosphate and α-naphthyl phosphate were preparations of Lachema, glycero 1-phosphate and glucose 6-phosphate were preparations of Calbiochem. Other substances used were prepared by procedures described earlier.

N^6-(6-Aminohexyl)adenosine

A mixture of 5·0 g (17·5 mmol) of 6-chloropurine riboside[3], 2·5 g (21·5 mmol) of 1·6-diaminohexane, 30 ml of dimethylformamide and 3·5 ml (25 mmol) of triethylamine was stirred at room temperature overnight and evaporated at 40°C/13 Pa. The residue afforded on crystallization from 90% ethanol (40 ml) 6·2 g of a crystalline product, R_F 0·70 in S1.

N^6-(6-Aminohexyl)adenosine 2′(3′)-Thiophosphate

A mixture of 2·5 g (6·85 mmol) of N^6-(6-aminohexyl)adenosine, 1·7 g (6·85 mmol) of di-n-butyltin oxide and 150 ml of methanol was refluxed for 90 min, cooled to 20°C, and 10 ml of tri-n-butylamine and 3 ml of thiophosphoryl chloride[4] were added gradually at this temperature. After 1 h standing at room temperature the mixture was evaporated in a vacuum and the residue allowed to stand in the presence of 100 ml of 10% lithium hydroxide at room temperature overnight. The mixture was neutralized with Dowex 50X8 in H^+-form, triethylamine was added until the pH of the mixture was 9, the ion exchanger was filtered off under suction, washed with 200 ml of water, and the filtrate concentrated *in vacuo* and applied on a column (500 ml) of Dowex 50X8 (H^+-form). After washing with water until UV absorption and conductivity of the filtrate dropped the column was eluted with 2 l of dilute (1 : 10) aqueous ammonia and the eluate evaporated in a vacuum (40°C/2 kPa). This residue was chromatographed on a 80 × 4 cm column of DEAE cellulose (Cellex, D, std capacity) using a linear gradient of triethylammonium hydrogen carbonate (pH 7·5) (0—0·3M, 3 l, elution rate 3 ml/min, fractions of 10 min each; the elution was monitored with Uvicord). Fraction 0·20—0·30M was evaporated in vacuum and rechromatographed on a column (100 × 4 cm) of Sephadex G-10 (fine), elution rate 30 ml/h. The fractions which contained the product were combined, evaporated in a vacuum and the residue was precipitated from methanol (5 ml) with ether (200 ml). The precipitated product was filtered off under suction and dried at 13 Pa over P_2O_5. Yield 0·60 g of triethylammonium salt of the product, chromatographically (R_F 0·38 in S_1, R_{Ap} 0·61 and 1·30 in S2) and electrophoretically (E_{Up} 0·41) pure. Content (determined spectrophotometrically): 90—95%.

Sepharose 4B Modified with N^6-(6-Aminohexyl)adenosine 2′(3′)-Thiophosphate ("AHAPS-Sepharose")

A solution of 0·5 mmol of triethylammonium salt of N^6-(6-aminohexyl)adenosine 2′(3′)-thiophosphate in 5 ml of 0·1M sodium hydrogen carbonate and sodium hydroxide (pH 9·0) was added to 30 ml of Sepharose 4B activated with cyanogen bromide[5] in 30 ml of the same buffer and the suspension was stirred at 4°C overnight. After filtration the product on the filter was washed with water and resuspended in 50 ml of 1M ethanolamine–HCl of pH 8·0. After 10 h stirring at room temperature the suspended material was filtered off under suction, washed with water and the product stored in a saturated sodium chloride solution at 0°C.

Methods

Paper chromatography (descending arrangement) was carried out on paper Whatman No 3 MM in the following solvent systems: 2-propanol–conc. ammonia–water (7 : 1 : 2) (S1), 2-propanol–conc. ammonia–0·2M triethylammonium borate (7 : 1 : 2) (S2), saturated ammonium sulfate–0·1M sodium acetate–2-propanol (79 : 19 : 2) (S3). Paper electrophoresis was carried out on the same paper at 20 V/cm in 0·1M triethylammonium hydrogen carbonate of pH 7·5 (1 h) (E1) or in 0·1M sodium citrate of pH 3·5 (2 h) (E2). The compounds were detected under UV light (Chromatolite).

Column chromatography, *a*) on PSPP-Sepharose-4B: column 1·8 × 21 cm (55 ml) of support[6] equilibrated with 0·02M of sodium acetate–acetic acid pH 5·0; elution rate 14 ml/h, fractions 15 min (Fig. 1), *b*) on AHAPS-Sepharose-4B: column 1·3 × 21 cm (30 ml) of support, equilibrated with 0·02M of sodium acetate–acetic acid pH 6·5, elution rate 14 ml/h, fractions 15 min (Fig. 2). The chromatographies were carried out at 5°C, the fractions dialyzed and concentrated by ultrafiltration on an Amicon UM 10 filter under nitrogen at 5°C.

Molecular weight determination was carried out on a thin layer of Sephadex G 100 SF (layer length 20 cm, 0·4 mm strong) in 0·02M sodium acetate–acetic acid (pH 5·5). After the development two parallel strips were copied onto two dry strips of paper Whatman No 3 MM one of which was previously impregnated with a 10^{-2}M solution of *p*-nitrophenyl phosphate and the other with a 10^{-2}M solution of adenosine 3′-phosphate. Both strips were incubated in a moist chamber at 37°C in horizontal position for 30 min and detected with ammonia vapours in the case of *p*-nitrophenyl phosphate (yellow spot at the site of activity), or with the molybdate reagent (described below) in the case of Ap (20 min at 60°C in a moist chamber; blue spot at the site of activity). The following proteins were used as standards: cytochrome C, MW 12400, chymotrypsinogen A 25000, ovalbumin 45000, serum albumin 67000 and aldolase 147000. They were detected directly on the plate using chlorine gas (15 min), short drying and spraying with 1% solution of potassium iodide and 1% of starch (blue spots at the sites of proteins).

Protein content determination. This was carried out according to Warburg (see[7]) from the ratio of absorbances at 260 and 280 nm.

Determination of optimum conditions for affinity chromatography. Tested solution (1 ml), and a corresponding buffer (3 ml) were added to 0·5 ml of carrier equilibrated with 0·02M buffer sodium acetate–acetic acid from pH 4—6·5 at 0·5 pH intervals, Tris-HCl from pH 7—9 at 0·5 pH intervals, and the suspension was shaken at 5°C for 1 h. After centrifugation (3000 rpm) an aliquot (25 µl) of supernatant was used for the determination of phosphomonoesterase activity. For PSPP-Sepharose-4B the maximum absorption of the enzyme is at pH 5·0, for AHAPS-Sepharose-4B at pH 5·5.

Determination of phosphomonoesterase activity. *a*) With *p*-nitrophenyl phosphate: the tested solution (25 µl) was added to 100 µl of a solution of 0·25 mg of disodium salt of *p*-nitrophenyl phosphate in 0·2M sodium acetate–acetic acid buffer of pH 5·5 and the mixture was incubated at 37°C for 15 min. Then 3 ml of 0·1M sodium hydroxide were added and absorbance was measured at 400 nm. A blank was carried out in the same manner in the absence of the enzyme and the content of the liberated *p*-nitrophenol was read from the calibration curve. *b*) With adenosine 3′-phosphate: the tested solution (25 µl) was added to 100 µl of a solution of 0·25 mg of adenosine 3′-phosphate (free acid) in 0·2M sodium acetate–acetic acid buffer of pH 5·5 and the mixture was incubated at 37°C for 15 min. The molybdate reagent (3 ml; prepared as 0·25% of ammonium molybdate and 1% of ascorbic acid in 0·66M sulfuric acid) was then added and the mixture allowed to stand at 27°C for 2 h. Absorbance was measured at 820 nm. A blank was carried out in the same manner in the absence of the enzyme and the liberated inorganic phosphate was read from the calibration curve estimated with potassium dihydrogen phosphate.

Determination of the effect of pH *on enzymatic activity* (Fig. 3) was carried out under standard conditions with *p*-nitrophenyl phosphate and adenosine 3′-phosphate in 0·2M buffers (see the determination of the binding onto modified Sepharose) at 0·5 pH intervals. Incubation time 30 min at 37°C with 2·8 µg of protein per assay.

Determination of influence of ions and further substances (Table I) was carried out under standard conditions in the presence of corresponding effectors, using 2·8 μg of protein per assay. Incubation was carried out at 37°C for 30 min.

Determination of the effect of temperature (Fig. 4) was carried out with adenosine 3′-phosphate under standard conditions, using 7 μg of protein per assay. Incubation lasted 15 min at 0°C, or at 20—70°C (by 10°C steps).

Determination of thermal stability (Fig. 5) was carried out with adenosine 3′-phosphate and 3·5 μg of protein per assay, under standard conditions. Preincubation from 0 to 120 min at 60°C in 0·02M buffer of pH 5·5, or 8·5, the samples were withdrawn at 10 min intervals (0—30min), or 30 min intervals (30—120 min), time of determination 15 min, at 37°C.

Determination of kinetic parameters (Fig. 6, Table II). The incubation mixture contained 0·5 to 20 μmol of substrate and 2·8 or 14 μg of protein in 125 μl of the 0·2M sodium acetate–acetic acid buffer of pH 5·5 and the incubation lasted 15, 30, and 60 min at 37°C. The extent of the cleavage was determined by measuring the liberated inorganic phosphate. In the case of adenosine 3′-phosphate methyl ester and adenosine 2′(3′)-methanephosphonate 100 μl of the mixture were separated by electrophoresis in system E1. The spots of the nucleoside were eluted with 5 ml of 0·10M-HCl and absorbance was measured ar 260 nm ($\varepsilon_{260} = 14000$).

Determination of substrate specificity (Table III—V) was carried out under standard conditions with 0·25 μg of lithium or ammonium salt of the substrate and 14 μg of protein per assay; incubation time 30 min, 1 h and 24 h, at 37°C. Aliquots of the mixtures were separated by electrophoresis in E1 and the spots of the starting substances and of the nucleosides were eluted with 5 ml of 0·1M-HCl. Absorbance was measured at 260 nm. Blanks were carried out in the same manner in the absence of enzyme.

Procedure of Isolating the Enzyme

Chromatographic separation of aqueous extracts of rape seedlings was described in the preceding paper[1]. Fraction 310—330 (400 ml, fraction B) of the extract from 1 kg of seedlings, containing phosphomonoesterase activity (after chromatography on modified Cellex P), was used for further purification steps: the fraction was saturated to 80% with ammonium sulfate, the precipitate was separated by centrifugation (30 min, 3000 rpm), dissolved in 100 ml of 0·02M sodium acetate–acetic acid buffer of pH 5·0, then dialysed continuously against 10 l of the same buffer and finally concentrated by ultrafiltration to 51 ml. This solution was applied onto a column of PSPP-Sepharose 4B and eluted first with 40 ml of the starting buffer, then with a linear gradient of 0—1M sodium chloride in the same buffer (2 × 500) and the elution course was followed by measuring absorbance at 280 nm and activity on Ap and *p*-nitrophenyl phosphate. Fractions 98—130 (126 ml) (fraction B1) were combined, dialysed continuously against 5 l of the starting buffer (pH 5·5) and concentrated by ultrafiltration to 20 ml. The elution course is shown in Fig. 1. This solution was applied onto a column of AHAPS-Sepharose-4B and eluted first with the starting buffer (pH 5·5, 63 ml) and then with 0—1M-sodium chloride gradient in the same buffer (2× 250 ml). The chromatography was monitored in the same manner as described above (Fig. 2). Fractions 45—57 were combined, dialysed continuously against the starting buffer (pH 5·5) and concentrated by ultrafiltration to 20 ml. This fraction (B2) was used for the determination of individual parameters and the substrate specificity of the enzyme. The course of the purification is shown in Table VI.

RESULTS AND DISCUSSION

Partial separation of crude aqueous extract of rape seedlings takes place on Cellex P modified with tert-butyl chloride. Elution with a linear gradient of sodium chloride affords the main part of phosphomonoesterase activity (identical both with respect to *p*-nitrophenyl phosphate and adenosine 3'-phosphate) at an approx. 0·3M-NaCl concentration. For further purification, the enzyme affinity towards thiophosphoric acid derivatives was made use of, which are according to preliminary experiments resistent to the enzyme. The first carrier used for this purpose is Sepharose 4B containing linked *p*-aminophenyl(phenyl) thiophosphate; this material was described and used earlier for the purification of phosphodiesterase from snake venom[6] and also in our preceding paper[1]. After removal of ballast protein by elution with the starting buffer, phosphomonoesterase activity was eluted with the linear gradient of sodium chloride (Fig. 1). This fraction was further purified by a second affinity chromatography on a new type of affinity support, Sepharose 4B with linked N^6-(6--aminohexyl)adenosine-2(3')-thiophosphate (AHAPS-Sepharose).

The appropriate ligand was prepared from N^6-(6-aminohexyl)-adenosine obtained from 6-chloropurine riboside[3] on reaction with hexamethylenediamine[8]. The nucleoside was first reacted with di-n-butyltin oxide[9] and cyclic intermediate formed then afforded on reaction with thiophosphoryl chloride in the presence of tri-n-butylamine and subsequent alkaline hydrolysis N^6-(6-aminohexyl)adenosine 2'(3')-thiophosphate. After deionization and chromatography on DEAE-cellulose the material was homogeneous in paper chromatography and electrophoresis, affording positive

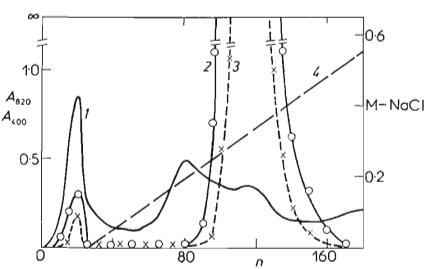

FIG. 1

Separation of Fraction B on PSPP-Sepharose 4B

1 Total protein (A_{280}), *2* cleavage of Ap (A_{820}), *3* cleavage of *p*-nitrophenyl phosphate (A_{400}), *4* NaCl concentration.

reaction for thiophosphates[10]. It was also completely resistent to the effect of bacterial phosphomonoesterase. Chromatography in a borate system (S2) showed the absence of the 5'-isomer. This material was then bound under the usual conditions onto Sepharose 4B, activated with cyanogen bromide[5]. Chromatography of the above partly purified fraction (B1) of phosphomonoesterase on this carrier (Fig. 2) eliminated further inactive protein fractions and afforded on elution with a sodium chloride gradient a preparation of phosphomonoesterase free of RNAse, DNase, phosphodiesterase, adenosine aminohydrolase and nucleoside hydrolase activities. The purification degree achieved was 270 with respect to the starting extract, the total yield corresponded to about 16% of the original activity. This material (B2) was also used for all further studies.

The molecular weight of the enzyme was determined by thin-layer chromatography on Sephadex G-100 (superfine) and the enzyme was localized by detection of the proteins on the one hand and, indirectly, by imprinting it on a chromatographic paper impregnated with a solution of both substrates and identification of *p*-nitrophenol or inorganic phosphate, respectively. On comparison with standard samples of proteins the molecular weight was estimated at 13000. According to the mentioned criteria the enzymatic activity is homogeneous, but the preparation contains inactive proteins.

The pH optimum is in the region of 5·0 for *p*-nitrophenyl phosphate and 5·5 for adenosine 3'-phosphate (Fig. 3), the temperature optimum of the enzyme (Fig. 4) is between 60 and 70°C. The thermal stability curve (Fig. 5) is almost identical at two

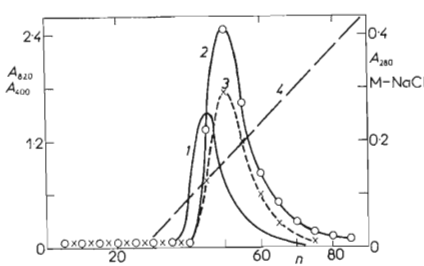

FIG. 2

Separation of Fraction B1 on AHAPS-Sepharose 4B

1 Total protein (A_{280}), *2* cleavage of Ap (A_{820}), *3* cleavage of *p*-nitrophenyl phosphate (A_{400}), *4* NaCl concentration.

selected pH values (5·5 and 8·5): the enzyme loses 50% of its activity at 60°C within about 20 min. At 4°C and pH 5·5 the enzyme is stable at least for 6 months without any appreciable loss of activity. Lyophilization does not cause any substantial loss in activity either (10%). The presence of magnesium ions or of other bivalent metals, or of EDTA, does not substantially activate or inhibit the enzyme. A strong inhibition is displayed only by fluoride ions, while inorganic phosphate has medium activity.

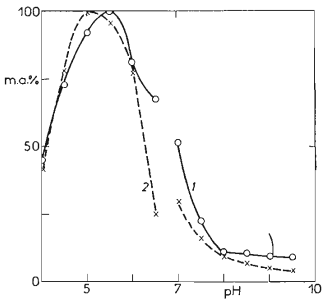

FIG. 3

pH-Dependence of the Cleavage Rate of Ap *1* and *p*-Nitrophenyl Phosphate *2* with Phosphomonoesterase

$v_{max} = 4{\cdot}35 \cdot 10^{-9}$ mol min^{-1} for [Ap] = $= 7{\cdot}2 \cdot 10^{-3}$M; $v_{max} = 5{\cdot}86 \cdot 10^{-9}$ mol . . min^{-1} for [*p*-nitrophenyl phosphate] = $= 9{\cdot}1 \cdot 10^{-3}$ m.

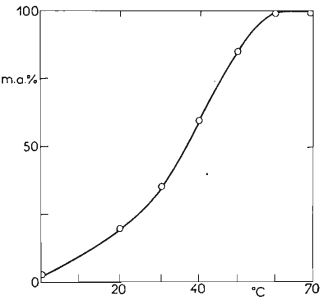

FIG. 4

Temperature Dependence of the Cleavage Rate of Ap

$v_{max} = 15{\cdot}5 \cdot 10^{-9}$ mol min^{-1} for [Ap] = $= 7{\cdot}2 \cdot 10^{-3}$M.

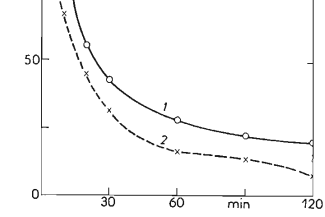

FIG. 5

Thermal Stability of Phosphomonoesterase at 60°C

1 at pH 5·5, *2* at pH 8·5; $v_{max} = 7{\cdot}1 \cdot 10^{-9}$ mol min^{-1} for [Ap] = $7{\cdot}2 \cdot 10^{-3}$M.

Nor does the presence of inactivating reagents of the type of S-reagents (*p*-chloromercuribenzoate, dithiothreitol and chloroacetic acid) and non-ionogenic detergents (Table I) have any effect whatever. The specificity of the enzyme was studied at approximately optimum conditions (pH 5·5). In a series of isomeric ribo- and 2′-deoxyribonucleotides (Table III) the enzyme is non-specific with respect to the character of the base and the position of the bound phosphoric acid residue. The reaction rate (extent of cleavage) is affected, however, and it is higher in ribo- than in 2′-deoxyribonucleotides. Also in the ribo series it applies that 5′-NMP = 2′-NMP < 3′-NMP,

TABLE I
The Effect of Effectors on Acid Phosphomonoesterase

Substance	—log M	Relative initial rate[a]
None		1·00
Cu^{2+}	3·7	0·63
Ag^{+}	3·7	1·06
Mg^{2+}	2·7	1·06
Ca^{2+}	2·7	1·07
Zn^{2+}	3·7	0·72
Hg^{2+}	3·7	0·77
Mn^{2+}	2·7	1·00
Fe^{3+}	3·7	1·11
Co^{2+}	3·7	1·05
NaF	2·7	0·42
NaN_3	2·7	0·99
KH_2PO_4	2·7	0·82[b]
	0·7	0·10[b,c]
EDTA	2·7	1·08
p-Chloromercuribenzoate	2·7	0·89[d]
Dithiothreitol	2·7	0·80[d]
Chloroacetic acid	2·7	0·99[d]
Tween 80	0·12%	0·97
Triton X-100	0·12%	0·90
Adenosine	4—2	1·00
Uridine 5′-thiophosphate	2	0·73[b]

[a] $v_0 = 3{\cdot}67 \cdot 10^{-9}$ mol min^{-1} for $s = 7{\cdot}2 \cdot 10^{-3}$M; [b] assayed with 14 μg of protein, separation in E1; [c] assayed in 0·2M phosphate pH 5·5; [d] enzyme preincubated in 0·02M-TRIS pH 8·0 for 4 h at 5°C.

while in the series of 2′-deoxyribonucleotides the dependence is reversed: 5′-dNMP > > 3′-dNMP. The nucleotide derivatives with a modified sugar residues (Table IV) are also hydrolysed by the enzyme. In addition to nucleotides, the enzyme also dephosphorylates aryl- and alkyl phosphates even though in this case the reaction rate is much slower than in the case of nucleotides. The achiral type of the interaction of the enzyme with the substrate also follows from the behaviour of L-nucleotide derivatives which are good substrates.

In the series of nucleotide analogues modified on the ester-bound phosphoric acid residue (Table V) the enzyme hydrolyses 3′-isomers only (the 5′-isomers are resistent): a cleavage takes place with esters of phosphorous acid and methanephosphonic acid, as well as with alkyl esters of 3′-nucleotides. Adenosine 5′-phosphate is cleaved under temporary formation of adenosine 5′-monophosphate, *i.e.* under cleavage of the diphosphate bond; the cleavage of inorganic pyrophosphate

TABLE II

Kinetic Parameters of Phosphomonoesterase Hydrolysis

Compound	Kinetic type[a]	$V_{max} \cdot 10^{10}$ mol/μh^{-1} min^{-1}	$K_m \cdot 10^3$ M	$S_{v_0 max} \cdot 10^3$ [b] M	$v_0^{max} \cdot 10^{10}$ [c] mol/μg^{-1} min^{-1}
Up	B	15·2	2	33·0	14·0
Cp	B	17·7	1·6	28·0	16·7
Ap	B	20·6	1·6	24·0	19·0
Gp	B	11·7	4·1	30·0	9·9
C-2′-p	A	6·3	10·1	—	—
UMP	B	2·3	5·0	17·0	1·7
CMP	A	3·7	6·0	—	—
AMP	A	3·5	3·4	—	—
GMP	B	3·2	4·0	15·0	2·4
dTp	B	1·6	3·9	27·0	1·3
dAp	B	2·6	3·5	17·0	2·1
dUMP	B	2·0	5·8	18·0	1·4
dTMP	B	1·4	3·3	23·0	1·2
dCMP	B	5·7	8·4	77·0	4·8
dAMP	B	2·9	3·6	13·0	2·2
ApMe[d]	A	2·7	8·1	—	—
Ap_cMe[e]	A	1·3	1·0	—	—
p-Nitrophenyl Phosphate	B	22·2	1·1	24·0	21·3

[a] A linear dependence $1/v \sim 1/s$; B substrate inhibition type [b] substrate concentration at v_0^{max}; [c] maximum v_0; [d] adenosine 3′-phosphate methyl ester; [e] adenosine 2(3′)-methanephosphonate

to orthophosphate also corresponds to this finding. Adenosine 5'-triphosphate also affords 5'-monophosphate as an intermediate, but an intermediary formation of ADP or inorganic pyrophosphate could not be demonstrated. Finally, the esters of thiophosphoric and sulfuric acids are completely stable against the effect of the enzyme.

The study of the kinetics of the hydrolysis of the phosphomonoester bond permits the classification of natural substrates into two types: a linear dependence $1/v \sim 1/s$ is displayed by 5'-CMP, 5'-AMP and 2'-CMP only (the analogues also include methyl ester of adenosine 3'-phosphate and adenosine 2'(3')- methanephosphonate) (Fig. 6c), while the absolute majority of nucleotides (all 3'-ribonucleotides, all 2'-deoxyribonucleotides, 5'-UMP and 5'-GMP) and *p*-nitrophenyl phosphate clearly

TABLE III

Substrate Specificity of Acid Phosphomonoesterase Towards Ribo- and 2'-Deoxyribonucleoside Derivatives

Compound	Splitting, % 30 min	Compound	Splitting, %		
			30 min	60 min	24 h
Ap	69	dAMP	22	—	—
Gp	60	dGMP	33	—	—
Up	65	dUMP	19	—	—
Cp	64	dCMP	28	—	—
C-2'-p	31	dTMP	11	—	58
A-2'(3')p	47[a]	dAp	22	—	—
G-2'(3')p	49	dGp	0	±	40
U-2'(3')p	43	dUp	0	0	38
C-2'(3')p	46	dCp	0	0	46
AMP	26	dTp	0	16	47
GMP	34	RNA yeast	0	0	0
UMP	30	DNA thymus	0	0	0
CMP	27				

[a] Products separated in S3; $v_0^{2'p} : v_0^{3'p} = 1 : 6$; v_0 initial velocity.

FIG. 6

Kinetics of the Cleavage with Phosphomonoesterase

a: *1* dTp, *2* UMP, *3* dAp, *4* dAMP, *5* GMP; *b*: *1* Gp, *2* Up, *3* Cp, *4* Ap, *5* *p*-nitrophenyl phosphate; *c*: *1* adenosine 2'(3')-methanephosphonate, *2* adenosine 3'-phosphate methyl ester, *3* CMP, *4* AMP, *5* C-2'-*p*, *6* dCMP; *d*: *1* dTMP, *2* dUMP.

TABLE IV

Specificity of Acid Phosphomonoesterase Towards Sugar Modified Nucleotides and Non-Nucleotidic Phosphomonoesters

Compound	Splitting,%	
	1 h	24 h
L-Adenosine 3′-phosphate[19]	32	73
L-Adenosine 5′-phosphate[20]	23	72
1-(α-L-Lyxofuranosyl)uracil 3′-phosphate[21]	±	76
1-(β-D-Arabinofuranosyl)cytosine 3′-phosphate[22]	53	76
1-(β-D-Arabinofuranosyl)cytosine 5′-phosphate[23]	±	100
1-(β-D-Ribopyranosyl)uracil 2′(3′)(4′)-phosphate[24]	42[a]	64
1-(β-D-Ribopyranosyl)adenine 2′(3′)(4′)-phosphate[24]	33[b]	87
1-(2,3-Dihydroxypropyl)cytosine 3′-phosphate	25	70
p-Nitrophenyl phosphate	56	
Phenyl phosphate	34	
α-Naphthyl phosphate	25	
Glycero-1-phosphate	10	
Glucose 6-phosphate	8	

[a] v_0 (2′ : 3′ : 4′) = 3·3 : 1 : 5; [b] v_0 (2′ : 3′ : 4′) = 2·4 : 1 : 3·3.

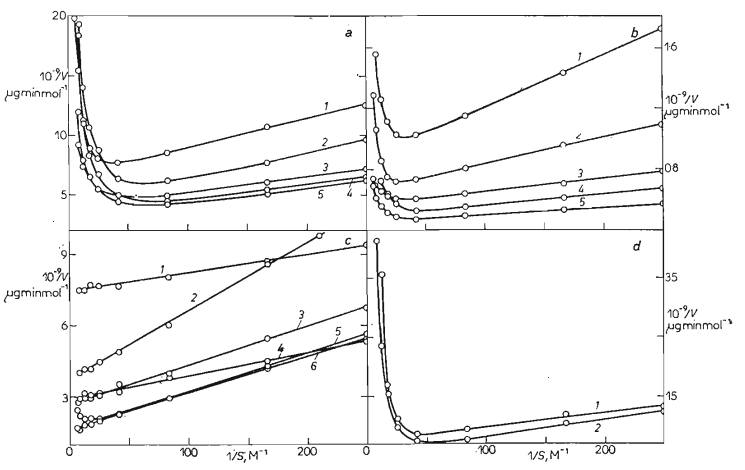

TABLE V

Substrate Specificity of Acid Phosphomonoesterase Towards Phosphorus-Modified Nucleotides

Compound	Splitting, %	
	1 h	24 h
Uridine 2'(3')-phosphite[25]	22	52
Cytidine 2'(3')-phoshite[25]	±	66
Adenosine 2'(3')-phosphite[25]	21	55
Adenosine 2'(3')-methanephosphonate[26]	22	66
Adenosine 5'-methanephosphonate[26]	0	0
Adenosine 5'-hydroxymethanephosphonate[26]	0	0
Adenosine 5'-aminomethanephosphonate[27]	0	0
Adenosine 5'-phosphoramidate	0	0
Adenosine 5'-phosphoromorpholidate	0	0
Adenosine 3'-phosphate methyl ester[23]	27	53
Adenosine 3'-phosphate n-butyl ester[23]	14	27
Adenosine 2'-phosphate methyl ester[23]	0	0
Adenosine 5'-phosphate methyl ester[23]	0	0
Uridine 3'phosphate methyl ester[23]	20	44
2'-Deoxyuridine 5'-phosphate methyl ester[23]	0	0
Adenosine 2'(3')-thiophosphate[28]	0	0
Uridine 2'(3')-thiophosphate[28]	0	0
Uridine 5'-thiophosphate[28]	0	0
Guanosine 5'-sulphate[29]	0	0
Adenosine 5'-diphosphate	72[a]	100[b]
Adenosine 5'-triphosphate	68[a]	100[b]
Inorganic diphosphate	80	—

[a] Products AMP and Pi separated in E2; [b] products AMP, Ado and Pi separated in E2.

TABLE VI

Course of the Purification of Acid Phosphomonoesterase

Degree	Protein mg	Activity EU	Spec. activity EU/mg	Yield %	Degree of purification
Crude extract	18 750	294	0·016	100	1
Modified Cellex P	306	183	0·61	62	38·2
PSPP-Sepharose 4B	25·8	88	3·42	30	214
AHAPS-Sepharose 4B	11·2	48	4·30	16	269

display inhibition at high substrate concentration (Fig. 6*a*,*b*,*d*). There is evidently a combined type of inhibition, since the plots obtained do not correspond to the forms derived mathematically for the inhibition of the substrate type[11,12]. From the results mentioned earlier (Table I) it seems probable that in this case we are not concerned with a combination with the product inhibition, because the inorganic phosphate is only a weak inhibitor, whereas nucleosides do not inhibit at all. Apparent extrapolated values of K_m and V_m, as well as the values of the concentrations of maximum attainable initial rate are shown in Table II.

From these data it follows that the affinity for the enzyme of all substances studied is approximately equal, while the initial rate of cleavage of 3'-ribonucleotides is distinctly higher in comparison with other nucleotides. A similar preference was also observed in the case of acid phosphomonoesterase from human prostate[13].

The results presented characterize the isolated enzyme as a non-specific acid phosphomonoester hydrolase (EC 3.1.2.2). The activity on alkyl- or aryl esters and nucleoside derivatives of phosphoric acid coincides in all steps of the purification, and the ratio of both activities does not change in the course of purification. Hence, it may be assumed that this is the dual activity of a single enzyme. The enzyme is thermolabile, its pH optimum is in the acid region and it evidently does not contain free SH groups or disulfidic bridges either in the region of the active centre. The bond with the substrate is of achiral type and in addition to monoester bonds of phosphoric acid the ester bonds of other acids of phosphorus with a small substituent on the phosphorus atom can also be hydrolysed, as for example in the esters of phosphorous and methanephosphonic acids, or also in phosphoric acid diesters with at least one alkyl group. However, the internucleotidic bonds are not cleaved by the enzyme.

In addition to specific nucleotidases (for example from wheat leaves[14] or seedlings[15] and from ryegrass[16]) several non-specific phosphomonoester hydrolases with an acid pH optimum were also isolated from plant material; the enzyme from lupine seedlings[17] and tobacco leaves[18] are strongly inhibited by fluoride ions, similarly as acid phosphomonoester hydrolase from rape seedlings, and they do not require the presence of metal cations. However, nothing is known regarding the substrate specificity of the two last mentioned enzymes[17,18], especially with respect to the esters of acids of phosphorus other than phosphoric acid. It cannot be excluded that in these enzymes the situation is similar as in the enzyme from rape seedlings.

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