NEW PYRIMIDINE (URIDINE) SPECIFIC CYCLIZING 2'-RIBONUCLEOTIDYLTRANSFERASE AND NONSPECIFIC DECYCLIZING 2',3'-PHOSPHODIESTERASE*

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Chromatography of an aqueous extract of rape seedlings on modified Cellex P, followed by chromatography on two types of modified Sepharose 4B, afforded two nucleolytic activities which split ribonucleoside 2',3'-cyclic phosphates. The first enzyme (Cl) affording 2'-nucleotides on splitting, was characterised as a decyclizing 2',3'-phosphodiesterase, nonspecific towards the nature of the heterocyclic base. The second enzyme (D2) splits uridine 2',3'-cyclic phosphate and cytidine 2',3'-cyclic phosphate with high preference for the uridine derivative, its specificity towards the heterocyclic base and the sugar moiety resembling that of pancreatic ribonuclease. The products are in both cases 3'-ribonucleotides. The enzyme splits also alkyl esters of uridine 3'-phosphate with the intermediary formation of uridine 2',3'-cyclic phosphate. The splitting rate decreases with growing alkyl moiety. This enzyme is classified as a new pyrimidine specific cyclizing 2-ribonucleotidyltransferase (EC 2·7·7). Both enzymes were isolated free of other nucleolytic activities. In the native state they are stabilised by an acidic protein.

In our previous communications[1-3] we described the isolation and identification of some nucleolytic enzymes contained in rape pollen extract. Among the most significant were the following activities: a non-specific ribonuclease, RNA-hydrolase, specific 3'-nucleotidase, and, as the main activity, a decyclizing phosphodiesterase, producing 2'-ribonucleotides. In connection with this study, it appeared of interest to compare nucleolytic activities found in various evolutional stages of the same plant, *i.e.* the rape (*Brassica napus* L.). We first analysed the nucleolytic enzymes present in the aqueous extract of rape seedlings. The weakly acidic aqueous extract contained three principal groups of nucleolytic activities: phosphomonoesterases, nucleases and decyclizing phosphodiesterases which were identified by splitting of uridine and adenosine 2',3'-cyclic phosphates. This work concerns isolation and characterisation of the last mentioned group of enzymes of the aqueous extract from rape seedlings.

EXPERIMENTAL

The paper chromatography was carried out on a paper Whatman No 3MM in the system 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2), paper electrophoresis on the same paper in 0·1M

triethylammonium hydrogen carbonate (pH 7·5) at 20 V/cm during 1 h. The compounds were detected by UV absorption (Chromatolight).

Preparation of Modified Cellex P (*cf.*[4])

Tert-butyl chloride (450 ml) was added dropwise during 2·5 h to a stirred suspension of Cellex P (Biorad; 180 g) in pyridine (3·6 l) and the mixture was refluxed for 1 h (bath temperature 130°C). Water (600 ml) was then added dropwise, the mixture was stirred for 30 min, filtered while hot (70—80°C) and the material on the filter washed with water until the filtrate exhibited no UV absorption. Preparation of the O-phenylthiophosphoryl-O-phenyl-*p*-NH-modified Sepharose 4B ("PSPP-Sepharose 4B") was carried out according to the described procedure[5].

Preparation of Uridine-5′-thiophosphoryl-O-phenyl-*p*-NH-Sepharose 4B ("USPP-Sepharose 4B")

Imidazole (3·0 g; 40 mmol) was added to a solution of *p*-nitrophenylthiophosphoryl dichloride[6] (2·72 g; 10 mmol) in acetonitrile (70 ml) and the mixture was stirred for 20 min at room temperature in a closed flask. 2′,3′-O-Isopropylideneuridine[7] (3·0 g; 10·6 mmol) and acetonitrile (30 ml) were then added and the stirring was continued for 2 days at room temperature. The suspension was filtered, the solid washed with acetonitrile (20 ml) and the filtrate taken down *in vacuo*. The residue was refluxed with 80% acetic acid (100 ml) for 45 min, the mixture was taken down, the residue coevaporated with water (3 . 50 ml), taken up in water (100 ml), made alkaline with triethylamine (pH 9) and extracted with ether (2 . 100 ml). The aqueous layer was concentrated *in vacuo* at 40°C to about 50 ml and applied to a column (80 × 4 cm) of DEAE cellulose (Cellex D, HCO_3-form). The column was washed with water (3 ml/min) till the absorption of the neutral fraction dropped and then eluted with 3 l of water and 3 l of 0·1M triethylammonium hydrogen carbonate pH 7·5 (linear gradient) under the above-mentioned conditions (fraction 10 min). The product fractions were combined, taken down *in vacuo*, the residue was coevaporated with ethanol (4 . 20 ml), dissolved in a minimum amount of water and applied to a column of Dowex 50X8 (Na^+-form; 50 ml). The UV-absorbing fraction was eluted and the solvent evaporated *in vacuo*, affording chromatographically (R_F 0·57) and electrophoretically (E_{Up} 0·45) uniform sodium salt of uridine 5′-thiophosphate *p*-nitrophenyl ester.

This product was hydrogenated at room temperature and ordinary pressure in water (100 ml) over 5% Pd/C (Merck; 1·6 g) until the hydrogen consumption ceased (130 ml). The suspension was filtered through Celite which was then washed with water and the filtrate (neutral reaction) was taken to dryness *in vacuo*, leaving chromatographically homogeneous (R_F 0·33) sodium salt of uridine 5′-thiophosphate *p*-aminophenyl ester (positive thiophosphate reaction[8]).

A solution of this product in ice-cold 0·1M sodium hydrogen carbonate solution (10 ml; pH 9·0) was added under stirring to Sepharose 4B (100 ml), activated with cyanogene bromide (15 g) under standard conditions[9], suspended in the same buffer (100 ml), and the stirring was continued at 4°C overnight. The suspension was filtered, washed with the same ice-cold buffer and equilibrated with 0·2M-Tris at room temperature overnight. The thus-obtained material was used in the affinity chromatography.

Column Chromatography

Modified Cellex P: column 50 × 4·5 cm (700 ml), equilibration with 0·02M sodium acetate–acetic acid, pH 5·0, elution rate 14·4 ml/h, fractions were taken at 1 h intervals.

O-*Phenylthiophosphoryl*-O-*phenyl*-p-NH-*Sepharose* 4B ("PSPP-Sepharose 4B"): column 21·5 × × 1·8 cm (55 ml), equilibration with 0·02M Tris-HCl buffer, pH 7·9, elution rate 13·2 ml/h, fractions taken at 15 min intervals.

Uridine-5'-thiophosphoryl-O-*phenyl*-p-NH-*Sepharose* 4B ("USPP-Sepharose 4B"): column 25 × × 1·8 cm (about 65 ml), equilibration with 0·02M-Tris-HCl, pH 7·4, elution rate 13·8 ml/h, fractions at 15 min intervals.

The chromatography was performed at 5°C and the fractions were dialysed and concentrated by ultrafiltration on a UM 10 (Amicon) filter under nitrogen at 5°C.

Enzyme Assays

Molecular weight determination by gel filtration was performed on a 100 × 2 cm column of Sephadex G-200 (40—120 μ) in 0·05M TRIS-HCl, pH 7·5, with 0·05M sodium chloride; elution rate 11 ml/h (Blue Dextran, $V_0 = 113·5$ ml).

Isoelectric focusation was carried out on an LKB Ampholine Electrofocusing Column (volume 110 ml, ampholite carrier CA 3·5—10, 36 h, end voltage 1 000 V, current 1 mA, 8°C).

Determination of enzymatic activity towards uridine or adenosine 2',3'-cyclic phosphates. The studied fraction (25 μl) was added to a solution of lithium salt (0·25 mg) of the substrate in 0·2M Tris-HCl, pH 7·5 (100 μl) (with 1 mM EDTA for the uridine derivative) and the mixture was incubated at 37°C. Incubation time: 30 min for uridine 2',3'-cyclic phosphate, 120 min for adenosine 2',3'-cyclic phosphate. The mixture was analysed by paper electrophoresis, the spots were eluted with 0·1M-HCl (10 ml) and the extinction at 260 nm was measured.

Determination of Phosphomonoesterase Activity

a) *Towards* p-*nitrophenyl phosphate*: The studied solution (25 μl) was added to a solution (100 μl) of disodium salt of *p*-nitrophenyl phosphate (0·26 mg) in 0·2M sodium acetate–acetic acid buffer, pH 5·5. After incubation at 37°C for 15 min, 0·1M sodium hydroxide solution (3 ml) was added and absorbance at 400 nm was measured. The control experiment was carried out similarly but in the absence of the enzyme.

b) *Towards uridine 3'-phosphate and adenosine 3'-phosphate*: The studied solution (25 μl) was added to a solution of adenosine 3'-phosphate (0·25 mg) or lithium salt of uridine 3'-phosphate (0·25 mg) in 0·2M sodium acetate–acetic acid buffer, pH 5·5 (100 μl) or in 0·2M Tris-HCl buffer, pH 8·5 (100 μl), and incubated at 37°C for 30 min. After addition of 0·25% ammonium molybdate and 2% ascorbic acid solution in 0·66M sulfuric acid (3 ml) the mixture was set aside for 2 h at 37°C and its absorbance at 820 nm was measured. Control experiments were performed in the absence of enzyme under otherwise identical conditions; the inorganic phosphate content was determined using a calibration curve based on potassium hydrogen phosphate.

Protein determination was carried out according to Warburg[10] from the ratio of absorbances at 260 nm and 280 nm.

Determination of optimum conditions for affinity chromatography. The studied solution (1 ml), followed by the appropriate buffer (3 ml) was added to the carrier (0·5 ml), which had been equilibrated with a 0·02M buffer (pH 4·0, 4·5, 5·0, 5·5, 6·0, 6·5: sodium acetate–acetic acid; pH 7·0, 7·5, 8·0, 8·5, 9·0: Tris-HCl). The suspension was shaken at 5°C for 1 h, centrifuged and an aliquot (25 μl) of the supernatant was used in the determination of activity by the above-mentioned procedure. For the modified Cellex P the maximum adsorbtion was at pH 5·0, for PSPP-Sepharose 4M and USPP-Sepharose 4B at pH 7·3—8·0.

Effect of ions and other compounds on the enzymatic activity was determined according to the general method (*vide supra*) with the protein (0·25 μg) of the pertinent fraction in the presence of the corresponding effectors. Incubation time 30 min.

Effect of EDTA on the enzymatic activity was determined under standard conditions in a buffer, 10^{-8} to 10^{-1}M in EDTA (final concentration), with 0·15 μg/ml of protein per assay. Incubation time 30 min.

Effect of pH on the enzymatic activity was determined under standard conditions with uridine 2′,3′-phosphate or uridine 3′-phosphate methyl ester in 0·2M buffers (0·5 pH steps; pH 4—6·5 sodium acetate–acetic acid, pH 7·0—9·0 Tris-HCl), contingently with 10^{-3}M-EDTA. Incubation time 30 min at 37°C with 0·18 to 14·4 μg of enzyme protein per assay.

Effect of temperature on the enzymatic activity was determined under standard conditions with uridine 2′,3′-cyclic phosphate (0·18 μg of protein per assay). Incubation time 30 min at 0°C, 20—70°C (in 10°C steps) in the presence of 10^{-3}M-EDTA.

Thermal stability was determined with uridine 2′,3′-cyclic phosphate under standard conditions at pH 7·5 in the presence of 10^{-3}M-EDTA and 0·22 μg of the enzyme protein in 0·2M buffer, pH 7·5 (25 μl) or in 0·02M buffer, pH 4·5, preincubated at 60°C for 0—90 min (the samples were withdrawn at 10 min intervals). Incubation time 30 min at 37°C.

Substrate specificity was determined with 0·25 mg of substrate (ammonium or lithium salt) under standard conditions with 0·31 μg or 0·62 μg of protein per assay, incubation time 60 min or 24 h at 37°C in the presence of 10^{-3}M-EDTA. Blanks were performed under identical conditions but in the absence of the enzyme.

RNA-*Depolymerizing activity* was determined with 1 mg of high-molecular yeast RNA or sodium salt of polyU (Calbiochem) in 0·17M Tris-HCl buffer (600 μl) containing 3 mM EDTA, with 2·48 μg of enzyme protein per assay; incubation 30 min at 37°C. After addition of 300 μl of 0·75% uranyl acetate in 25% perchloric acid, the mixture was set aside for 2 h at 0°C, centrifuged, the supernatant diluted with 15 parts of water and the extinction at 260 nm measured. The blank was carried out under the same conditions but with addition of the enzyme after precipitation with uranyl acetate.

Determination of kinetic parameters: The incubation mixture (125 μl) was 10^{-2}M to $1{\cdot}5.10^{-1}$M in the substrate (uridine 2′,3′-cyclic phosphate. cytidine 2′,3′-cyclic phosphate, or methyl ester of uridine 3′-phosphate), 0·16M in Tris-HCl, pH 7·5, 0 or 10^{-3}M in EDTA, and contained 0·036 μg to 1·80 μg of protein per assay. Incubation time 10—45 min (the extent of splitting did not exceed 30%). The concentration of the substrate was determined spectrophotometrically at 260 nm (at 280 nm for the cytidine derivative).

Isomer Determination in the Products of Ribonucleoside 2′,3′-Cyclic Phosphates Splitting

a) *Cytidine 2′,3′-cyclic phosphate*: The analysis was performed on a 29 × 1·5 cm column of Dowex 1X8 ($HCOO^-$), equilibrated with 0·02M formic acid; elution rate 84 ml/h. The eluate (0·02M formic acid) was monitored continuously using a Uvicord (LKB) instrument. The amount applied was 3 mg of cytidylic acid, together with 5 mg of CMP as internal standard. The elution course is given in Fig. 9. The control chromatography of cytidine 3′-phosphate and cytidine 2′(3′)-phosphate was carried out under identical conditions.

b) *Adenosine 2′,3′-cyclic phosphate*: Paper chromatography in saturated ammonium sulfate–0·1M sodium acetate–2-propanol (79 : 19 : 2).

c) *Uridine 2',3'-cyclic phosphate*: Lithium salt of uridine 2',3'-cyclic phosphate (100 mg) was degraded by the enzyme fraction of phosphotransferase (10·7 μg of protein) in 0·1M Tris-HCl (1·5 ml), pH 7·5, at 37°C for 40 h. The mixture was chromatographed on two sheets of Whatman No 3MM paper and the bands of uridylic acid were eluted with dilute (1 : 10) ammonia (100 ml). The eluate was taken down *in vacuo*, dried at 0·1 Torr over phosphorus pentoxide and the residue was mixed with dimethylformamide (3 ml) and dihydropyran (1 ml). Then, 6M-HCl in dimethylformamide was added until the reaction was strongly acidic (moistened pH-paper), the mixture was stirred for 3 h at room temperature, made alkaline by addition of triethylamine and chromatographed on two sheets of paper Whatman No 3MM. The bands of the bis-tetrahydropyranyl derivative (R_F 0·50) were eluted with dilute ammonia (1 : 10; 100 ml), the eluate was taken down and the yield (145 μmol) determined spectrophotometrically. The residue was dissolved in 50% methanol (10 ml), triethylamine (0·5 ml) was added, the mixture was taken down and the residue dried at 0·1 Torr over phosphorus pentoxide. 2',3'-Di-O-benzoyluridine[11] (0·22 g; 0·5 mmol) and pyridine (2 ml) were added, the mixture was homogenised by shaking with glass beads, and treated with 2,4,6-triisopropylbenzenesulfonyl chloride (0·2 g; 0·66 mmol). After standing for 5 h under exclusion of moisture, 50% pyridine (3 ml) was added, followed after 30 min by 30% methanolic ammonia (100 ml). The mixture was set aside overnight, taken down and the residue was partitioned between water (50 ml) and ether (25 ml). The aqueous layer was concentrated *in vacuo* and chromatographed on two sheets of Whatman No 3MM paper. The product bands (UpU) (R_F 0·27, E_{Up} 0·36) were eluted with dilute (1 : 10) ammonia (50 ml), the eluate was evaporated *in vacuo* and the yield determined spectrophotometrically (94 μmol;65%).

This product (10 μmol) was incubated with pancreatic ribonuclease (3× crystallised; 100 μg) in 0·2M Tris-HCl (100 μl), pH 8·0, at 37°C for 60 min and the mixture was analysed by paper electrophoresis (*vide supra*). Degradation (94·6%) to uridine 3'-phosphate and uridine in the ratio 1 : 1·04.

Proof of Uridine 2',3'-Cyclic Phosphate Formation in the Splitting of Uridine 3'-Phosphate Methyl Ester by the Enzyme D2

The incubation mixture contained 0·25 mg of ammonium salt of uridine 3'-phosphate methyl ester and 14·4 μg of the protein D2 in 125 μl of 0·16M Tris-HCl buffer (pH 7·5). After incubation at 37°C for 30 min the mixture was analysed by paper electrophoresis (*vide supra*) using uridine 3'-phosphate, its methyl ester and uridine 2',3'-cyclic phosphate as standard samples (E_{Up}: uridine 2',3'-cyclic phosphate 0·60, UpMe 0·50). According to spectrophotometric analysis of the eluted spots of the products, the mixture contained 15·4% of Up, 16·0% of uridine 2',3'-cyclic phosphate and 68·6% of the starting compound.

Uridine 3'-Phosphate Methyl Ester

N,N'-Dicyclohexylcarbodiimide (4 g) was added to a solution of pyridinium salt of 2',5'-di-O-(1-ethoxyethyl)uridine 3'-phosphate[12] (prepared from 2 mmol of the calcium salt and dried by coevaporation with pyridine) in pyridine (20 ml) and methanol (20 ml) and the mixture was set aside in a stoppered flask at room temperature for 5 days. Water (3 ml) was added and after 30 min the mixture was taken down *in vacuo*. The residue was diluted with water (100 ml), filtered, extracted with ether (3 × 25 ml) and the aqueous phase was concentrated *in vacuo*. Chromatography on a column (80 × 4 cm) of DEAE cellulose (linear gradient 0—0·2M triethylammonium hydrogen carbonate, pH 7·5; 2 l) afforded 0·05—0·08M product which, after evaporation, was dried by coevaporation with ethanol and rechromatographed on 2 sheets of paper Whatman

No 3MM. Elution of the product bands (R_F 0·53, E_{Up} 0·52) with dilute ammonia (1 : 10, 50 ml), followed by concentration *in vacuo* and lyophylisation, gave ammonium salt of the product (400 mg) which was chromatographically and electrophoretically pure.

Preparation of Aqueous Extract of Rape Seedlings

Rape seeds (1 kg) were germinated for 4 days at room temperature in the dark on a moist filter paper (about 80% germination) after one day of washing by flowing tape water. Thus-obtained material was disintegrated in an ice-cold 0·3M sodium acetate–acetic acid buffer, pH 5·5 (3 l), using a Homogenisator 324 (Poland) disintegrator (14000 rpm, 5 min). The homogenisate was centrifuged first at 2000 × *g* (30 min at 2—3°C) and the supernatant at 10000 × *g* (30 min at 2—3°C). The supernatant was subjected to continual dialysis against 0·02M sodium acetate–acetic acid buffer, pH 5·0 (50 l). The obtained dialysed extract (1500 ml) was again centrifuged at 10000 × *g* (30 min, 2—3°C).

Isolation of Decyclizing Phosphodiesterase Activities from Aqueous Extract of Rape Seedlings

A) *Chromatography on modified Cellex P*: The aqueous extract from the preceding experiment (1500 ml) was applied on a column of modified Cellex P (*vide supra*) and eluted successively with 0·02M sodium acetate–acetic acid buffer, pH 5·0 (2500 ml), linear gradient of sodium chloride (0—0·7M) in the same buffer (2 × 2000 ml) and finally with 1M sodium chloride, again in the same buffer (2000 ml). UV-Absorbance (at 254 nm) of the eluate was followed and the fractions were tested on the splitting of uridine 2′,3′cyclic phosphate, adenosine 2′,3′-cyclic phosphate, uridine 3′-phosphate, adenosine 3′-phosphate and *p*-nitrophenyl phosphate at the above-mentioned pH values, using the standard methods. The distribution of the UV absorbances and activities towards the single substrates is shown in Fig. 1.

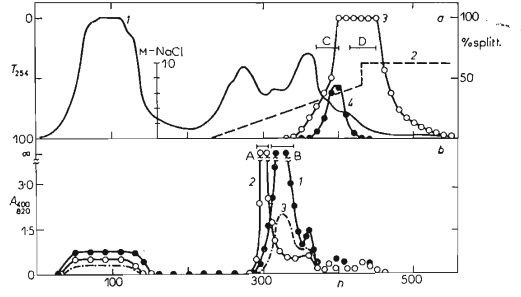

FIG. 1

Separation of Nucleolytic Activities on Modified Cellex P

a) *1* Total protein (T_{254}), *2* concentration of NaCl, *3* splitting of uridine 2′,3′-cyclic phosphate (pH 7·5), *4* splitting of adenosine 2′,3′-cyclic phosphate (pH 7·5); *b*) *1* splitting of Ap, Up (pH 5·5), *2* splitting of Ap, Up (pH 8·5), *3* splitting of *p*-nitrophenyl phosphate (pH 5·5).

Fractions 370—400 (600 ml; fraction C) exhibited an activity towards uridine 2',3'-cyclic phosphate and adenosine 2',3'-cyclic phosphate, fractions 414—450 (720 ml; fraction D) were active towards uridine 2',3'-cyclic phosphate. Both these fractions were precipitated with ammonium sulfate up to 80% saturation, left overnight at 0°C, the sediments were centrifuged at 2000 × *g* (30 min, 2—3°C), dissolved in 0·02M Tris-HCl buffer, pH 7·9 (100 ml), continually dialysed against the same buffer (20 l) and concentrated by ultrafiltration on a UM 10 filter (*vide supra*) to about 50 ml.

B) *Chromatography of the fraction* D *on* PSPP-*Sepharose* 4B: The concentrated dialysed fraction D (50 ml) was applied to a column of PSPP-Sepharose 4B (*vide supra*) and eluted, first with 0·02M Tris-HCl, pH 7·9 (160 ml), then with linear gradient 0—1M sodium chloride in the same buffer (2 × 500 ml). The fractions were tested on splitting uridine 2',3'-cyclic phosphate. Fractions 65—87 (78 ml) were combined (fraction D1), continuously dialysed against 0·02M Tris-HCl, pH 7·4 (10 l), and concentrated by ultrafiltration to 20 ml. The course of elution is depicted on Fig. 2.

C) *Chromat of ographythe fraction* D1 *on* USPP-*Sepharose* 4B: The fraction D1 from the pre-P ceding preparation was applied to a column of USPP-Sepharose 4B (*vide supra*). The elution was performed with 0·02M Tris-HCl, pH 7·4 (160 ml), followed by linear gradient 0—1M sodium chloride in the same buffer (2 × 500 ml). The elution course is depicted in Fig. 3; the eluate was tested for the activity towards uridine 2',3'-cyclic phosphate. Fractions 105—135 (130 ml, fraction D2) were combined, continuously dialysed against 10 l of 0·02M Tris-HCl, pH 7·5, and concentrated to 20 ml by ultrafiltration. This solution was used in kinetic measurements, study of specificity, pH and temperature dependences, molecular weight, and isoelectric point. The isoelectric focusation of the fraction D2 is given in Fig. *4a* and the overall purification procedure in Table I.

D) *Chromatography of the fraction* C *on* USPP-*Sepharose* 4B: A concentrated dialysed portion of fraction C (50 ml) was applied to a column of USPP-Sepharose 4B (*vide supra*) and eluted first with 0·02M Tris-HCl (190 ml), pH 7·4, and then with linear gradient 0—1M sodium chloride

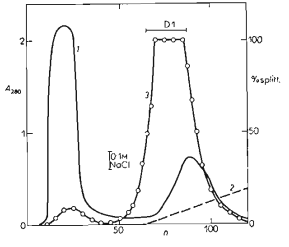

FIG. 2

Separation of the Fraction D on PSPP--Sepharose 4B

1 Total protein (A_{280}), *2* molarity of NaCl, *3* splitting of uridine 2',3'-cyclic phosphate (pH 7·5).

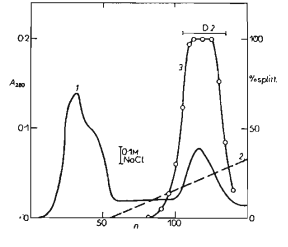

FIG. 3

Separation of the Fraction D1 on USPP--Sepharose 4B

1 Total protein (A_{280}), *2* molarity of NaCl, *3* splitting of uridine 2',3'-cyclic phosphate (pH 7·5).

TABLE I

The Overall Procedure of the Purification of Decyclizing Phosphodiesterase Activities of the Rape Seedlings Extract

Purification	Fraction	Total protein mg	Total activity EU[a]	Specific activity EU/mg	Yield %	Degree of purification
Dialysed extract	—	18 750	2 555	0·14	100	1
Modified Cellex P	D	285	982	3·4	38·4	24
PSPP-Sepharose 4B	D1	24·4	623	26·0	24·4	186
USPP-Sepharose 4B	D2	6·4	473	74·0	18·5	529
Isoelectric focusation	(D3)	<0·05	14	>287	>0·6	>2 050
Dialysed extract	—	18 750	282	0·01	100	1
Modified Cellex P	C	155	21	0·13	7·4	13
USPP-Sepharose 4B	C1	20·3	7·8	0·38	2·8	38
Isoelectric focusation	C2	3	1·9	0·62	0·7	62

[a] 1 EU represents the amount of enzyme which hydrolyses 1 μmol of uridine 2′,3′-cyclic phosphate per min under conditions given in the Experimental, in the presence of EDTA.

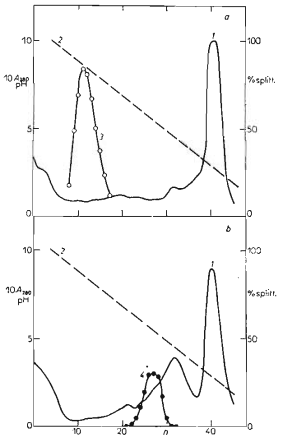

FIG. 4

Isoelectric Focusation of the Fractions D2 (*a*) and C1 (*b*)

1 Total protein (A_{280}), *2* pH, *3* splitting of uridine 2′,3′-cyclic phosphate, *4* splitting of adenosine 2′,3′-cyclic phosphate.

in the same buffer (2 × 500 ml). The fractions were checked for the splitting of uridine and adenosine 2′,3′-cyclic phosphates. The elution course is given in Fig. 5. Fractions 20—30 (45 ml) were combined, dialysed continuously against 0·02M Tris-HCl, pH 7·5 (5 l) and the dialysate concentrated to 20 ml (fraction C1). This fraction was used in the determination of the isoelectric point (Fig. 4*b*) and other properties. The overall course of purification of this fraction is given in Table I. Fractions 105—115 (45 ml) contained the activity D2, identical with that isolated as described under *C*.

Isolation and Properties of the Acidic Protein from the Fraction D2

The acidic protein (pI 2·8) was separated by isoelectric focusation (*vide supra*) of the fraction D1 and isolated by dialysis against 0·02M triethylammonium hydrogen carbonate, pH 7·5 (5 l) and lyophilisation. Yield 6·4 mg, negative test for splitting of uridine 2′,3′-cyclic phosphate; $s_{20,w}$ 1·7, mol.w. 16300 for $\bar{v} = 0{\cdot}72$ (method according to Yphantis, 39460 rpm). N-Terminal amino acids: Gln, Ile (1 : 1). Amino acid analysis: mol.w. 16600, after acidic hydrolysis 23·8 mol% Glx, 7·1 mol% Pro, 7·9 mol% Leu.

Deactivation of the Enzyme D2 by Dithioerythritol

A solution of the protein D2 (640 μg) and dithioerythritol (8 μmol) in 0·16M Tris-HCl, pH 8·0 (1 ml), was incubated overnight at 5°C until the activity toward uridine 2′,3′-cyclic phosphate disappeared completely (determined by the standard procedure). The mixture was continuously dialysed against 0·2M Tris-HCl, pH 7·5 (1 l), under nitrogen. Air was bubbled through this solution and 25 μl aliquots were taken in one-hour intervals in order to determine the activity toward uridine 2′,3′-cyclic phosphate. Even after 24 h at room temperature, no reactivation of the enzymatic activity was observed.

RESULTS AND DISCUSSION

Preliminary analysis of weakly acidic aqueous extract of rape seedlings revealed the presence of phosphomonoesterase, nuclease and decyclizing phosphodiesterase activities (as shown by splitting of two different ribonucleoside 2′,3′-cyclic phosphates, two different nucleotides, *p*-nitrophenyl phosphate and high-molecular RNA).

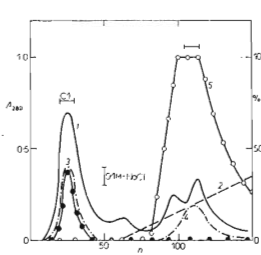

FIG. 5

Separation of the Fraction C on USPP-Sepharose 4B

1 Total protein (A_{280}), *2* molarity of NaCl, *3* splitting of adenosine 2′,3′-cyclic phosphate (without EDTA), *4* splitting of uridine 2′,3′-cyclic phosphate (without EDTA), *5* splitting of uridine 2′,3′-cyclic phosphate (in the presence of EDTA).

Since in the separation of the mixture the fractions exhibiting activity towards RNA coincide with some of phosphomonoesterase activities, the RNA-test was omitted in the course of the analysis. All activities were tested in acidic as well as alkaline region and it can be assumed that they represent an absolute majority of nucleolytic activities present in the extract.

The initial separation of the extract was carried out on a modified Cellex P, obtained by esterification with tert-butyl chloride in pyridine. This material was successfully used in the isolation of enzymes from mustard seedlings[4]. On this column, the starting buffer eluted the greater part of the ballast proteins whereas the nucleolytic activities remained adsorbed. (In this isolation step, no preliminary concentration of the crude extract is necessary.) Washing with linear gradient of sodium chloride resulted in successive elution of the single activities, depicted on Fig. 1. Fractions A and B contained phosphomonoesterase activities which will be dealt with elsewhere, the last fractions, C and D, contained activities, splitting uridine and adenosine 2′,3′-cyclic phosphate to the corresponding mononucleotides. These fractions were contaminated with a small amount of phosphomonoesterase activities. The minor fraction C which splits both uridine and adenosine 2′,3′-cyclic phosphates was, however, not completely separated from the main activity, fraction D, capable of splitting only the uridine derivative. The main portion, fraction D, was subjected to affinity chromatography on Sepharose 4B, modified with O,O-diphenylthiophosphoryl group (PSPP-Sepharose 4B), which has been already used in purification of snake venom exonuclease[5]. This carrier removed most of the residual contaminating activities and ballast proteins (Fig. 2). The fraction, active towards uridine 2′,3′-cyclic phosphate (D1), was further purified on a new type of affinity carrier, which had been prepared by reaction of cyanogen bromide activated Sepharose 4B with *p*-aminophenyl ester of uridine 5′-thiophosphate.

This ligand was obtained by reaction of 2′,3′-O-isopropylideneuridine with *p*-nitrophenyl ester diimidazolide of thiophosphoric acid, prepared *in situ* by reaction of *p*-nitrophenyl ester thiophosphoryl dichloride with imidazole. Acidic hydrolysis of the obtained product removed the protecting dioxolane group in the nucleoside and the free *p*-nitrophenyl ester of uridine 5′-thiophosphate was transformed to the *p*-aminophenyl ester by hydrogenation on palladium.

Thus-obtained carrier (USPP-Sepharose 4B; Fig. 3) exhibits activity towards various nucleolytic enzymes. In this, as well as in other cases, the optimum conditions for enzyme sorption and resorption were determined in advance by batch experiments. Chromatography of the fraction D1 afforded a very pure enzyme fraction D2, free of all other nucleolytic activities; this fraction was further used in the more detailed studies.

The basic properties of this enzyme, further designated as the enzyme D2, were studied using uridine 2′,3′-cyclic phosphate as substrate; since the enzyme is strongly activated in the presence of EDTA, the corresponding tests were carried out in the

presence of this activator. The molecular weight of the enzyme D2 was determined by gel filtration on Sephadex G-200 using Blue Dextrane (MW = $2 . 10^6$), bovine serum albumine (MW = 67000) and cytochrome C (dog heart; MW = 13000) as internal standards. The maximum activity towards uridine 2',3'-cyclic phosphate coincides with that of cytochrome C and we can therefore assume that the molecular weight of the enzyme lies in the region of 13000 daltons. The pH-optimum of the enzyme D2 is in the region 6·8–7·2 in the presence of EDTA (Fig 6), the temperature optimum at 50°C. The temperature stability curves (Fig. 7) show that even at 60°C the enzyme is relatively stable at the pH-optimum (30% decrease after 90 min), and even more at low pH values (10% drop after 90 min). The isoelectric point of the enzyme D2 was determined by isoelectric focusation (Fig. 4) to be pI 8·5. It was found that the fraction D2 is accompanied with a strongly acidic protein (pI 2·8) without any nucleolytic activity. The presence of this protein, which contains a significantly high portion of glutamic acid (or glutamine) and consists probably of two

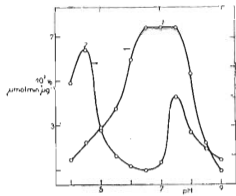

FIG. 6

pH-Dependence of the Splitting Rate of Uridine 2',3'-Cyclic Phosphate with the Enzyme D2

1 In the presence of EDTA, *2* in the absence of EDTA.

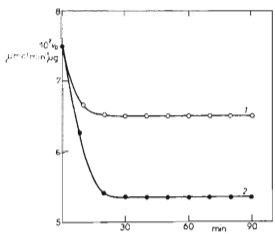

FIG. 7

Thermal Stability of the Enzyme D2 at 60°C

Splitting rate of uridine 2',3'-cyclic phosphate after preincubation: *1* pH 4·5, *2* pH 7·5.

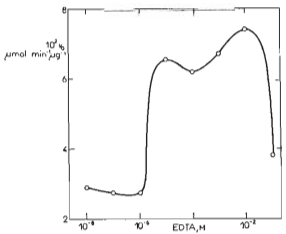

FIG. 8

The Effect of EDTA on the Initial Rate of Splitting of Uridine 2',3'-Cyclic Phosphate with the Enzyme D2

chains (two N-terminal amino acids in the same molar ratio), probably stabilises the enzyme D2. Its removal destroyed the enzyme activity (fraction D3) towards uridine 2',3'-cyclic phosphate within several hours.

Inhibitors and activators of the enzyme D2 (Table II). The studied metal cations can be divided into three groups: activating ions (Fe^{2+}, Fe^{3+}), weak inhibitors (Cu^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Ag^{+}) and strong inhibitors (Zn^{2+}, Cd^{2+}, Hg^{2+}). The inhibitory activity does not depend on the cation valency; an extraordinary behaviour is exhibited by divalent cations of the second sub-group metals, which show a decreasing effect with increasing nucleon number.

Since this fact, together with the strongly activating effect of EDTA, indicated that the enzyme D2 can be a metalloprotein, we analysed the protein of the fraction D2 for heavy metals by semi-quantitative method of emission spectrographic analysis. The analysis revealed a high copper content in the protein fraction (by two orders

TABLE II

Activators and Inhibitors of the Enzyme D2 in the Splitting of Uridine 2',3'-Cyclic Phosphate

Compound	Concentration [M]	Relative initial rate	Compound	Concentration [M]	Relative initial rate
—	—	1·00[a]	NaN_3	10^{-3}	0·89
Cu^{2+}	10^{-4}	0·52	Na_2HPO_4	10^{-2}	>1·50[c]
Ag^{+}	10^{-4}	0·89	NaCl	1	0·23
Mg^{2+}	10^{-3}	0·69	urea	1	0·74
Ca^{2+}	10^{-3}	0·91	EDTA	10^{-3}	>1·50[c]
Zn^{2+}	10^{-4}	0·15	dithioerythritol	10^{-3}	0·95[d]
	10^{-3}	0[b]	chloroacetic acid	10^{-3}	0·72[d]
Cd^{2+}	10^{-4}	0·20	tween 80	0·02%	0·79
	10^{-3}	0·08	triton X-100	0·02%	0·86
Hg^{2+}	10^{-4}	0·40	*p*-nitrophenyl phosphate	10^{-2}	1·34
Mn^{2+}	10^{-3}	0·62	uridine 3'phosphate	$6{\cdot}4 \cdot 10^{-3}$	1·11
Fe^{2+}	10^{-3}	>1·50[c]		$3{\cdot}2 \cdot 10^{-2}$	>1·50[c]
Fe^{3+}	10^{-4}	1·10		$6{\cdot}4 \cdot 10^{-2}$	>1·50[c]
	10^{-3}	>1·50[c]	uridine 5'-phosphate	$6{\cdot}4 \cdot 10^{-3}$	1·03
Co^{2+}	10^{-4}	0·66		$3{\cdot}2 \cdot 10^{-2}$	0·77
Ni^{2+}	10^{-4}	0·71	uridine	$6{\cdot}4 \cdot 10^{-3}$	0·79
NaF	10^{-3}	0·91		$3{\cdot}2 \cdot 10^{-2}$	0·60

[a] $v_0 = 1{\cdot}7 \cdot 10^{-8}$ mol min^{-1} for [S] = $6{\cdot}4 \cdot 10^{-3}$M; [b] the inactivation is reversible, addition of an equimolar amount of EDTA fully restores the enzymatic activity; [c] quantitative splitting [d] before addition of the substrate, the enzyme was incubated with the given inhibitors for 20 h at 5°C.

TABLE III

Substrate Specificity of D2 Enzyme

Compound	Substrate	Splitting %[a]	Pancr. RNAse (ref.[13–16])
	Nucleoside 2′,3′-cyclic phosphates		
I	uridine	87	+
II	5-chlorouridine	100	+
III	5-bromouridine	100	+
IV	5-aminouridine	68	+
V	5-dimethylaminouridine	50	+
VI	5-diethylaminomethyluridine	71	·
VII	5-ethoxycarbonyluridine	49	+
VIII	5-methyluridine	70	+
IX	6-methyluridine	0/0	—
X	3-methyluridine	0/0	--
XI	cytidine	31	+
XII	5-methylcytidine	21	+
XIII	N^4-dimethylcytidine	0/0	+
XIV	isocytidine	0/0	—
XV	1-(β-D-ribofuranosyl)-2-pyrimidinone	0/0	+
XVI	6-azauridine	24	+
XVII	3-(β-D-ribofuranosyl)-4-pyrimidinone	0/0	--
XVIII	3-(β-D-ribofuranosyl)uracil	0/0	—
XIX	3-(β-D-ribofuranosyl)-6-methyluracil	0/0	—
XX	3-(β-D-ribofuranosyl)-1,6-dimethyluracil	0/0	--
XXI	adenosine	0/0	--
XXII	guanosine	0/0	—
XXIII	8-hydroxyguanosine	0/0	+
XXIV	isoguanosine	0/0	—
XXV	3-(β-D-ribofuranosyl)uric acid	0/0	—
	Sugar-modified nucleoside 2′,3′-cyclic phosphates		
XXVI	5′-deoxyuridine	77	+
XXVII	1-(α-L-lyxofuranosyl)cytosine	0/0	+
XXVIII	1-(β-D-ribopyranosyl)uracil[b]	0/0	
XXIX	L-uridine	0/0	—
XXX	L-cytidine	0/0	—
XXXI	α-uridine	0/0	—
XXXII	1-(*S*)-(2,3-dihydroxypropyl)uracil	0/0	—
XXXIII	1-(*S*)-(2,3-dihydroxypropyl)thymine	0/0	—
XXXIV	1-(*R*)-(2,3-dihydroxypropyl)thymine	0/0	—

TABLE III
(*Continued*)

Compound	Substrate	Splitting	Pancr. RNAse (ref.[13-16])
	Oligonucleotides and other compounds		
XXXV	uridine 3′,5′-cyclic phosphate	0/0	–
XXXVI	uridylyl-(3′→5′)-uridine	0/0	+
XXXVII	uridylyl-(3′→5′)-adenosine	0/0	+
XXXVIII	uridylyl-(3′→5′)-uridine 2′,3′cyclic phosphate[c]	87	+
XXXIX	yeast RNA[d]	0/0	+
XL	polyuridylic acid[d]	0/0	+

[a] 0/0 zero splitting after 1 h, confirmed by 24 h treatment at 37°C; [b] mixture with 3′,4′-cyclic phosphate; [c] product determined as UpUp which affords UpU on bacterial phosphomonoesterase treatment; [d] estimated by Kunitz's assay (*cf.* Methods).

of magnitude higher than the background and than the usual content in the natural material) but no significantly high content of zinc or other metal ions. It seems thus that either the enzyme D2 or its complex with the acidic protein is stabilised in its native form by copper ions; it possesses, however, a higher affinity towards other ions, particularly towards the zinc and cadmium ions (an impregnation of seeds by copper compounds or a contamination during the isolation procedure is excluded).

Fluoride or azide ions in usual inhibitory concentrations have no substantial effect on the enzyme D2, neither has 1M urea or surface active compounds. On the other hand, high ionic strength of a neutral electrolyte (NaCl) has been found to exhibit strongly inhibitory effect. Inorganic phosphates, as well as monoesters of phosphoric acid (*p*-nitrophenyl phosphate and uridine 5′- or 3′-phosphate), show a distinct activating effect. As already mentioned, the presence of EDTA activates markedly the enzymatic activity. This manifests itself also in the determination of other properties of the enzyme, *e.g.* pH-optimum which in the presence of EDTA is shifted by 0·3 – 0·5 pH to the acidic region. A plot of the initial splitting rate against the EDTA concentration (Fig. 8) shows a dramatic change between the concentrations $5 \cdot 10^{-5}$M and 10^{-6}M of the complexing agens, *i.e.* at unusually low concentrations.

Neither monochloroacetic acid nor dithioerythritol show strong inhibitory effect on the enzyme activity. After longer exposure, higher concentrations of dithioerythritol suppress the enzyme activity to zero. After dialysis of this mixture, however,

we did not succeed in reactivation of the enzyme by aerial oxidation. If the disulfidic bridges are split at all, they recombine probably under formation of another molecular structure, contingently also with the accompanying acidic protein.

Mechanism of the enzymatic reaction: Studies of splitting of various model compounds with the enzyme D2 have shown that this enzyme is not capable of splitting internucleotidic phosphodiester bonds: neither RNA, nor polyuridylic acid nor dinucleoside phosphates with 5'- or 3'-terminal uridine nor uridine 3',5'-cyclic phosphate is degraded. The enzyme exhibits no nucleotidase or nonspecific phosphomonoesterase activity. The only type of substrate among the nucleic acid metabolites are uridine and cytidine 2',3'-cyclic phosphates whereas purine 2',3'-cyclic phosphates

TABLE IV
Splitting of Nucleotides and Their Alkyl Esters by the D2 Enzyme

Compound	Nucleotide	Alkyl ester	Splitting %[a]
XLI	uridine 3'-phosphate[b]	—	0/0
XLII		methyl	49
XLIII		ethyl	23
XLIV		n-propyl	3
XLV		n-butyl	0
XLVI		2-hydroxybutyl	0
XLVII	uridine 2'-phosphate	methyl	0/0
XLVIII	2'-deoxyuridine 3'-phosphate	methyl	0/0
XLIX	cytidine 3'-phosphate	—	0/0
L		methyl	0/0
LI		ethyl	0/0
LII		n-propyl	0/0
LIII	cytidine 5'-phosphate	methyl	0/0
LIV	adenosine 3'-phosphate	—	0/0
LV		methyl	0/0
LVI		ethyl	0/0
LVII		n-propyl	0/0
LVIII		isopropyl	0/0
LIX		n-butyl	0/0
LX	adenosine 2'-phosphate	methyl	0/0
LXI	adenosine 5'-phosphate	methyl	0/0
LXII	2'-deoxyadenosine 3'-phosphate	methyl	0/0
LXIII	guanosine 3'-phosphate	methyl	0/0

[a] 0/0 zero splitting after 1 h, confirmed by 24 h treatment at 37°C; [b] degradation products: uridine 3'-phosphate and uridine 2',3'-cyclic phosphate.

are resistant. The absolute preference of the enzyme for a 2',3'-cyclic phosphodiester bond follows unequivocally from the splitting of uridylyl-(3'→5')-uridine 2',3'-cyclic phosphate which affords quantitatively the dinucleotide UpUp without affecting the internucleotide linkage (constitution of the product was proved by degradation with alkaline bacterial phosphomonoesterase, which afforded UpU as the sole product).

Further study proved that also alkyl esters of uridine 3'-phosphate can be substrates of the enzyme D2: whereas esters of the isomeric 2'- and 5'-nucleotides (Table IV) are resistant, aliphatic ester of uridine 3'-phosphate are split to uridine 3'-phosphate. However, the extent of the splitting in this series decreases in the order methyl > ethyl > n-propyl, the n-butyl ester being resistant. Since an increase in hydrophilic character of the n-butyl group (in the 2-hydroxybutyl derivative) brings about no effect, it can be assumed that the affinity of the esters towards the enzyme depends on the steric requirements of the uridine 3'-phosphate moiety. The sterically demanding nucleosidic moiety bonded by an ester bond to uridine 3'-phosphate in oligonucleotide derivatives hinders therefore splitting with the enzyme. Under mild conditions and in the absence of EDTA it was possible to prove that the cleavage of uridine 3'-phosphate methyl ester proceeds *via* the intermediate uridine 2',3'-cylic phosphate which then undergoes a rapid splitting by the enzyme.

Both uridine and cytidine 2',3'-cyclic phosphates are split to 3'-nucleotides: in the case of the cytidine derivative this was proved by separation on an ion exchange resin (Fig. 9), in the case of the uridine compound by a more complicated procedure, *viz.* isolation of the product, its blocking with tetrahydropyranyl group and condensation with 2',3'-di-O-benzoyluridine in the presence of TPS. Alkaline and acid deblocking afforded uridylyl-uridine, 94% degradable with pancreatic RNase. We can

TABLE V
Michaelis Parameters for D2 Enzyme

Compound	$K_m \cdot 10^3$ [a] M	$V_{max} \cdot 10^9$ [a] [mol min^{-1}] per μg protein
Uridine 2',3'-cyclic phosphate	4·9 (20·0)[b]	118·0 (2·22)[b]
Cytidine 2',3'-cyclic phosphate	20·0	8·96
Uridine 3'-phosphate methyl ester	20·0	15·90

[a] With 10^{-3} M EDTA; [b] in the absence of EDTA.

thus conclude that uridine 2',3'-cyclic phosphate affords pure uridine 3'-phosphate by splitting with the enzyme D2.

The kinetic parameters for some substrates (Table V, Fig. 10) show that the enzyme D2 exhibits a high preference for uridine 2',3'-cyclic phosphate: this is reflected by the K_m value which for uridine 3'-phosphate methyl ester and cytidine 2',3'-cyclic phosphate is 4 times higher than that for uridine 2',3'-cyclic phosphate, as well as by the V_{max} value which is by about an order of magnitude lower for both former compounds than for uridine 2',3'-cyclic phosphate. In the absence of EDTA the binding of uridine 2',3'-cyclic phosphate is 4 times weaker and the rate 50 times slower than in the presence of EDTA. The dependence of V_{max} on the concentration of the enzyme is linear within the whole concentration region used.

The specificity of the enzyme D2 towards ribonucleoside 2',3'-cyclic phosphates, modified in the heterocyclic moiety (Table III) proved that its requirements are unusually similar to those of pancreatic ribonuclease. In almost all respects, the properties of both these enzymes are parallel: *e.g.* the negative effect of substitution in the position N^3 or $C_{(6)}$, the necessary presence of N^3—CO-grouping in the α-position to the nucleoside bond, no effect of substitution at $C_{(5)}$, *etc.* An exception is represented by the resistance of the 2-pyrimidone derivative *XV* and the N^4-dimethylcytidine compound *XIII*, both of which are split by the RNase A. If we consider also the low affinity of both other cytidine derivatives *XI*, *XII*, it is obvious that the enzyme D2 prefers a uracil type arrangement. The specificity of the enzyme D2

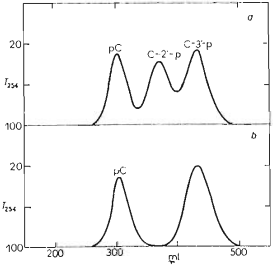

FIG. 9

Separation of Isomeric Cytidine Monophosphates on Dowex 1X8 (formate form)

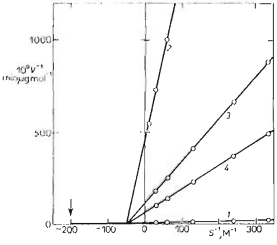

FIG. 10

Lineweaver-Burke Plot for the Enzyme D2

1 Uridine 2',3'-cyclic phosphate (with EDTA), *2* uridine 2',3'-cyclic phosphate (without EDTA), *3* cytidine 2',3'-cyclic phosphate (with EDTA), *4* uridine 3'-phosphate methyl ester (with EDTA).

towards cyclic nucleotides with modified sugar moiety is also analogous to that of the pancreatic RNase A. The resistance of both the L-nucleotide derivatives *XXIX*, *XXX* proves the binding of the whole chiral substrate molecule to the enzyme which is demonstrated also by the resistance of the derivative *XXXI*. The sugar pyranoside derivative *XXVIII* and the acyclic derivatives, isosteric with the part of the sugar moiety (*XXXII*–*XXXIV*), are also not degraded. A behaviour, different from the RNase A, was observed in the case of the lyxofuranosyl derivative *XXVII* which has an opposite configuration in the position 4′ and which is resistant towards the enzyme D2. However, since the 5′-deoxyuridine derivative *XXVI* represents a good substrate, the failure of reaction with the compound *XXVII* is not due to an unsuitable orientation of the hydroxyl, but rather due to the presence of a sterically bulky hydroxymethyl group in the vicinity of the binding and catalytic site of the enzyme. The enzyme of the fraction D2 is thus a pyrimidinespecific 2′-nucleotidyltransferase with a high preference for uridine 3′-phosphate derivatives containing a sterically little demanding ester-bonded substituent. Concerning the specificity and requirements for the substrate properties, this enzyme closely resembles pancreatic ribonuclease. As far as we know, such enzyme has not been hitherto described and it can be classified as a new type of the group 2·7·7. Its physiological role is not sufficiently obvious. Such an enzyme was not found in the rape pollen.

The minor decyclizing phosphodiesterase activity, accompanying the above-mentioned cyclizing 2′-nucleotidyltransferase D2, is contained in the fraction C (Fig. 1). It was purified by chromatography on USPP-Sepharose 4B, on which this enzyme, characterised by cleavage of adenosine 2′,3′-cyclic phosphate, is eluted already with the starting buffer (Fig. 5). The thus-obtained preparation was sufficiently pure but its total amount was not enough to allow a more detailed study. This enzyme (C1) splits all the four 2′,3′-cyclic phosphates derived from the natural ribonucleosides, pH-optimum being about 7·5. Contrary to the enzyme D2, the presence of EDTA has no effect on its splitting rate. Its pI, determined by isoelectric focusation (Fig. 4*b*), is 5·3–5·4; also in this case the isoelectric focusation separated the strongly acidic protein without nucleolytic activity, probably stabilising the activity of C1: removal of this protein and dialysis of fractions with the C1 activity resulted in rapid disappearance of enzymatic activity. Judged from course of the isolation (chromatography on USPP-Sepharose 4B), it is probable that this protein is not an artefact arising from the fraction D1 since this fraction is eluted only by a higher ionic strength of the starting buffer.

The enzyme C1 splits 2′,3′-cyclic phosphates of ribonucleosides to mononucleotides; in the case of adenosine (paper chromatography), as well as of cytidine (chromatography on Dowex 1), the exclusive presence of 2′-nucleotides was proved. The fact that the enzyme shows neither phosphomonoesterase activity towards 2′,3′- or 5′-ribonucleotides, nor splits RNA or model oligoribonucleotides, proves unequivocally that it is a decyclizing 2′,3′-phosphodiesterase, producing 2′-nucleotides, analogous

to the enzyme found in the aqueous extract of rape pollen[1,2]. A proof of identity of both these activities, however, could not be given within the scope of this work.

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